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Bachelor of Science in Biomedical Engineering

Quantitative analysis of contamination of biological origin in direct detection *in-situ*

Dissertation to obtain the Master Degree in
Biomedical Engineering

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Abstract

Fast detection of biological contaminations is of great importance in numerous areas of human health, such as, toxicology, environmental health and medicine. Currently, this is one of the top concerns of space agencies, since the duration of manned missions is becoming increasingly longer and the conventional methods for biological contaminations and treatment are unavailable while in space.

Microorganisms have been shown to proliferate under punishing environments as the ones suffered in space. This, associated with the astronauts' weakened immune system, may originate serious health problems.

The need for finding sample retrieving methods *in situ* and techniques for fast and online detection of biological contamination is very important, not only for preventing infections but also for pursuing the indicated treatment. The technique used in this work was the Ion Mobility Spectrometry coupled with Gas Chromatography (GC-IMS). This is an analytical technique that identifies organic compounds in the gaseous phase.

In order to check if it is possible to identify bacteria based on the volatile organic compounds (VOCs) released by them, two bacteria were studied: *Escherichia coli* (ATCC 25922 pCU18) and *Pseudomonas aeruginosa* (ATCC 27853). Cultures of each bacteria individually and in a mixture were made and a bacterial sample was retrieved by swabbing. The cotton swab was then sealed in a vial. The vial's headspace was analysed with the IMS equipment.

The objective of this thesis was to find a pattern of released bacterial VOCs that allowed their identification. This work indicates that it is possