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**Faculty of Environmental Science  
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**Detection of some bacterial markers by Ion  
Mobility Spectrometry**

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**PhD thesis summary**

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*The experimental results that represent the basis for achieving this PhD thesis were all obtained at Loughborough University, United Kingdom.*

*The research extended over 11 months, during which the PhD student Ileana-Andreea Rațiu worked in the Analytical Chemistry Laboratory of the Department of Chemistry, Loughborough, under direct supervision of Prof. Univ. Dr. C.L. Paul Thomas and Dr. Victor Bocoș-Bințișan, whom she addresses heartfelt thanks.*

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## Abstract

The purpose of this research project was to investigate the feasibility of bacterial markers' detection using Ion Mobility Spectrometric techniques.

The reason for choosing the theme "*Detection of bacterial markers by Ion Mobility Spectrometry*" was to explore a relatively new concept, in which the potential of IMS (Ion Mobility Spectrometry) is used for microorganisms' detection.

Thus, **the first chapter** of the thesis, "*Methods for detecting microorganisms. Bio-medical and pharmaceutical applications of Ion Mobility Spectrometry*" includes an introduction part and the techniques available for microorganisms' detection, with their performances, approached comparatively. These will be related to IMS - through its applications, particularly those concerning microorganisms and biogenic compounds, therefore IMS operating principle and instrumentation will also be discussed here.

In **the second chapter**, "*Detection of biological markers using Ion Mobility Spectrometry techniques*", particular aspects of the two types of bacterial markers - the enzymatic markers, and those produced by pyrolysis - will be presented.

In **the third chapter**, entitled "*Sampling and analysis, data processing and interpretation of results*" is presented the experimental, original part, which focuses on a series of measurements and tests for biogenic markers at trace level in the headspace atmosphere. This part will present the results obtained, the instrumentation used and will briefly describe the experimental conditions. So, the third chapter will include the obtained experimental outcomes and related discussions.

At the end of each chapter are drawn a series of conclusions, concerning the investigations performed and the results obtained. The conclusions are summarized in **chapter four**, where the possible future investigations are also indicated.

The PhD thesis ends with a set of references that aim precisely on microorganisms' detection by Ion Mobility Spectrometry techniques.

### **Keywords:**

**bacterial markers**  
**Ion Mobility Spectrometry**  
**Gas Chromatography**  
**Mass Spectrometry**  
**detection of microorganisms**  
**headspace air samples**  
**"Principal Components Analysis".**

## Introduction

The detection and rapid identification of bacteria, particularly the pathogenic ones, remains an important and challenging task when it comes to food security, drinking water quality control, combating infectious diseases or preventing bio-terrorism. It is noteworthy that, every year, about 1.5 billion people suffer from a bacterial infection. Therefore, bacterial agents must be treated with maximum care.

Discussing about testing effectively the bacteria, this requires analytical methods that have to obey a series of restrictive criteria. Thus, the most important limitations are the time required for analysis and the sensitivity. It is also highly desirable to have available analytical methods as selective as possible, since a small number of pathogenic species are often present in the complex biological and environmental matrix, together with non-pathogenic microorganisms.

Ion Mobility Spectrometry (IMS) is a modern analytical technique which, due to its remarkable sensitivity, fits perfectly to traces detection of chemicals present in air, but also in liquid or solid samples. This technique involves two stages: (a) ionization of chemical species at atmospheric pressure, followed by (b) subsequent separation of generated ions, based on mobility differences in a neutral drift gas and under the influence of an electric field with relatively low intensity.

Applications of Ion Mobility Spectrometry (IMS) are very diverse: military applications (detection of chemical warfare agents), security applications (detection of drugs and explosives), environmental and industrial applications (control and monitoring of different pollutants), as well as medical and pharmaceutical applications (diagnosis of disease, control and quality assurance and authenticity of pharmaceutical products). As a rule of thumb, any chemical which may be ionized is detected using Ion Mobility Spectrometry.

In the last two decades years, Ion Mobility Spectrometry has been in a continuous development and expansion - as well as its new applications, particularly those related to microorganisms (cells, bacteria, fungi) detection, medical applications (diagnosis, for example respiratory tests, therapy and medication control), food quality control, safety monitoring and characterizing the control processes in the chemical and pharmaceutical industries. For example, the researchers from Centre for Analytical Science (Loughborough University) and ISAS (Institute for Analytical Sciences) in Dortmund have performed a series of feasibility studies with biological and medical purposes, including the detection of bacteria, fungi and metabolites in the human breath. For all these characteristic samples, it was proved that the analysis pattern can be used to identify the cell species, fungi and bacteria, as well as for screening various diseases. Also, the

quantification of such data could be used to obtain information about the process state (such as bacterial culture growth, the disease development, the medication level and the stage of cancer).

In the international literature, an increasing number of studies on the instrumentation, operating principles and applications of the Ion Mobility Spectrometry have been lately available. Thus, it appears that the applications of this analytical technique are most complex, being extremely useful and necessary, particularly due to the concentrations of extremely varied organic and inorganic chemicals that can be detected at very low limits (traces levels - ppb<sub>v</sub>), actually from any type of samples (liquid, solid or gaseous).

In Romania, Dr. Bocoş-BinţiŃan Victor is the author of the first monographic book on Ion Mobility Spectrometry - published in 1998, after only two other monographic books on this theme had been published in 1984 and 1994 in the United States (the last one has been reprinted in 2005).

### **The main objective**

The purpose of this research project was to investigate the feasibility of bacterial markers' detection using Ion Mobility Spectrometric techniques.

The reason for choosing the theme "*Detection of bacterial markers by Ion Mobility Spectrometry*" was to explore a relatively new concept, in which the potential of IMS (Ion Mobility Spectrometry) is used for microorganisms' detection.

## Summary of the thesis

The PhD thesis contains four chapters, i.e.: the first chapter, presenting “*Methods for detecting microorganisms. Bio-medical and pharmaceutical applications of Ion Mobility Spectrometry*”, the second chapter, where the “*Detection of biological markers using ion mobility spectrometry*” is discussed and the last chapter, presenting methodologies used for “*Sampling and analysis, data processing and interpretation of the results*”, a *description of instrumentation, and experimental design*, as well as *experimental results and discussions*, in a detailed subchapter.

**The first chapter** of the thesis, "*Methods for detecting microorganisms. Bio-medical and pharmaceutical applications of Ion Mobility Spectrometry*" includes an introduction part and the techniques available for microorganisms' detection, with their performances, approached comparatively. These will be related to IMS - through its applications, particularly those concerning microorganisms and biogenic compounds, therefore IMS operating principle and instrumentation will also be discussed here.

In **the second chapter**, "*Detection of biological markers using Ion Mobility Spectrometry techniques*", particular aspects of the two types of bacterial markers - the enzymatic markers, and those produced by pyrolysis - will be presented.

In **the third chapter**, entitled "*Sampling and analysis, data processing and interpretation of results*" the experimental, original part will be presented, focusing on a series of measurements and tests for biogenic markers at trace level in the headspace atmosphere. This part will present the results obtained, the instrumentation used and will briefly describe the experimental conditions. So, the third chapter will include the obtained experimental outcomes and related discussions.

At the end of each chapter are drawn a series of conclusions, concerning the investigations performed and the results obtained. The conclusions are summarized in **chapter four**, where the possible future investigations are also indicated.

The PhD thesis ends with a set of references that aim precisely on microorganisms' detection by Ion Mobility Spectrometry techniques.

# 1. Methods for detecting microorganisms.

## Bio-medical and pharmaceutical applications of Ion Mobility Spectrometry

Analytical techniques employ different principles through which compounds at trace level – with concentrations of the order of parts per million (ppm) or even smaller, i.e. part per billion (ppb), or parts per trillion (ppt) – found in different environments /samples could be detected on the basis of a well-established property of the analyte.

The fundamental tool in the analysis of microorganisms is, from the microbiological perspective, testing of the intracellular and extra cellular enzymes. For several decades, enzyme tests have helped microbiologists to perform the taxonomy, detection and the identification of the microorganisms. Currently, high performance analytical equipment may be used to analyze enzymes, thus providing complex information about the organisms from which they originate.

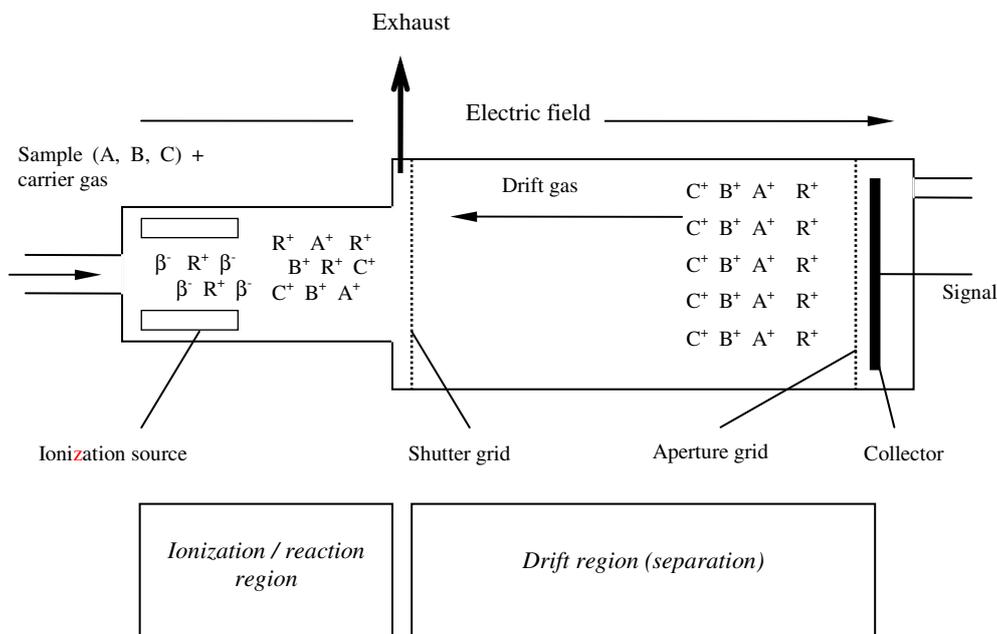
### **Ion Mobility Spectrometry - brief description**

In Ion Mobility Spectrometry, the chemical separation and detection are achieved by:

1. ionization of a gas or vapors;
2. separation of ionic species in a drift tube, under the influence of an electric field with relatively low intensity, at (or near) atmospheric pressure;
3. conversion of ionic clouds in ionic currents at the end of the drift tube (where the detector is);
4. signal processing of the resulted ion current, in order to provide useful information on chemical identification and on quantification [*Bocoş-Binţinţan, 2004*].

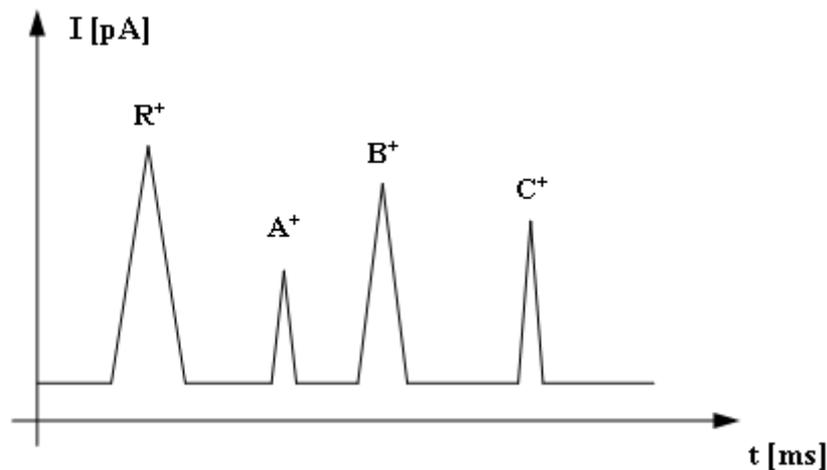
Inside the IMS instrument (Figure1) the experimental steps are as follows: primary ions are produced in a carrier gas by an ionization source (usually a radioactive source, using the beta isotope  $^{63}\text{Ni}$ ), then these primary ions (called reactant ions) begin a sequence of fast collisional ion-molecule reactions that generate product ions, which include the target analyte molecules. The ions formed in the reaction region are then periodically introduced into the drift region by using a shutter grid, where they are moved by an electric field through a neutral drift gas (usually nitrogen or air at

atmospheric pressure) and finally reach the detector (a Faraday plate). Both positive and negative ions can be studied. The transit time values through the drift region are registered, in milliseconds or tens of milliseconds. Obviously, the arrival time (called drift time) of a peak of current quantifies the drift rate and consequently is closely related to the mobility of the ions from this peak [Eiceman, 2002; Bocoş-Binţinţan, 2004].



**Figure 1. Schematic of an ion mobility spectrometer cell [Bocoş-Binţinţan, 2004]**

After the separation in the drift tube, the ions collide with the detector and so, the so-called “ion mobility spectrum” is generated (Figure 2), where  $R^+$  represents the peak of the reactant ions while  $A^+$ ,  $B^+$ ,  $C^+$  are the peaks of the product-ions.



**Figure 2. Ion mobility spectrum [Bocoş-Binţinţan, 2004].**

## **Bio-medical and pharmaceutical applications of Ion Mobility Spectrometry**

Quick identification of bacteria is essential in increasingly more fields. For example, if it is possible to identify a pathogenic bacterium, an appropriate antimicrobial therapy may be implemented, and the necessary epidemiological studies may be performed.

The Ion Mobility Spectrometry has been continuously developing in recent decades, as well as its new applications related to microorganisms, medicine, food quality control, safety monitoring and the characterization of control processes in the chemical and pharmaceutical industry.

In this respect, many feasibility studies have been conducted in biological and medical purposes, including the detection of bacteria, fungi and metabolite molecules in the human breath. All these have shown that this analytical technique can be used to identify cell species as well as many diseases. Also, the quantification of such information may serve to obtaining information about the status of the process (the disease level, the necessary medication level, to ensure quality control in the pharmaceutical industry).

It has been known for a long time that the odorant vapors derived from urine or breathing process reflect the respective person's diseases. Employment of appropriate analytical techniques has replaced the classical examination of patients by simply measuring the chemicals [*Vautz et al, 2008; Prabha et al, 2008*].

More specifically, Karpas proposed new methods for quick and more precise diagnosis of the vaginal infections, compared to the classical methods [*Karpas, 2002*].

The employment of IMS for the detection, identification and monitoring of the volatile compounds such as halothane, enflurane, isoflurane - used as exhaled anesthetic during surgery has been studied by Eiceman (2005). In the same time, preliminary studies proved that there are differences between the chemical composition of air exhaled by persons having pulmonary diseases, compared to the chemical composition of air exhaled by healthy persons. These assumptions are based on the fact that blood reflects the concentration of volatile organic compounds in the breathing process, due to the gas exchange occurring in the lungs [*Karpas et al, 2002; Eiceman, 2005*].

During the manufacturing process of pharmaceuticals, monitoring the chemicals is critical to ensure quality control. The pharmaceutical companies have experienced for a long time the need of a quick, efficient and inexpensive instrumentation, to guarantee quality control and to ensure the quality of their products. The classical techniques employed to ensure quality control in the pharmaceutical industry have some deficiencies related to the low speed and limited precision that they can provide. The techniques based on Ion Mobility were tested as alternatives for quality

control in the pharmaceutical industry, proving to be convenient due to the cheap instrumentation that lends itself very well to miniaturization, providing excellent sensitivity and response in real time [Ryan et al, 2008].

## Summary

Instrumental or microbiological analytical methods are employed to exploit a well-established property of the analyte. Thus, the o-nitrophenol property of having a relatively high vapor pressure was employed, which allows the direct analysis of these vapors using Ion Mobility Spectrometry. This way, by detecting the o-NP we have a sensitive, relatively compact and simple algorithm for the detection of bacteria; this algorithm may be successfully applied both to monitor drinking and waste water, as well as to quickly detect microorganisms in the medical facilities.

As any other analytical technique, Ion Mobility Spectrometry's employment for a particular application must be approached strictly on an individual basis. Factors to be considered include detection limits, response time, matrix interferences, cost, calibration time, portability, etc.

The systems of samples introduction are essential for IMS, particularly if the analytes are not entirely extracted from the sample, or if they are transferred to more devices coupled between each other. Sample input systems are thus employed depending on various characteristics of the equipment, but especially on the state of aggregation of the studied sample.

Bio-medical and pharmaceutical applications are based on the property of odorant vapors from metabolic processes to reflect the diseases of the respective person. Thus, the metabolites found in exhaled air can be directly correlated with the existence of different diseases. Some metabolites are biomarkers, e.g. diabetes occur with acetone, nitric acid is correlated with severe asthma, ammonia shows the existence of liver problems, while others indicate the presence of bacteria.

Employment of Ion Mobility Spectrometry allowed efficient and quick detection of various types of vaginal infections, successful detection, identification and monitoring of volatile compounds such as halothan, enfluran, isofluran – used as anesthetic inhalants during surgery, and also a direct diagnosis of lung damage, through a simple human breath sample.

Portable equipment, low limits of detection, real-time response and the easy employment of the IMS instrumentation allow the monitoring, quality assurance / quality control of pharmaceuticals, but also ensure the health and safety of employees of pharmaceutical companies.

## 2. Detection of biological markers employing IMS techniques

There are two main methods to detect biological markers with Ion Mobility Spectrometry techniques: detection of microorganisms employing markers produced by enzymatic processes and detection of microorganisms employing markers produced by pyrolysis [Snyder *et al*, 2001; Snyder *et al*, 2004].

### Detection of microorganisms employing markers produced by enzymatic processes

In many fields, quick identification of microorganisms is essential. For example, the possibility to identify pathogenic bacteria will allow the application of appropriate antimicrobial therapy and development of appropriate epidemiological studies [Creaser *et al*, 2004; Snyder *et al*, 1991; Strachan *et al*, 1995].

Detection of orthonitrophenol (ONP) - a bacterial marker common to most bacteria and generated by biochemical enzymatic reactions - has been described very clearly by Bocoş- Binţinţan and Raţiu (2009). By detecting headspace ONP vapors in the ambient air, detection limits less than ppm have been achieved, in a few seconds (Figure 3), so a quick response ("real time response"). For this purpose, an ion mobility spectrometer produced by the German company I.U.T (*Institut für Umwelt Technologien*) GmbH Berlin, IMS-Mini model was employed (Figure 4), a portable instrument that can be operated independently, without needing any kind of utilities or chemical reagents.

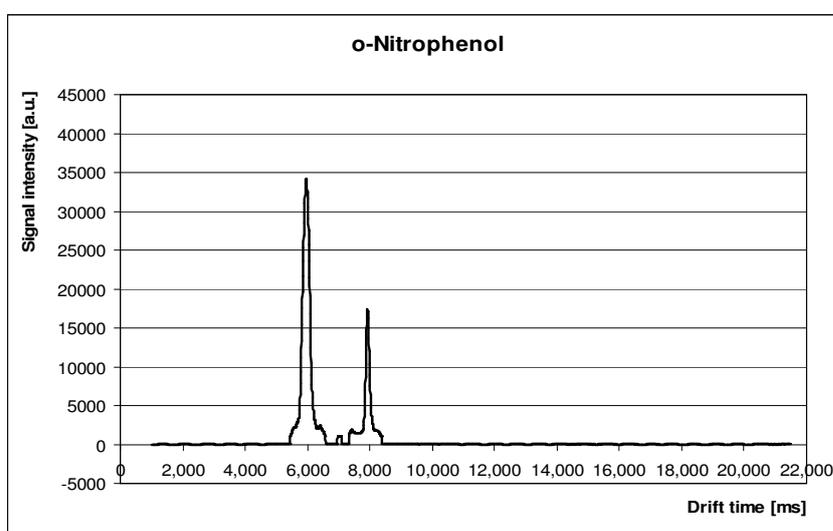


Figure 3. Ion mobility spectrum of o-nitrophenol [Bocoş-Binţinţan and Raţiu, 2009]



**Figure 4. Ion mobility spectrometer IMS-MINI (I.U.T. GmbH Berlin)**

The *Salmonella typhimurium* bacteria were determined employing the ELISA combined method (Enzyme-Linked Immunosorbent Assay), then employing a final step mediated by the phosphatase enzyme, and by detection of the obtained phenol (as a result of the ELISA reaction), employing Ion Mobility Spectrometry. Detection limits were about 10,000 bacteria in a 10 mL aliquot of sample [Smith et al, 1997].

Räsänen et al (2010) used an IMS detector - type ChemPro-100i, equipped with 16 detectors (IMS channels), 5 semiconductor sensors (MOS) and a one FET (field effect transistor) sensor - for monitoring and detection of volatile organic compounds derived from the colonies of mold. Thus, the differences between the headspace samples containing mold and the blank ones have were monitored. The statistical results proved a clear separation/differentiation between the samples containing mold and the blank samples, the same way the confirmation method (GC-MS) proved the existence of different compounds in the samples with mold and in the blank samples [Räsänen. et al, 2010].

Vinopal and colleagues have used two devices manufactured by Barringer (model 350A and 400A IONSCAN<sup>®</sup>). The objective of their study was to investigate the utility of the IMS techniques in differentiating the bacterial strains by direct analysis of entire bacterial cells, and also in differentiating bacterial strains and species in real time, without special testing programs and without using reagents. The distinct reproducibility of charts for different growing conditions proved the feasibility of using the IMS response as a characteristic "fingerprint" of bacteria, to identify the differences between species of bacteria [Vinopal et al, 2002].

## Detection of microorganisms employing markers produced by pyrolysis

Pyrolysis Mass Spectrometry (Py-MS) is a sensitive analytical technique that works on the principle of rapid thermal degradation (pyrolysis). Pyrolysis takes place before ions get separated in the mass spectrometer. The technique is intended for analyzing non-volatile compounds in complex matrices. Pyrolysis is responsible for the formation of volatile fragments in complex molecules, whose masses are then displayed as a mass spectrum [Snyder et al, 2004].

The possibility of detecting several hundreds nanograms of endospores of *Bacillus* using *picolinic acid* and *pyridine* as biochemical markers (characteristic compounds of dipicolinic acid - present in the cellular walls of spores) was experimentally proved by Jacek and colleagues (1997). Their instrumentation consisted of a pyrolizer coupled with a Mobility Spectrometer model EVM (Environmental Vapour Monitor - manufactured by Graseby Ltd. & FemtoScan Inc companies), which is actually a GC / IMS tandem system [Jacek et al, 1997].

The products derived from the bacteria endospores were mainly dipicolinic acid and pyridine (the 2,6 – piridin-dicarboxilic acid) - resulted from the thermo analysis of the spores' cellular walls. Picolinic acid could be detected by pyrolysis of less than one hundred nanograms of *Bacillus subtilis*, by bringing it to the inferior limit of detection [Dworzanski et al, 1997].

The research group consisting of Cheung, Xu, Thomas and Goodacre investigated in 2008, three types of bacteria - two species of *Bacillus subtilis* and one of *Bacillus megaterium* - in order to assess the possibility of their differentiation, employing the instrumental chain Py-GC-DMS (Pyrolizer - Gas Chromatograph - Differential Mobility Spectrometer). After data processing based on multiple statistical approaches, the authors managed to successfully prove the differentiation of bacteria species belonging to the same genus [Cheung et al, 2009].

Prasad and colleagues published a series of articles related to the analysis of various species of bacteria and the influence of growth temperature on chemical components generated by these bacteria, by Pyrolysis Gas Chromatography and Differential Mobility Spectrometry (Py-GC/DMS). Thus, these authors employed a Py-GC/DMS analyzer, investigated the possibility of analyzing bacterial species on eight types of bacteria, and obtained detailed biochemical information such as topographical representations (3D) of ion current intensity, retention time and compensation voltage, by simultaneous detection of both modes of operation. After pyrolysis, the bacteria-specific biomarkers were found at characteristic retention time and compensation voltage, and were confirmed with additional standards by GC-MS techniques, thereby achieving differentiation between Gram-negative and Gram-positive types [Prasad et al, 2006; Prasad et al, 2007, Prasad et al, 2008].

Finally, there were also attempts to detect *entire microorganisms* employing Ion Mobility Spectrometry. In this respect, Rodacy, Sterling and Butler (1999) tried to investigate the entire microorganisms with IMS. The experimental results have shown that it is possible to introduce whole viruses in an Ion Mobility Spectrometer (employing the electrospray method), and that a decrease in the reactant ions' peak could be observed. The lack of virus peaks may be due to a variety of effects – from the processes leading to cluster formation, their multiple loading, to the limitations due to the injection process (because of the very low virus mobility).

However, the experiments conducted by Rodacy and colleagues (1999) have shown that through electrospray, very large biological ions (e.g. viruses) may be successfully injected in the IMS spectrometer. The problem with this design is that it is not ideal to detect viral particles, since the high tension of electrospray unloading and the electrospray process itself cause huge increase in the noise level. Therefore, the authors support the need for a method to introduce the sample in vapor state [Rodacy et al, 1999].

## Summary

There are two possibilities for microorganisms' detection employing Ion Mobility Spectrometry techniques, namely: 1) detection of microorganisms with markers produced by enzymatic processes and 2) detection of microorganisms with markers produced by pyrolysis [Snyder et al, 2004; Snyder et al, 2005].

For microorganisms' detection with markers produced by enzymatic processes, there are also two alternatives: 1) employing a growth substrate, to which a certain nutrient is intentionally added - which is metabolized to produce a chemical that is known and detectable with the employed device (e.g. ortho-nitrophenyl- $\beta$ -D-glucopyranoside will generate ortho-nitrophenol, while urea will generate ammonia), or 2) volatile organic compounds generated in the headspace atmosphere may be directly monitored.

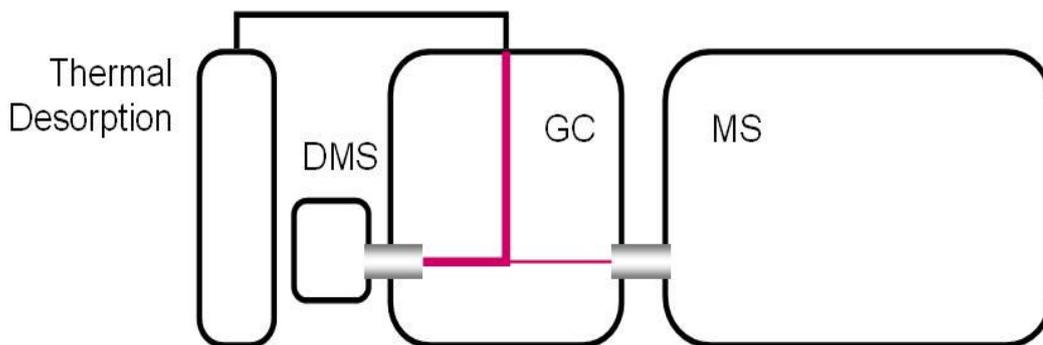
The microorganisms' detection using markers produced by pyrolysis works by the principle of quick thermal degradation which takes place before ions get separated in the Mass Spectrometer. Thus, pyrolysis may be employed to classify or identify bacteria using the constituents' derivatives of the digestive enzymes or other cellular constituents.

However, there were also attempts to achieve similar results by introducing entire bacteria in the pyrolyzer, and the results were promising.

### 3. Sampling and analysis, data processing and interpretation of the results

#### Instrumentation and experimental design

Cultures of three bacterial species with relatively low pathogenic character - *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (NCTC 10073) and *Staphylococcus aureus* (NCIMB 8625) - were prepared at the Department of Chemistry, Loughborough University, United Kingdom. The specialist in biology inoculated the bacterial cultures in glass vials with a volume of 30 ml, each containing 5 ml agar growth medium. Headspace air samples with a volume of 1 L each were collected on Tenax-Carbotrap desorption tubes (Markes International™, Cardiff, UK), at different incubation times, respectively 24, 48 and 72 hours after the initial incubation. Two datasets were obtained for each of the three species of bacteria, from the analytical instruments used: gas chromatograph coupled to mass spectrometer and to differential mobility spectrometer (GC/MS - DMS) (Figure 5, and an ion mobility spectrometer with transversal electric field (Environics IMS) (Figure 6) - from which resulted the second dataset.



**Figure 5. Conceptual diagram of the TD/GC/MS+DMS (Gas Chromatograph coupled to Mass Spectrometer and Differential Mobility Spectrometer).**

In both approaches, both for the samples analyzed with TD - GC/MS - DMS and for the samples analyzed with the Envionics Ion Mobility Spectrometer IMS, the same samples (cultures of bacteria) were employed; the samples analyzed with GC / MS-DMS were taken in the morning, and direct analysis with Envionics IMS was performed after approx. 8 hours.

This thesis will focus on the experimental data obtained employing Ion Mobility Spectrometer with transversal electric field (Envionics IMS) - which represents, in fact, the objective of this research project - while the data from TD / GC / MS will be used as a method for

validating the first outcomes. There is little information available on the data obtained using Differential Mobility Spectrometer (DMS), a technique related to IMS, but processing and interpretation of this aspect are still ongoing.

### **Transverse IMS functionality**

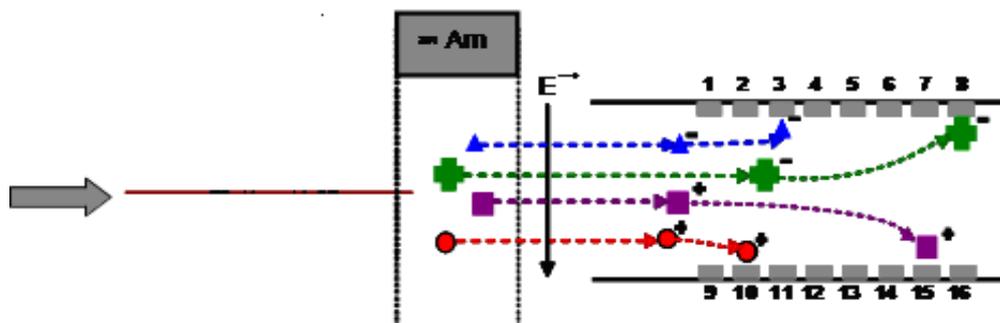
The spectrometer used in this study was a 16-channel dual polarity transverse IMS (EnviroNics Oy, Finland). A snapshot of it is shown in Figure 6.



**Figure 6. Snapshot of Ion Mobility Spectrometer with transversal electric field (EnviroNics IMS)**

The instrument is a parallel plate device with a unidirectional flow of transport gas with two arrays of eight detectors, one positive and one negative, aligned orthogonally to the inlet flow enabling the simultaneous detection of positive and negative product ions. The plates are separated by a distance of 0.5 mm. The total sensor length is 6 mm. The electric field of the spectrometer is  $5 \text{ kV m}^{-1}$ . The instrument uses a  $\alpha$ -radioactive source from the decay of  $^{241}\text{Am}$  (activity of 5.9 MBq). Ion detection works on the principle that ions of differing mobilities are deflected into different trajectories by the transverse electric field, and this results in the fractionation, by mobility, of ions into the different detector channels. Different analytes generate different profiles across the mobility channels and signal processing systems similar to those used for sensor arrays are used to assign responses to different analytes [Moll, 2011]. Data acquisition rate is fixed at 1 scan/s. The drift gas is recirculated purified air maintained at a flow rate of  $1300 \text{ cm}^3 \text{ min}^{-1}$  and 273 K. The pressure in the IMS cell is 101 kPa. Sensor temperature, pressure and flow rate is continuously monitored in the cell. [Moll, Rațiu et al, 2010; Huo, Rațiu et al, 2011; Rațiu et al, 2012].

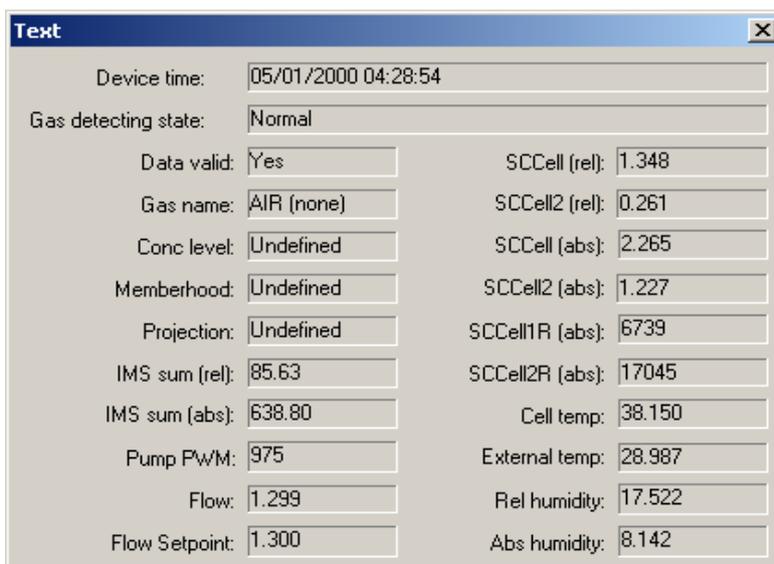
The separation principle of ChemPro100i IMS, which can be seen in Figure 7, is as follows: ambient air is pumped inside the ChemPro100i detector, molecules are ionized by radioactive ionization source and cluster ions are carried by gas flow drift along the cell and turned in IMS detectors by the transversal electric field  $E$ .



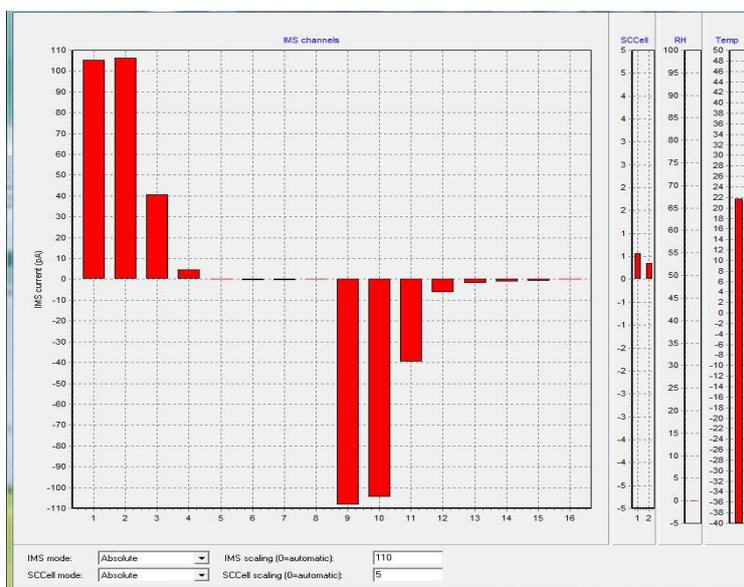
*Figure 7. Separation principle in ChemPro100i Environics IMS.*

IMS cell contains 8 pairs of electrodes (channels). Cluster ions with different mobilities, carried by the drift gas and deviated by the electric field, will kick the detectors (electrodes), so ions with greater mass will reach the last electrode, while the lower mass ions, being more easily diverted, will stop at the first electrode. Detection takes place simultaneously in both the positive and negative mode of operation. The result / IMS response is actually a distribution of ionic clusters along the cell, which is converted to ionic currents, measured by the eight positive detectors and eight negative detectors simultaneously [Moll, Rațiu et al, 2010; Räsänen et al, 2010; Rațiu et al, 2012].

Spectrometric functions (cell temperature, flow rate) and data acquisition are controlled through the accompanying software package, Chempro™, version 1.02 (Environics Oy, Finland), transmitted via a COM connection to the IMS cell. For this study, the software was run from a Dell Studio 1737 laptop. The software comprises two units: one for viewing the cell parameters, such as pressure, temperature and humidity, and the other representing the detector channel responses. Screenshots for these sections are shown in Figures 8 and 9. Data is recorded by default in .txt format, which is converted to Microsoft® Excel .XLS file type. The processing of all data in this study was carried out in Excel 2003 [Moll, Rațiu et al, 2010; Rațiu et al, 2012].



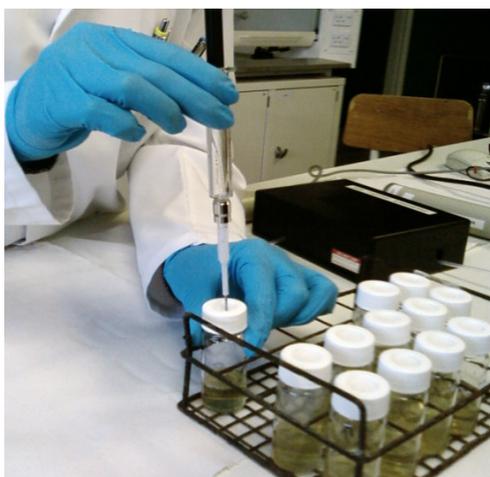
**Figure 8. Screenshot of the window in the ChemPro100 software showing the physical and electrical parameters in the transverse ion mobility cell**



**Figure 9. Screenshot of the detector responses from the ChemPro100 software. The observed response patterns relate to water-based reactant ion chemistry arising from the IMS transport gas operating at  $1300 \text{ cm}^3 \text{ min}^{-1}$  through the IMS cell.**

## Samples analyzing using ChemPro100i IMS

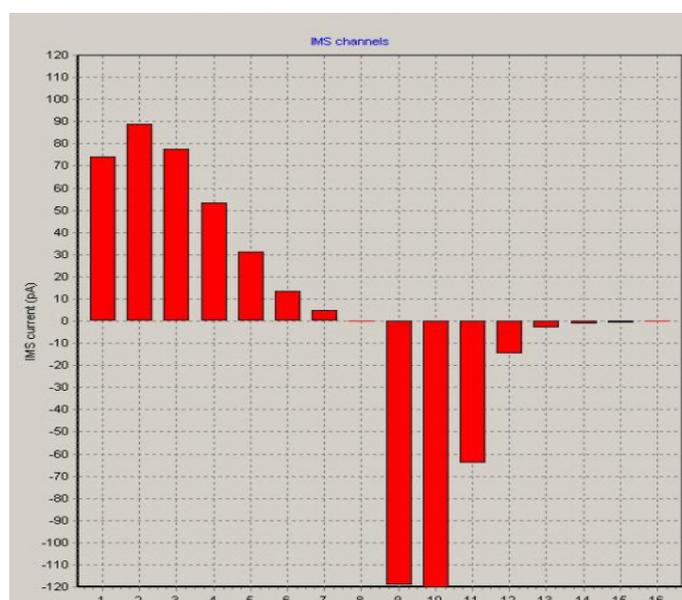
Using a 5 ml glass syringe for gases and PTFE piston, through the rubber septum cap, air samples were taken from the atmosphere of each vial headspace (Figure 10). Samples taken were immediately injected into the device, at a distance of about 1 cm of IMS cell (Figure 11). The answer can be observed after about 1 second from the sample injection (Figure 12).



**Figure 10. Headspace sampling procedure**



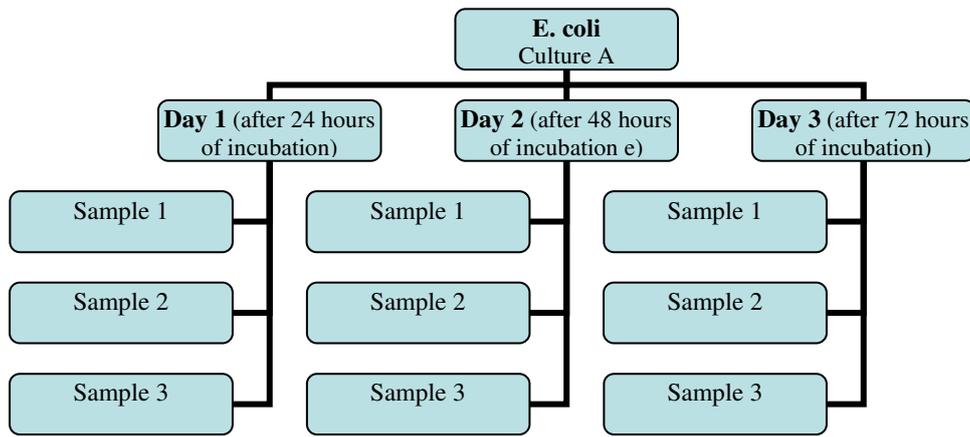
**Figure 11. Injection of samples in ChemPro100i IMS device**



**Figure 12. A transverse IMS spectral profile of a headspace air sample from *Escherichia coli*. Responses in channels 1-2 correspond to the reactant ion peak (RIP) in the positive mode,  $[H_2O]_n^+$ , and channels 9-10, the RIP in the negative mode,  $[O_2]$ .**

Samples analyzed using IMS Environics were taken from three strains of bacteria: *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. For each species were prepared 10 cultures of bacteria, from which samples were taken in triplicate at three incubation times, (after 24, 48 and 72 hours) following the model shown in Figure 13.

Thus, for each monitored species were collected and analyzed samples for three days, reaching therefore a total of 90 samples for each of the three species, and finally to reach a total of 540 samples headspace analysis (270 samples containing three species of bacteria incubated and 270 blank samples, which were inoculated culture medium only).

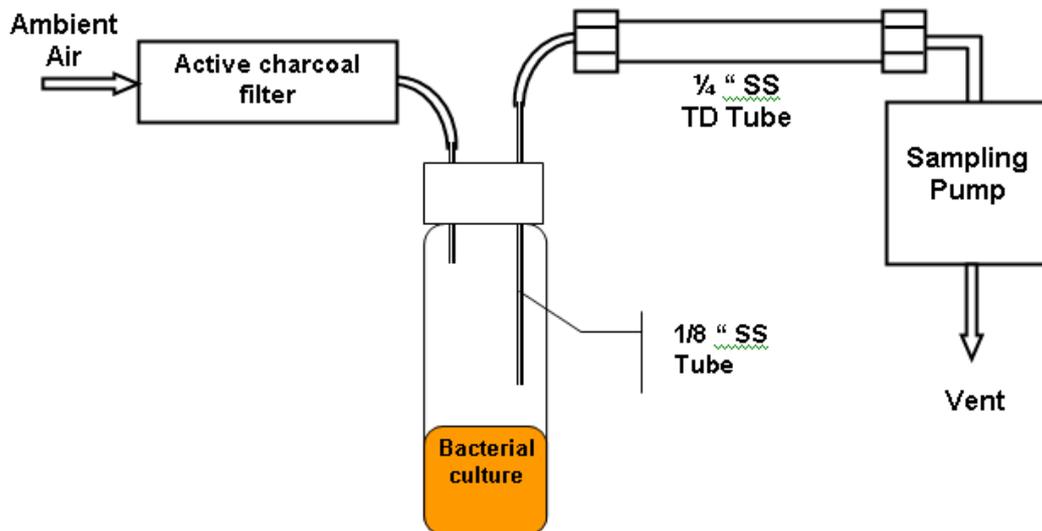


**Figure 13. Schematic of headspace air sampling analysed with Environics IMS, for an individual sample with Escherichia coli specie.**

### **The TD / GC - MS system (thermodesorber / gas chromatograph / mass spectrometer)**

A total number of 90 headspace air samples from bacterial cultures (30 from each species) were processed by TD-GC-MS, together with 30 blanks.

The sampling system was a custom built sampling unit based upon a portable air sampling pump. A schematic diagram of the sampling system is given in Figure 14.



**Figure 14. Sampling system for headspace air above the bacterial cultures [Rațiu. et al, 2011].**

A glass vial with a volume of 30 cm<sup>3</sup> (plastic cap with silicone septum), containing bacteria incubated in growth medium, was connected through a 100 cm<sup>3</sup> charcoal filter (Agilent

Technologies, CA, USA) to the ambient air, then with a trap containing adsorbent material (Tenax™ TA 35-60 mesh and Carbotrap™ 20-40 mesh). The adsorbent trap was manufactured by Markes International, type C2-AXXX-5032 Tube, Stainless Steel, 1/4" i.d., length 9 cm. Using a portable pump model MSA ESCORT ELF (Mine Safety Appliances, Inc., USA), a total volume of 1 L of air, obtained by sampling during 2 minutes with a gas flow of 0.5 L min<sup>-1</sup>, was passed through the trap. This method achieved dynamic headspace sampling of the chemicals associated with a bacterial strain [Rațiu. et al, 2011]

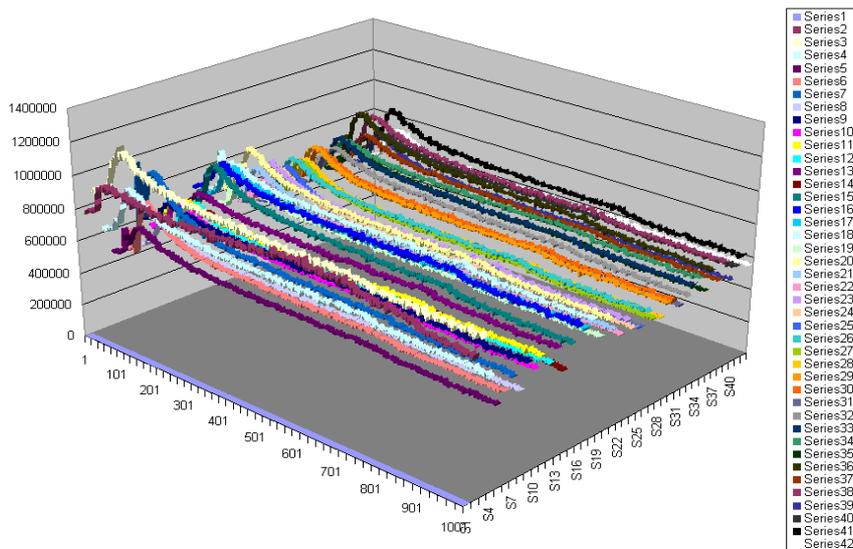
The samples were stored in a refrigerator at 4°C for maximum 72 hours, and then analyzed using the hyphenated TD-GC-MS instrumentation.

The TD-GC-MS system incorporates a double-stage thermal desorption unit (manufactured by Markes International, UK), coupled to a Varian 3800 gas chromatograph equipped with a Varian 4000 ion trap mass spectrometry detector. Table 1 summarizes the instrumental operating parameters that were employed.

**Table 1. Summary of experimental parameters**

<b>Markes Double Stage TD:</b>	<b>Varian-3800 GC:</b>	<b>Varian-4000 Ion Trap MS:</b>
Primary desorption flow: 50 cm <sup>3</sup> min <sup>-1</sup>	Column: 30 m × 0.25 mm × 0.25 μm DB-5	Scan type: Full
Primary desorption temperature: 280°C	Carrier gas flow: He @ 2.0 cm <sup>3</sup> min <sup>-1</sup>	Mass range: 40 to 445 Da
Primary desorption time: 5 min	Initial oven temperature: 40°C	Tune type: Auto
Cold trap volume: 0.019 cm <sup>3</sup>	Initial hold time: 0 min	Ionization type: EI
Cold trap temperature: -10°C	Oven temperature program: 3.3°C min <sup>-1</sup> to 90°C	Target TIC: 20000 counts
Cold trap packing: U-T2GPH (General purpose hydrophobic)	2.5°C min <sup>-1</sup> to 140°C	Max ion time 25000 μs
Secondary desorption flow: 2 cm <sup>3</sup> min <sup>-1</sup>	10°C min <sup>-1</sup> to 300°C - hold for 8.85 min	Emission current: 10 μA
Secondary desorption temperature: 300°C	Total run time: 60 min	Total run time: 60 min
Secondary desorption time: 5 min		Scan time: 0.82 s
Trap heating rate: 100°C min <sup>-1</sup>		Transfer line temperature: 270°C
Transfer line temperature: 140°C		Trap temperature: 150°C
		Manifold temperature: 50°C

Before running each sample, a cleaning method (blank trap) which consisted of heating up to 310°C and purging with helium through the GC capillary column, was carried out in order to avoid memory effects from previous sampling cycles. Cleaning was considered adequate if the intensity of the total ion current remained constant between the same limits during the whole analysis process and if the operating conditions of the GC-MS instrument were unchanged. The intensity of the total ion current for the trap blank remained within the above mentioned limits over the measurements campaign of 42 days (Figure 15) [Turner, 2009; Rațiu. et al, 2011].



**Figure 15.** A 3D representation of the response resulting from the method of cleaning performance "blank trap." It may be noted that the representation of the baseline, total ion current signal, (TIC) for 10 minutes (duration of the "trap blank" sequence) for a period of 42 days, during which the total ion current signal intensity remained constant between 400 V and 800 V [Rațiu. et al, 2011]

### Primary Retention Index

At the beginning of each day and after the consecutive analysis of five samples, a "retention index" mixture was analyzed in order to determine the proper functioning of the TD-GC-MS chain.

A primary retention index ladder was generated using a mixture containing 17 known chemicals, which produced peaks that remained in the same position (retention time) in all chromatograms. A modified version of Kovats retention index  $I$  equation, which allows for temperature programming of the gas chromatography system, was used (Equation 1).

$$I = \frac{t_{R(Unknown)} - t_{R\mu}}{t_{RN} - t_{R\mu}} \cdot 100 \cdot z \cdot \mu \quad (\text{Eq. 1})$$

, where  $t_{R(Unknown)}$  is the retention time of the compound of interest [min],  $t_{R\mu}$  is the retention time of the previous lower molecular weight component [min],  $t_{RN}$  is the retention time of the next higher molecular weight component [min],  $z$  is the difference in C atom number, and  $\mu$  is the number of C atoms of the lower M known component [Turner M.A. , 2009; Rațiu I.A. et al, 2011].

Using the known straight chain hydrocarbons from the retention index standard mixture, the values for retention index RI were assigned based on the number of C atoms for each component. The values assigned to each compound were then plotted against their respective retention times to produce a linear RI ladder. The equation obtained from the trend line produced was then used to

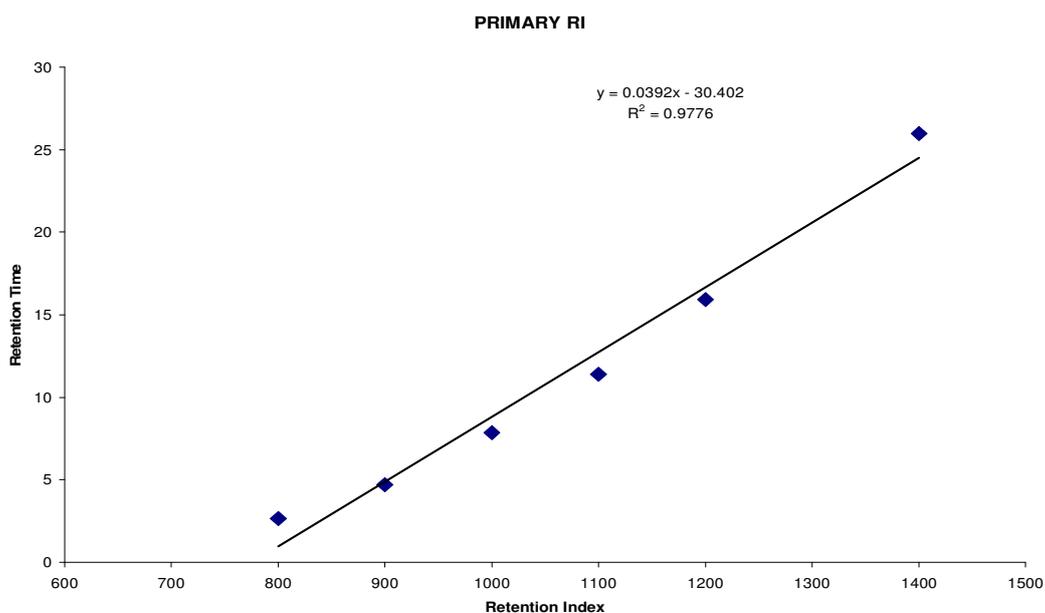
assign retention index values to the known components that were present in all sample chromatograms, forming this way a secondary RI. The secondary RI was then used to align all sample data.

Retention Index assignment was achieved by designating a RI value to each of the straight chain hydrocarbon components in the RI standard mixture. The RI for each of the 6 hydrocarbons is based on the carbon number of the component. The assignments used in this method to align data are given in Table 2 [Turner, 2009; Rațiu et al, 2011].

**Table 2. Component list of straight chain hydrocarbons identified peaks in the retention index standard**

<i>Compound</i>	<i>Retention time (RT) [min<sup>-1</sup>]</i>	<i>Retention Index (RI)</i>
Octane	2.647211	800
Nonane	4.708737	900
Decane	7.861105	1000
Undecane	11.41037	1100
Dodecane	15.92647	1200
Tetradecane	25.97974	1400

A plot of retention times against assigned RI values for octane, nonane, decane, undecane, dodecane and tetradecane for these studies is shown in Figure 16. The intercept (C= -30.402) and gradient (M= 0.0392) of the trend line generated were obtained.

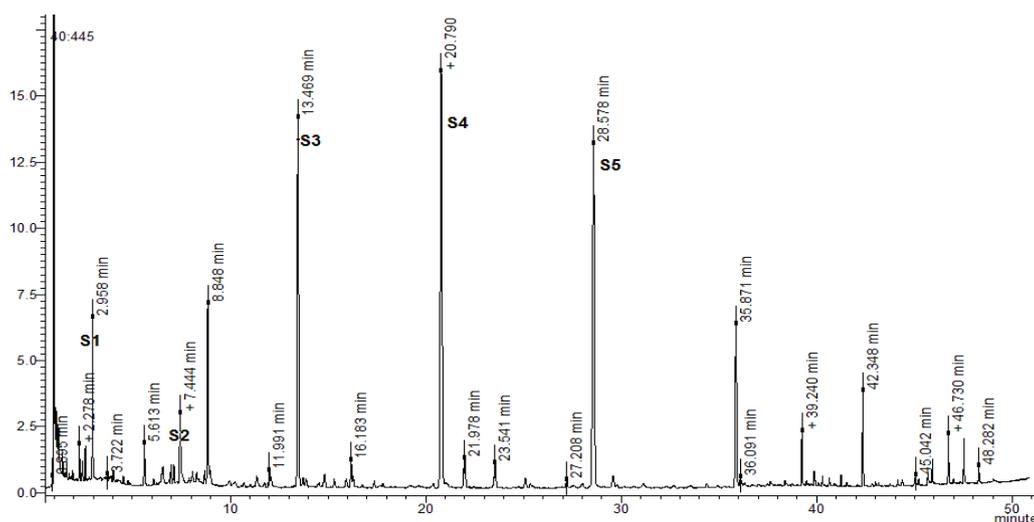


**Figure 16. Plot of the retention times and the assigned RI values** [Rațiu. et al, 2011]

## Secondary Retention Index

It is not always practical to add numerous RI compounds to a sample at point of collection for the purpose of aligning samples and to aid analyte prospecting. In such instances a secondary retention index can be employed. Under the analysis conditions, a number of siloxane components that result from the interactions of moisture found in headspace samples and in the thermal desorption tube were selected. These siloxane components are continually present in all sample chromatograms and have been selected to build a secondary RI [Turner , 2009; Rațiu. et al, 2011].

Using the headspace sample chromatograms, the five selected siloxane components were identified and their retention times recorded. The retention times from the identified siloxane components were then used to calculate the RI value for each of the siloxane compounds (Table 3). Figure 17 indicates an example of 5 siloxane peaks (S1 to S5) observed in all the headspace samples.

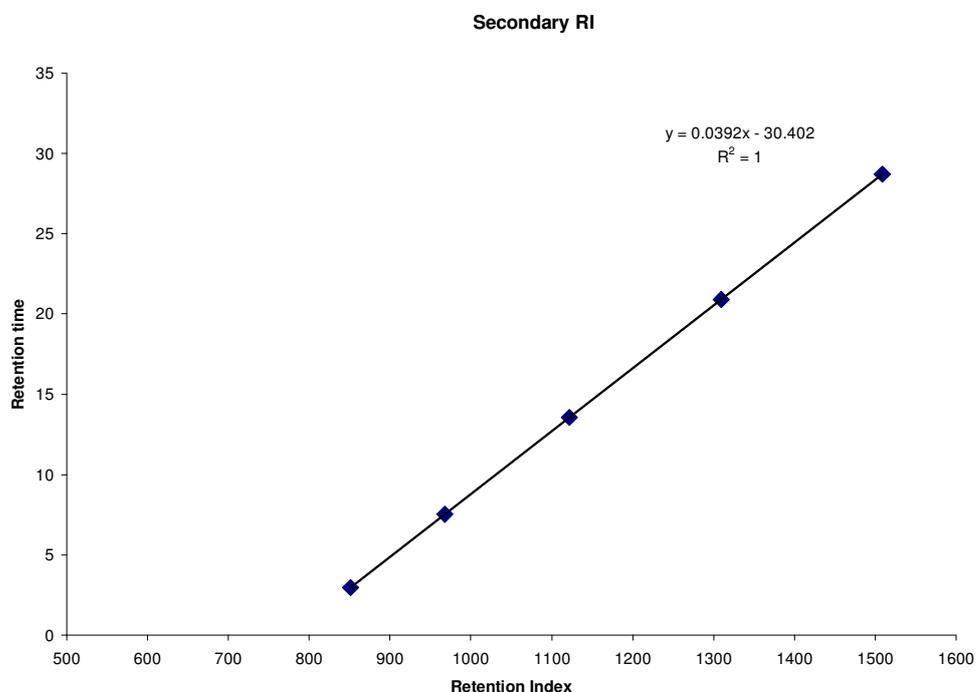


**Figure 17. Example of headspace sample chromatogram with endogenous siloxane peaks (S1 to S5) highlighted [Turner, 2009]**

**Table 3. Static RI values and approximate retention times for siloxane peaks used in secondary retention index**

Siloxane compound	Retention time (RT) (minutes)	Retention Index (RI)
S <sub>1</sub>	2.9552	850.949
S <sub>2</sub>	7.5181	967.3495
S <sub>3</sub>	13.5745	1121.849
S <sub>4</sub>	20.9233	1309.319
S <sub>5</sub>	28.733	1508.546

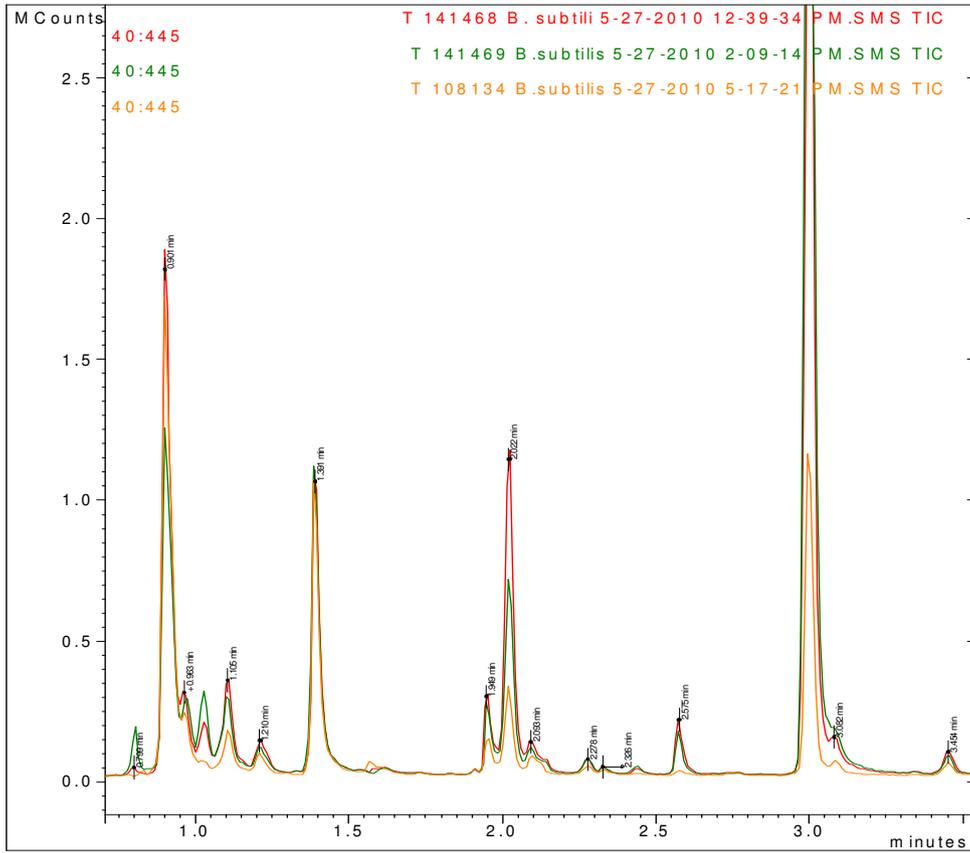
Using the headspace samples chromatograms, five siloxane compounds were identified and selected (Figure 17) whose retention time was "watching" in all the samples. Equivalent siloxane peak values (Table 3) was used for building the "Secondary Retention Index ". Secondary Retention Index graph can be seen in Figure 18.



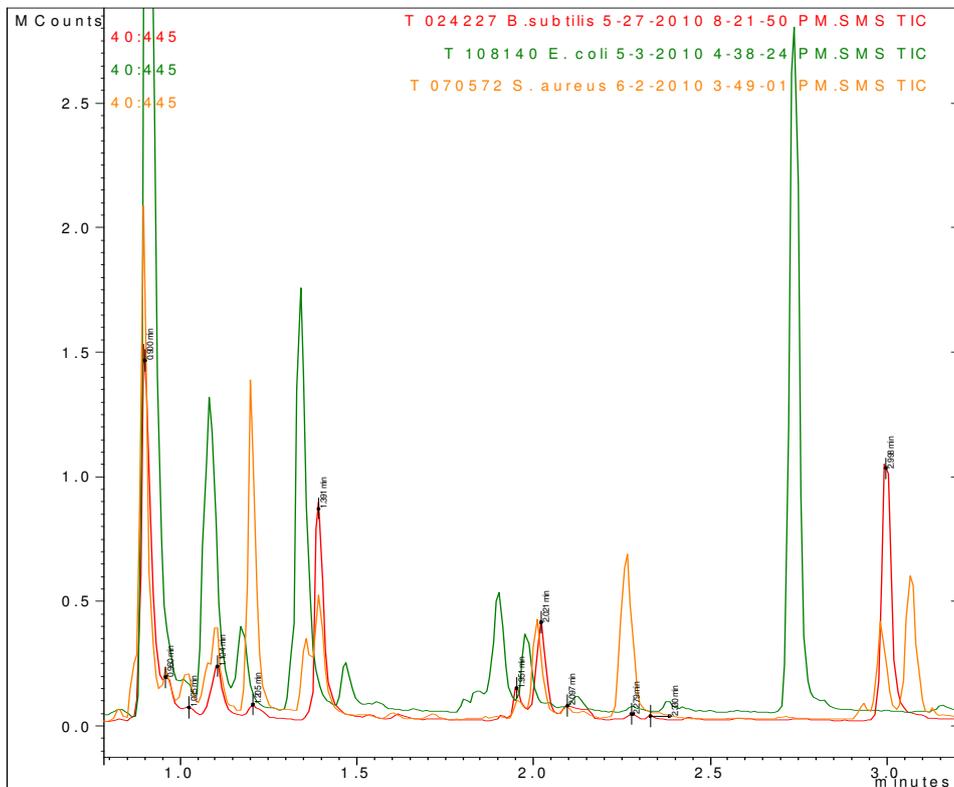
**Figure 18. Plot of the secondary retention times and the assigned RI siloxane peaks values.**  
[Rațiu et al, 2011]

### Creating compounds libraries

Libraries of compounds for headspace samples were created with the aim of summing up compounds found in the samples and, also, of checking for any differences found in samples with different species of bacteria, or samples incubated after different incubation times (different days). Illustrative examples could be considered as those in Figures 19 and 20, where (i) similarities for different tubes with samples taken after the same incubation time, from the same species of bacteria and, respectively, (ii) differences between samples where different species of bacteria were incubated (*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*) have been observed [Rațiu et al, 2011].



**Figure 19. GC-MS Chromatograms of *Bacillus subtilis* species resulting from samples taken after 72 hours from incubation. In the first 3 minutes, samples from the same cultures display similar profiles.**



**Figure 20. Chromatograms of *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*, after 72 hours from incubation. After the first 3 minutes, samples from different species of bacteria present different profiles**

## Results and discussions

Assuming that *there is no difference* between our samples, PCA was applied to check:

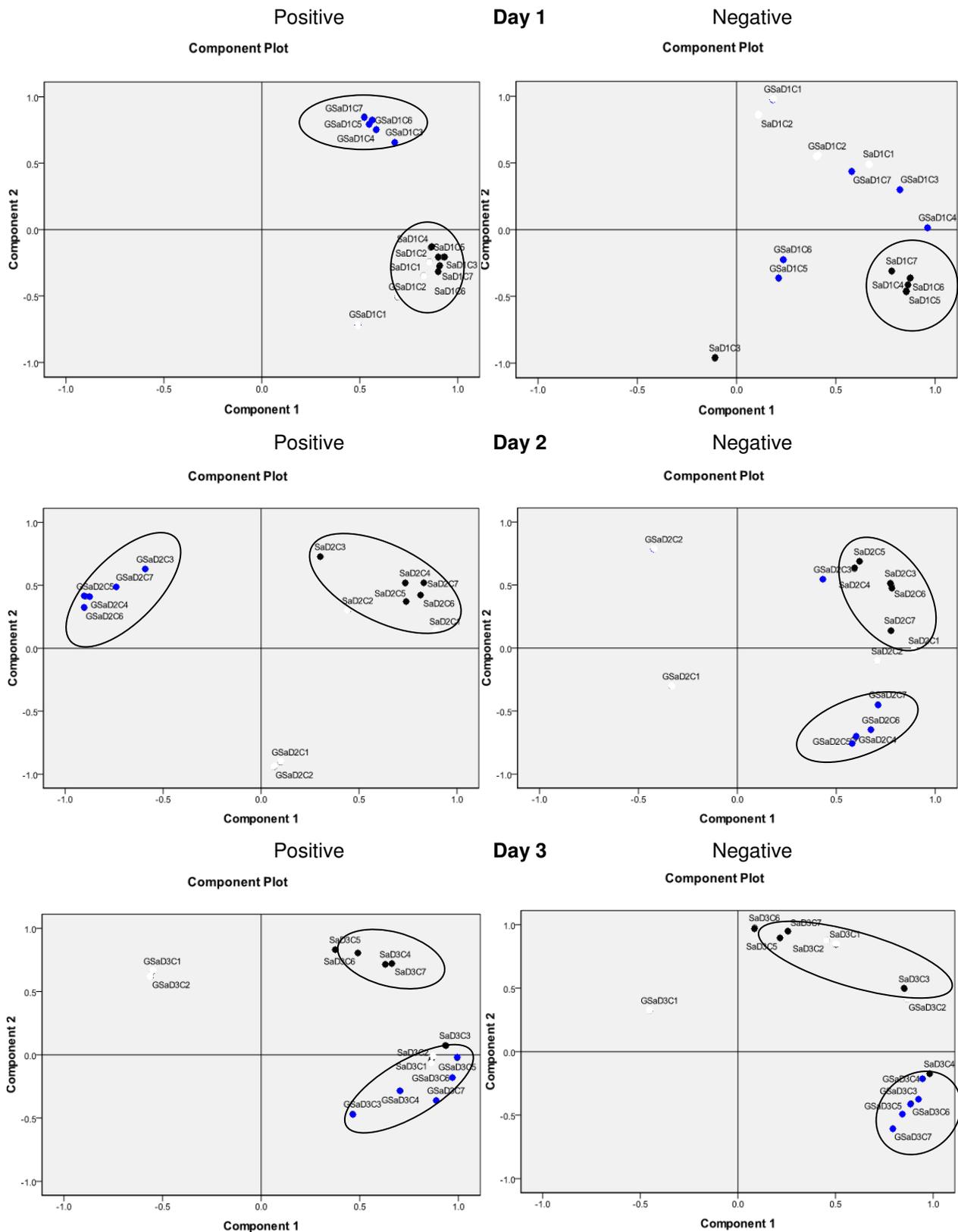
- if we have differences between samples where bacteria and blanks (only culture medium) were incubated;
- if ChemPro100i IMS senses any differences between different species of bacteria taken after the same incubation time;
- if there are differences between the samples where the same species were inoculated, but taken at different incubation times;
- if each channel / detector individually analyzed presents a distinct profile.

### Differentiation between bacteria samples and blank samples

The crosshairs that delimitate the four quadrants divide the plots from the chart into positive and negative charge. We have four quadrants (upper left quadrant - called "Quadrant I", upper right quadrant - called "Quadrant II", lower right quadrant – called "Quadrant III" and lower left quadrant - "Quadrant IV"). The crosshairs are set at 0 on both PC1 ("principal component 1") and PC2. So, the four quadrants represent positive and / or negative charge, for both PC1 and PC2. Practically, quadrant I (QI) has positive charge in PC2 and negative in PC1, quadrant II (QII) has a positive character for both PC1 and PC2, quadrant III (QIII) is positive for PC1 and negative for PC2 and finally, quadrant IV (QIV) is negative for both PC1 and PC2.

Channels C1 and C2 - corresponding mainly to the signal of reactant ions' peak that, as expected, does not show significant responses - were grouped separately from the other channels (C3 - C7), and C8 - that is used only for checking some conformity parameters, so it does not show any visible response ("0" is displayed on channel 8) - was excluded from the chart points "Component Plot". However, to highlight regularly the existence of channels C1 and C2 in the chart points, but mostly since they show a visible response (decrease in signal intensity while the others C3 - C7 increase); these channels were not removed from the chart "Component Plot", but were marked in white.

Following the six graphs in Figure 21, a differentiation between samples containing incubated the *Staphylococcus aureus* species and blank samples could be observed; thus, it was possible to notice that the samples with bacteria occur separately, clustered, from those that had only growth medium incubated.



**Figure 21. PCA on the Environic responses for *Staphylococcus aureus* & Growth medium during three days of incubation in the positive mode and negative mode. Each point represents the response associated with an individual detector/channel from 10 biological replicates, each sampled in triplicate, where: Sa - *Staphylococcus aureus* Gm – growth medium; D1 – Day 1; D2 – Day 2; D3 - Day 3; C3...C7 – Channels / detectors. [Rațiu et al, 2012]**

The positive mode shows partition both for samples with bacteria and for samples with growth medium. Therefore, by analyzing the point charts where positive ions were detected, cluster ions derived from samples with bacteria could be observed, initially in QIII, then - after 48 hours of incubation – they will move to QII where they will remain throughout the monitored period of time.

Meanwhile, clusters derived from samples containing culture medium only, moved from QII, where they originally occurred (after 24 hours of incubation) to QI (after 48 hours of incubation), and then to QIII (after 72 hours of incubation).

In the negative mode of operation a differentiation between samples with bacteria and samples with growth medium could be observed. The points corresponding to cluster ions derived from the *Staphylococcus aureus* bacteria occurred initially (after 24 hours of incubation) in QIII, after 48 hours of incubation they moved to QII, where they were observed also in the third day, with the exception of C4, which returned to QIII.

Clusters derived from the growth medium show a chaotic arrangement after 24 hours of incubation, appearing divided between QII (C3, C4, C7) and QIII (C5, C6), but then, after 48 hours, they could be observed in QIII (except for C3, that remains in QII), and still there, after 72 hours of incubation.

Results similar to the example above were observed by applying SPSS to the samples with *Escherichia coli* and *Bacillus subtilis* inoculated. Given the facts mentioned above, we considered the discrimination between samples incubated with one of bacteria monitored species (*Escherichia coli*, *Bacillus subtilis* or *Staphylococcus aureus*) and the blank samples, by employing an Ion Mobility Spectrometer from Environics as being feasible.

### **Discrimination between samples containing different bacterial species (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*)**

After applying the statistical test "Principal Components Analysis" (PCA) to the samples analyzed employing an Ion Mobility Spectrometer with transverse electric field (Environics IMS) we found that the respective device could discriminate between all three species of bacteria (*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*):

- after three days since incubation started, if we follow the negative mode;
- after two days, if we consider the clusters detected in positive mode.

On the other hand, watching all six graphs of the points in Figure 22 we could observe that *Staphylococcus aureus* has remained separate from the other two *Escherichia coli* and *Bacillus subtilis*, even after the first 24 hours of incubation.

Other relevant issues that will be highlighted here are:

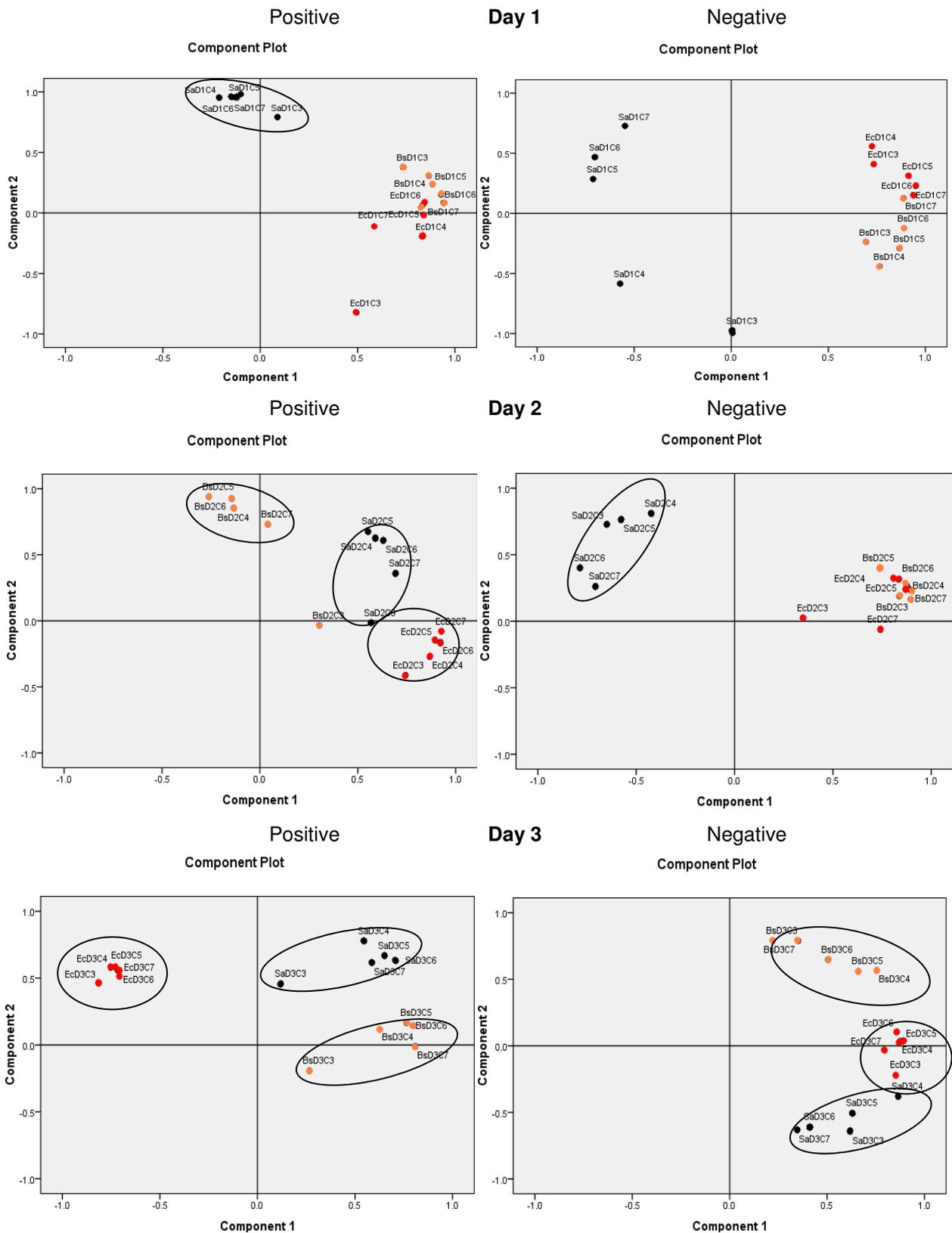
- samples with *Staphylococcus aureus* inoculated have a fairly extensive and constant separation from all three incubation times for both positive and negative modes;
- *Escherichia coli* displays (after 48 hours in the positive mode and only after 72 hours in the negative mode) a clear separation and a better grouping over time passing;
- *Bacillus subtilis* samples cluster separately from the other two after 48 hours in the positive mode and after 72 hours in the negative mood, showing a relatively constant group.

Channels 1 and 2 - corresponding to the signal of reactant ions - showed no significant responses, as expected, and noted also in the previous cases. To avoid overcrowding of graphs, C1 and C2 were removed from the chart points.

More specifically, by separately analyzing the three cases (three incubation times) we found that:

- After the first day (after 24 hours of incubation):
  - In the positive mode the grouping of *Staphylococcus aureus* species in QI has been observed and, apart from this, the other two (*Escherichia coli* and *Bacillus subtilis*) were assigned between QII and QIII;
  - In the negative mode, samples with *Staphylococcus aureus* were distributed between QI and QIV, but their clustering as a group could not be observed, although they remained separately from the other two (*Escherichia coli* and *Bacillus subtilis*) that were distributed parallel to the first ones, between QII and QIII.

In addition, we could say that although a clear grouping of the three species could not be observed at this stage (after 24 hours of incubation), the samples with *Staphylococcus aureus* remained isolated from those with *Escherichia coli* and *Bacillus subtilis*, being separated by PC1.

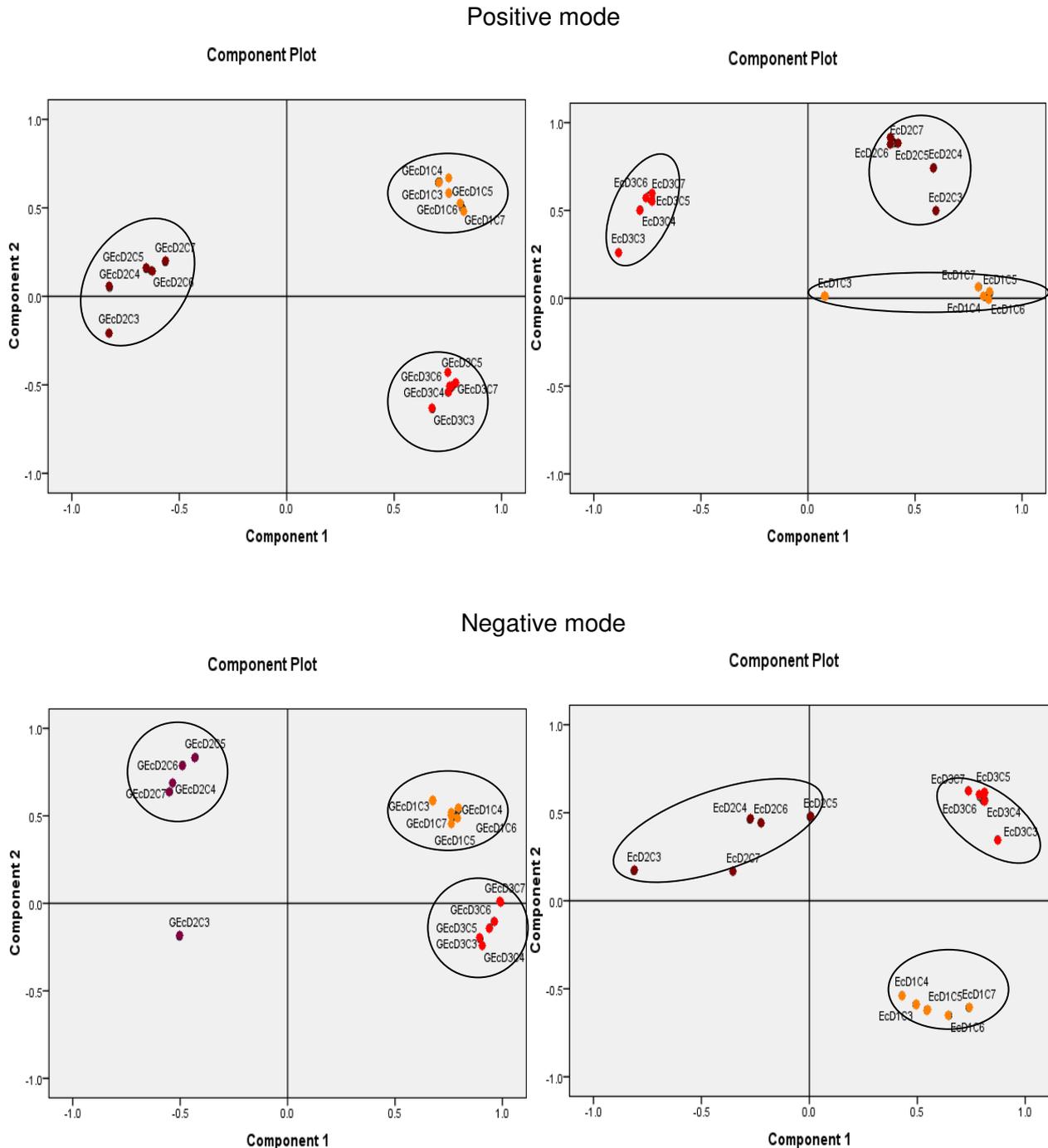


**Figure 22. PCA on the Environics responses for *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* during three days of incubation in the positive mode and negative mode. Each point represents the response associated with an individual detector/channel from 10 biological replicates, each sampled in triplicate, where: Ec - *Escherichia coli*, Bs - *Bacillus subtilis* Sa - *Staphylococcus aureus*; D1 – Day 1; D2 – Day 2; D3 - Day 3; C3...C7 – Channels / detectors. [Rațiu. et al, 2012]**

- After the second day (after 48 hours of incubation):
  - in the positive mode we could notice a difference between all three monitored species, that were distributed as follows: *Bacillus subtilis* occurred in QI, thus presenting positive charge in PC2 and negative in PC1, *Staphylococcus aureus* occurred in QII, being positively charged for both PC1 and PC2, while *Escherichia coli* occurred in QIII, presenting positive charge for PC1 and negative for PC2.
  - in the negative mode, grouping of the species *Staphylococcus aureus* as a cluster was observed in QI and separately, *Escherichia coli* and *Bacillus subtilis* species grouped in QII.
  
- After the third day (after 72 hours of incubation):
  - in the positive mode, separate grouping of samples from all three species of bacteria was observed, i.e. the samples with *Escherichia coli* species were found in QI, those with *Staphylococcus aureus* in QII, while samples with *Bacillus subtilis* were also grouped separately from the other two, standing at the boundary between QII and QIII (mainly the QII).
  - in the negative mode, clusters coming from all three species of bacteria studied were observed to be separately grouped. Thus, the points corresponding to the *Bacillus subtilis* were grouped into QII, showing net positive charge, those with *Escherichia coli* were located between QII and QIII, while the clusters derived from *Staphylococcus aureus* were grouped in QIII, being positively charged for PC1 and negatively for PC2.

As a conclusion, we can say that using SPSS statistical software, i.e. applying the "Principal Components Analysis" (PCA) to the samples analyzed with Ion Mobility Spectrometer, ChemPro100i, it was found that the device can perceive differences between all three species of bacteria (*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*) after three days of the beginning of incubation - in the negative mode of operation, and after two days - if we consider the positive mode of operation. On the other hand, studying all six graphs of points in Figure 22 we could see that *Staphylococcus aureus* has remained separately from the other two (*Escherichia coli* and *Bacillus subtilis*), even after the first 24 hours of incubation.

## Differentiation of the analysed bacteria depending on incubation time



**Figure 23. PCA on the Environics responses for Escherichia coli, comparatively with growth media from three different incubations time in the positive mode and negative mode. Each point represents the response associated with an individual detector/channel from 10 biological replicates, each sampled in triplicate, where: Ec - Escherichia coli, Gm – growth medium; D1 – Day 1; D2 – Day 2; D3 - Day 3; C3...C7 – Channels / detectors. [Rațiu et al, 2012]**

By applying the statistical method "Principal Components Analysis" (PCA) - for samples taken after 24, 48 and 72 hours since incubation, samples inoculated with *Escherichia coli* species, and analyzed employing an Ion Mobility Spectrometer Environics IMS - differences between culture media taken at different times, and between all three days when samples with *Escherichia coli* were monitored were obtained (Figure 23).

Detectors (channels) C1 and C2, corresponding to the signal of reactant ions do not provide significant responses, which we consider normal, and thus, they were removed from the chart points.

A more detailed assessment of the relevant chart points corresponding to the samples with *Escherichia coli* species allows us to conclude the following:

- in the positive mode, the samples with *Escherichia coli* species were grouped separately, according to the three incubation times of sampling. More specifically, the corresponding points of the third day occurred in the first quadrant (QI) of the chart points, those of the second day were observed in QII, while clusters corresponding to samples taken in the first day were actually located on the line between QII and QIII.
- in the negative mode, the same as for positive mode, a differentiation between samples taken at different incubation times was observed. Therefore, the points corresponding to the samples collected after 48 hours of incubation occurred in QI, the clusters derived from samples taken after 72 hours of incubation were grouped in QII, while the points corresponding to samples taken in the first day occurred in QIII.

Regarding the blank samples, the points corresponding to the samples taken after different incubation times remained separately from each other and were grouped similarly for both the positive and the negative mode.

In the following paragraphs, final conclusions regarding the differentiation according to incubation time of the three monitored bacteria species (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*), and of the corresponding blank samples will be exposed:

- the device employed (ChemPro100i IMS) senses differences between growth media (blank samples) of all the three species of bacteria taken at different incubation times for both positive and negative mode;

- Analyzing the *Bacillus subtilis* species:
  - in the positive mode, there was differentiation between clusters derived from samples taken in the third day, that appeared separately grouped from the clusters resulted from the samples taken in the first and in the second days.
  - in the negative mode, it was observed that the points corresponding to samples taken on the first day were in a group, and separately, that the points from day one and day two were grouped together, without differences between them.
  
- Analyzing the *Staphylococcus aureus* species:
  - in the positive mode, we could differentiate the samples from the first day and the samples from the other two days that occurred, but grouped together.
  - in the negative mode of operation, statistical tests revealed discrimination only between samples collected in the second day, that occurred separately grouped from those taken in the first and in the third day.
  
- Analyzing the *Escherichia coli* species:
  - in both positive and negative operating modes, differences between the points corresponding to the different incubation times and occurring separately from each other were observed.
  
- C1 and C2 channels / detectors, corresponding mainly to the signal of reactant ions showed no significant responses, as actually expected, therefore these points were removed from the graphs, to avoid extra agglomeration.

On other hand, we could finally conclude that employing the SPSS software, more specifically by applying the "Principal Components Analysis" (PCA) to the samples analyzed with an Ion Mobility Spectrometer Environics IMS it was possible to remark differences between blank samples taken at different incubation times (between samples collected after 24, 48 and 72 hours of incubation). The device also sensed discrimination between all three days (three times of incubation) when the samples with *Escherichia coli* species were analyzed, while for the samples with the other two species monitored, *Bacillus subtilis* and *Staphylococcus aureus* there was evidenced a clear discrimination only between two of the three days.

## Comparative evaluation of *specific* and *common* chemical compounds of the three monitored bacterial species

Using the data obtained from GC-MS analysis (gas chromatography coupled with mass spectrometer) and processed employing the Pro Analyzer software and database NIST (*National Institute for Science and Technology*) as a method of confirmation/validation, we have identified a large number of chemical compounds. The profile of the observed headspace air samples - which was very complex - showed the presence of the same chemicals in all the three days of monitoring but also showed the occurrence of various chemicals in one or two of the sampling days, however, most often we met chemicals identical for samples taken in similar conditions (same bacterial samples inoculated, identical incubation time).

Therefore, we have identified four chemicals characteristic to *Bacillus subtilis* bacteria and two chemical compounds specific to *Escherichia coli* and *Staphylococcus aureus* species. At the same time, we found compounds common to all of the three monitored species (such as dimethyl disulfide - found in all the analyzed samples, but was not found in the blank samples) and chemicals common for two of the three species monitored (e.g. trichloromethane - common for the samples inoculated with *Escherichia coli* and *Staphylococcus aureus* species, while toluene was found in both samples where *Bacillus subtilis* and *Staphylococcus aureus* bacteria were incubated) [Rațiu et al, 2011].

**Table 4. Chemicals characteristic for each monitored species - *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* – obtained employing GC-MS data, AnalyzerPro software and NIST spectral library**

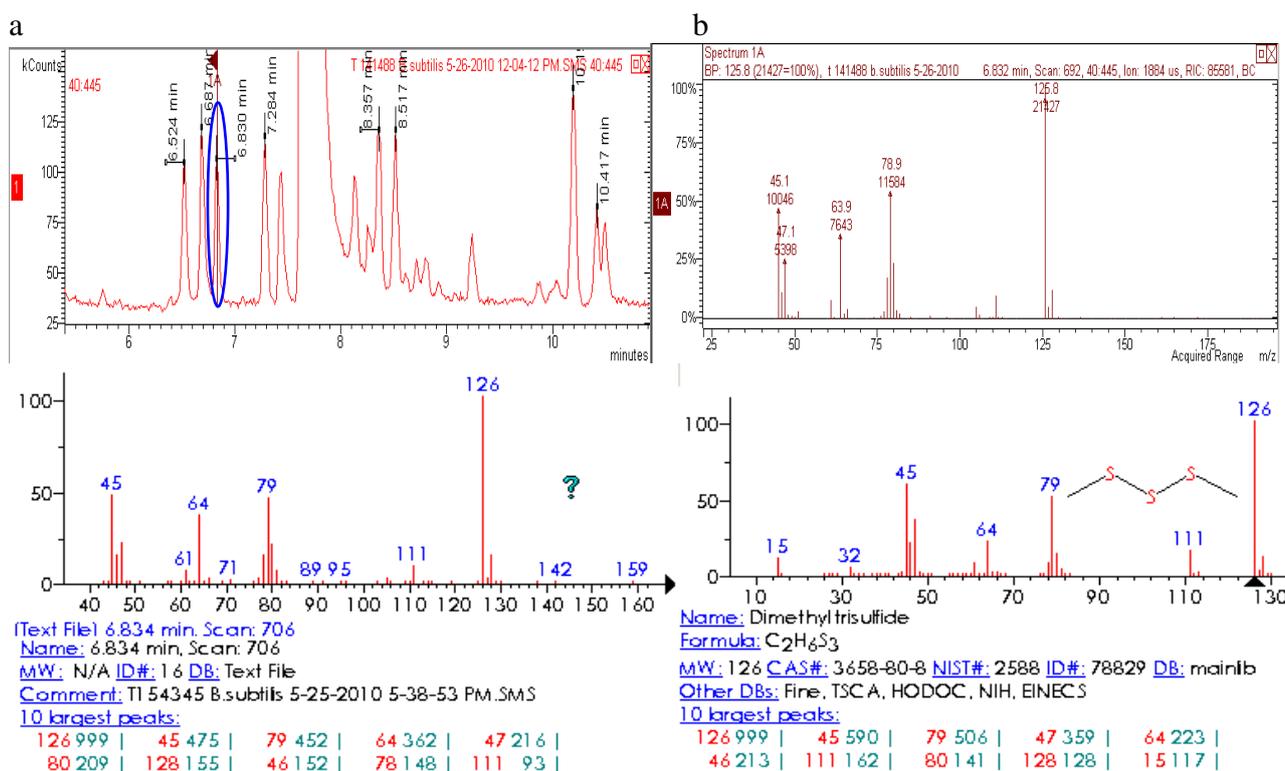
Compounds characteristic only for <i>Bacillus subtilis</i>		Compounds characteristic only for <i>Escherichia coli</i>		Compounds characteristic only for <i>Staphylococcus aureus</i>	
Chemical	RI	Chemical	RI	Chemical	RI
4-Pentene-2-ol, 2-methyl	799	Guanidine	799	Propanoic acid, 2-hydroxy-2-methyl-, methyl ester	800
Heptane, 3-ethyl-5-methyl-	825	Citrazinic triTMS	1122	Acetamidoacetaldehyde	804
Phenylglyoxal	942				
Dimethyl trisulfide	946				

We considered the chemicals found in all three days of monitoring at the same retention time as being characteristic compounds of each of the three species. They were listed in Table 4 and in Figures 24, 25, 26 (showing examples of substances considered specific to each bacterial species

monitored) where the mass spectrum of each chemical compound found in the headspace samples associated with a substance is presented in comparison with the mass spectrum of the substance found in the database (NIST). Siloxane compounds - present in the samples - occur naturally in all samples collected in Tenax - Carbotrap tubes type, since they result from the process of tubes desorption. These compounds were not considered as compounds specific to any bacteria.

Looking at Table 4 we can remark the following aspects:

- 4-Penten-2-ol, 2-Methyl Heptane 3-ethyl-5-methylene-, Phenylglyoxal, dimethyl trisulphide compounds are considered characteristic of the *Bacillus subtilis* species. Their retention indices can be observed in Table 4.
- Citrazinic triTMS and Guanidine were found in samples in which the *Escherichia coli* bacterium was incubated. Retention indices are presented in Table 4.
- Propanoic acid 2-hydroxy-2-methyl-, methyl ester and Acetamidoacetaldehyde were considered specific for *Staphylococcus aureus*. They occurred in all samples which housed the *Staphylococcus* at the incubation times listed in Table 4.



**Figure 24. Dimethyl trisulfide identified as a characteristic compound of the *Bacillus subtilis* species using the gas chromatogram (a) and mass spectrum (b), viewed with the software tool used VARIAN GC-MS and confirmed using NIST database (bottom) [Rațiu et al, 2011].**

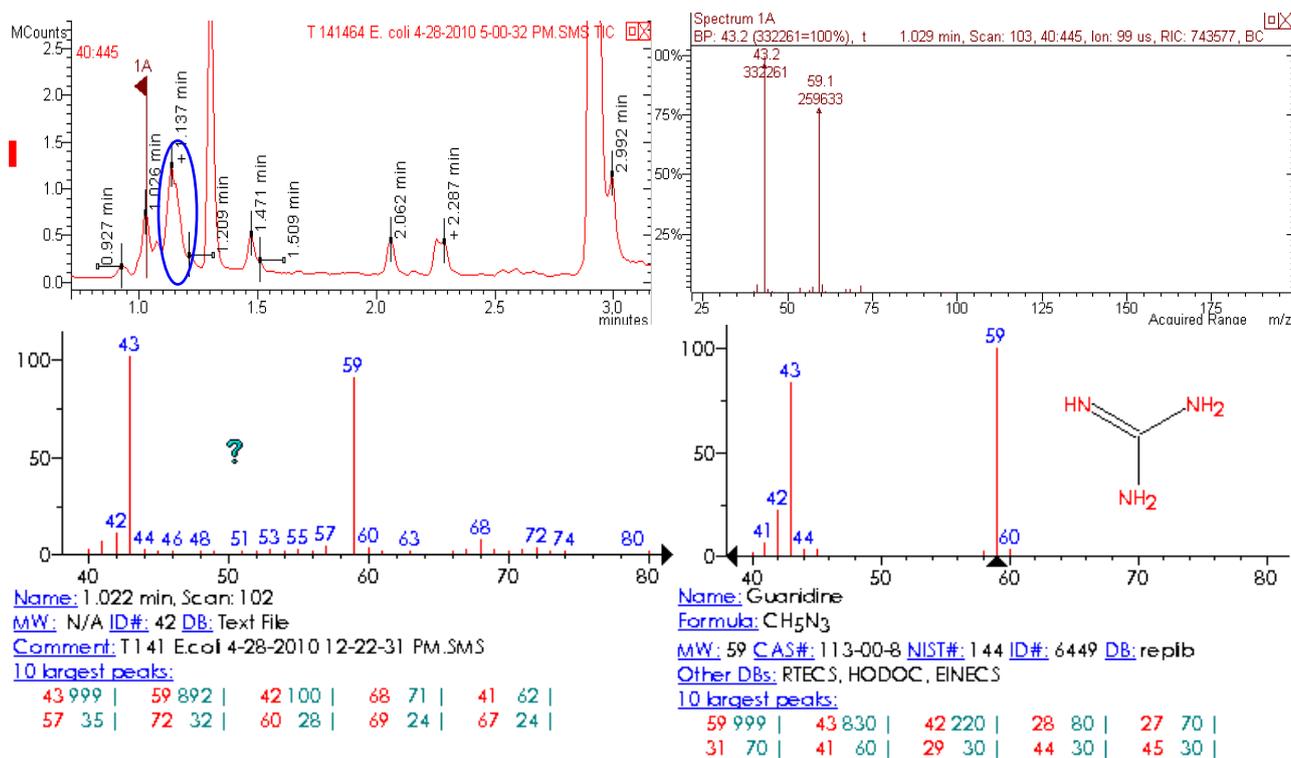


Figure 25. Guanidine identified as a characteristic compound of the *Escherichia coli* species using the gas chromatogram (a) and mass spectrum (b), viewed with the software tool used VARIAN GC-MS and confirmed using NIST database (bottom) [Rațiu et al, 2011] .

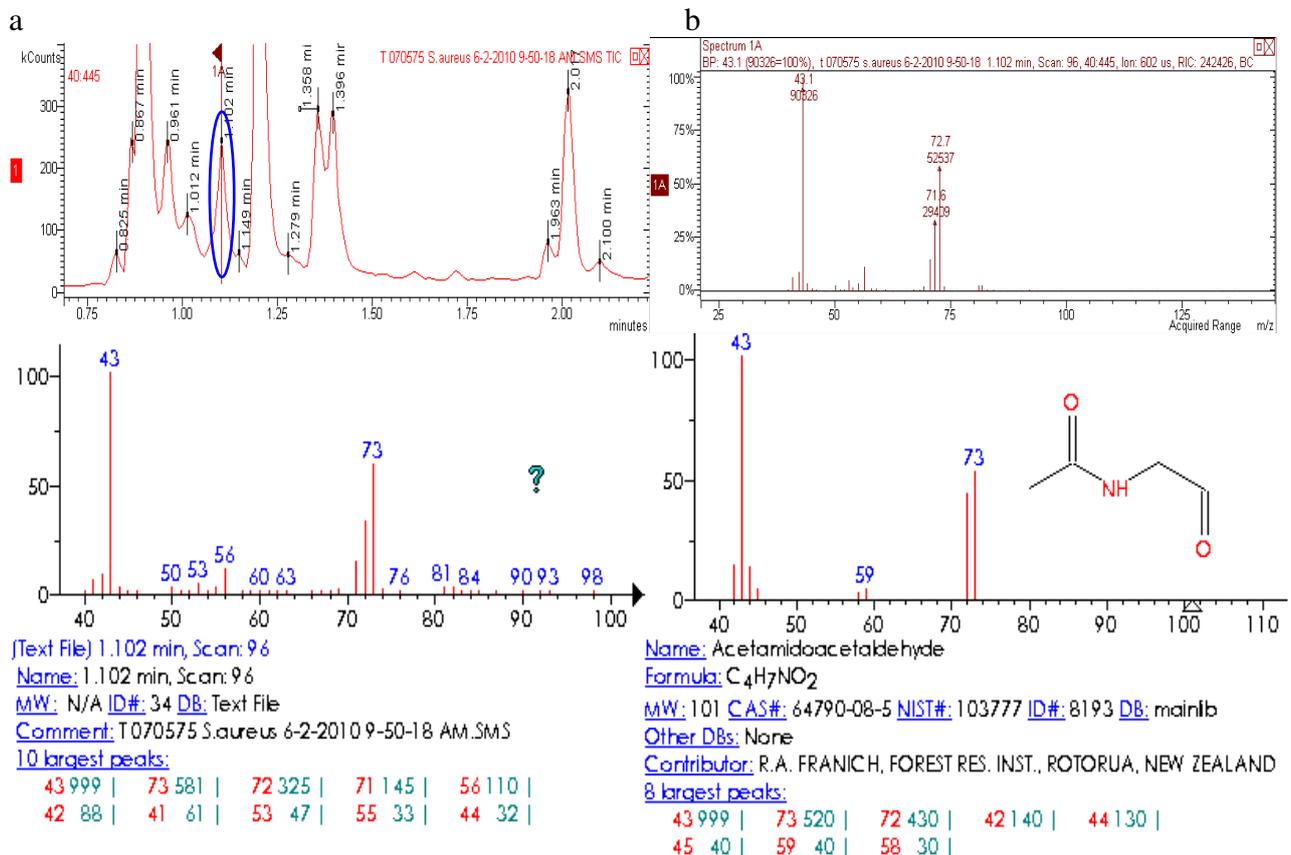


Figure 26. Acetamidoacetaldehyde highlighting as a characteristic compound of the *Staphylococcus aureus* species using the gas chromatogram (a) and mass spectrum (b), viewed with the software tool used VARIAN GC-MS and confirmed using NIST database (bottom) [Rațiu et al, 2011] .

In addition to the compounds characteristic to each individual species, chemical compounds common to all three monitored species, or only for two species of the monitored bacteria were also highlighted, as we could see in Table 5.

**Table 5. Chemicals common to two or to all three monitored species – *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* – obtained employing GC-MS data, Analyzer Pro software and NIST spectral library**

Compounds common to <i>Bacillus subtilis</i> , <i>Escherichia coli</i> and <i>Staphylococcus aureus</i>		Compounds common to <i>Escherichia coli</i> and <i>Staphylococcus aureus</i>		Compounds common to <i>Bacillus subtilis</i> and <i>Staphylococcus aureus</i>	
Chemical	RI	Chemical	RI	Chemical	RI
Disulfide, dimethyl	827 / 828	Trichloromethane	807 / 808	Toluene	833

Therefore, the conclusions that could be drawn are:

- dimethyl disulphide was found in samples where all three species: *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* were incubated (Table 5) but was not present in samples incubated only with growth medium, as shown in Figure 27, that is why this substance was considered common for the three bacterial species studied.
- the samples where *Escherichia coli* and *Staphylococcus aureus* species were inoculated had trichloromethane as common compound. It appeared at retention times listed in Table 5. Trichloromethane was not present in blank samples, which was also shown in Figure 28.
- toluene could be detected in samples with *Bacillus subtilis* and *Staphylococcus aureus* (Table 5); it was not present in samples containing culture medium, as shown in Figure 29.

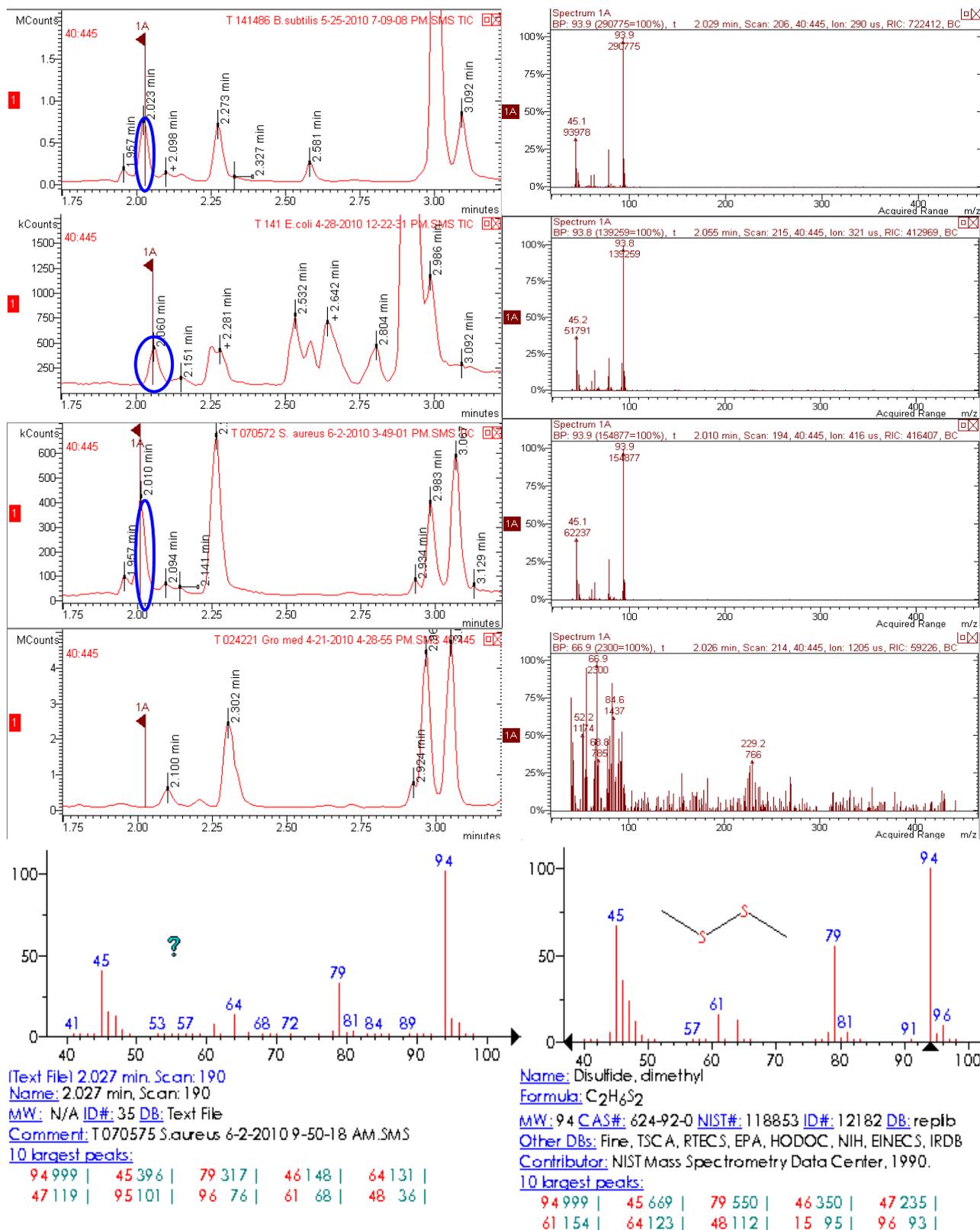
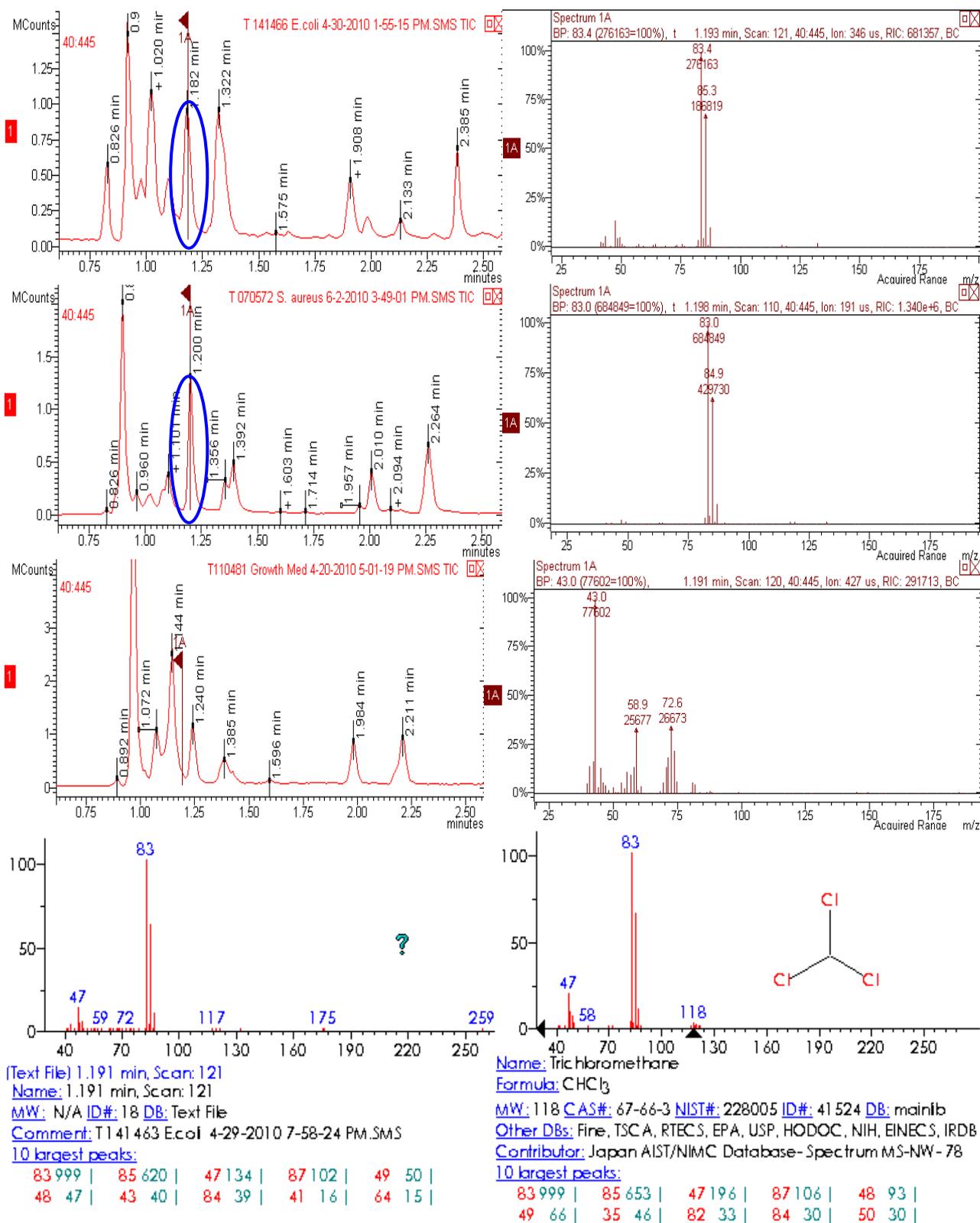
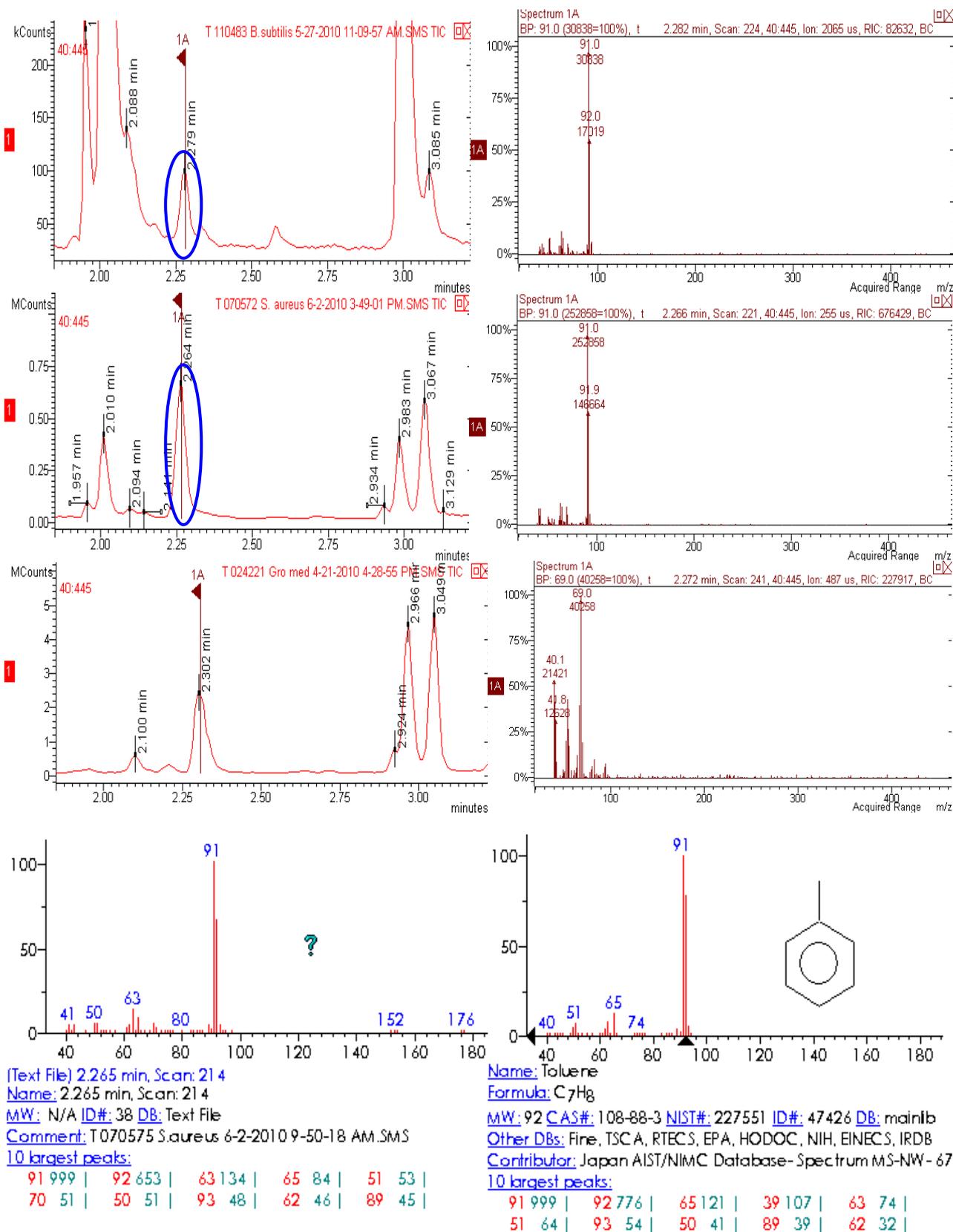


Figure 27. Disulfide dimethyl found as a characteristic compound of all three bacterial strains – *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* using the gas-chromatograms (left side) GC and the mass spectra (right side) viewed with Varian GC-MS software tool used, and confirmed using NIST database. The marker disulfide dimethyl appears in the bacterial samples, but not in blank samples [Rațiu et al, 2011].



**Figure 28. Trichloromethane found as a characteristic compound of *Escherichia coli* and *Staphylococcus aureus* using the gas-chromatograms (left side) GC and the mass spectra (right side) viewed with Varian GC-MS software tool used, and confirmed using NIST database. The marker disulfide dimethyl appears in the bacterial samples, but not in blank samples [Rațiu et al, 2011].**



**Figure 29.** Toluene found as a characteristic compound of *Bacillus subtilis* and *Staphylococcus aureus* using the gas-chromatograms (left side) GC and the mass spectra (right side) viewed with Varian GC-MS software tool used, and confirmed using NIST database. The marker disulfide dimethyl appears in the bacterial samples, but not in blank samples [Rațiu et al, 2011] .

## 4. Conclusions

Instrumental and microbiological analytical methods are employed to exploit a well-established property of the analyte. Thus, o-nitrophenol's property - of having relatively high vapor pressure, allowing direct analysis of these vapors by Ion Mobility Spectrometry - was employed. This way, detecting o-NP provides a sensitive, relatively compact and simple algorithm for bacteria detection. As with any analytical technique, Ion Mobility Spectrometry usefulness for a particular application must be dealt with strictly on an individual basis. Factors to be considered include detection limits, response time, matrix interference, cost, time calibration, portability, etc.

The systems samples' introduction are essential for IMS, particularly if the analytes are not completely extracted from the sample, or if they are transferred to more devices that are coupled to each other. Thus, sample input systems are employed depending on various characteristics of the equipment, but especially on the state of aggregation of the sample used.

Bio-medical and pharmaceutical applications are based on odorant vapors' property to reflect metabolic diseases from the respective person. Thus, the metabolites found in the exhaled air may be directly correlated with the existence of different diseases. Some metabolites are biomarkers, e.g. diabetes occur with acetone, nitric acid is correlated with severe asthma, ammonia shows the existence of liver problems, while others indicate the presence of bacteria.

Ion Mobility Spectrometry could be employed for efficient and quick detection of various types of vaginal infections, identification and monitoring of volatile compounds used as inhalant anesthetic during surgery and may directly diagnose lung damage from a simple human breath sample taken. Portable equipment, low limits of detection, real-time response and the ease of employing IMS instrumentation, allows monitoring and quality assurance of pharmaceutical products, but also ensures the health and safety employees of pharmaceutical companies.

There are two main methods to detect biological markers on the basis of Ion Mobility Spectrometry techniques, i.e.: 1) detection of microorganisms with markers produced by enzymatic processes and 2) detection of microorganisms with markers produced by pyrolysis.

For detection of microorganisms with markers produced by enzymatic processes, there are also two alternatives: 1) you can use a growth substrate, in which is a certain nutrient is intentionally added - which is metabolized to produce a chemical known and detectable with the respective device, or 2) the volatile organic compounds generated in the headspace atmosphere may be directly monitored.

Detection of microorganisms employing markers produced by pyrolysis work on the principle of rapid thermal degradation which occurs before ions get separated in the Mass Spectrometer. Thus, pyrolysis may be a useful tool for classifying and identifying bacteria by the means of derivatives constituent of digestive enzymes or other cellular constituents.

However, there were also attempts to achieve similar results by introducing entire bacteria in the pyrolizer, and the results are promising.

For this research project, bacteria from the headspace atmosphere were sampled at different times of incubation, respectively after 24, 48 and 72 hours, thus obtaining two sets of data for each bacterial culture, from the devices we worked with: Thermodesorber coupled with Gas Chromatograph and coupled with Mass Spectrometer (TD - GC / MS) and independent of this instrumental chain, with an Ion Mobility Spectrometer with transverse electric field (EnviroNics IMS). The samples analyzed with the TD - GC / MS and analyzed by EnviroNics IMS were taken from the same bacterial culture after the same time passed from the beginning of incubation.

After direct analysis of headspace air samples, followed by data processing obtained by EnviroNics IMS device, we could notice differentiation between:

- samples containing bacteria and those who had only growth medium (agar) incubated;
- samples containing different species of bacteria;
- samples that had inoculated the same species of bacteria, but were taken after different incubation times.

Using data from GC-MS as a confirmation/validation method, it was observed that the profile of headspace air samples was very complex. Nevertheless, the presence of the same chemical substance in all the three days of monitoring was shown, as well as the occurrence of various chemicals in one or in two of the sample days, but most often chemicals identical for the samples taken in similar conditions (same species of bacteria inoculated, identical incubation time) were observed. Therefore, we have identified four chemicals characteristic to *Bacillus subtilis* bacterium and two chemical compounds specific to *Escherichia coli* and *Staphylococcus aureus* bacteria.

Our results are consistent with those published by groups of researchers from Finland - which focused on the differentiation of air samples containing volatile organic compounds from moulds in buildings [Räsänen et al, 2010] and from Germany - which focused on metabolites produced by fungi [Tiebe et al, 2010].

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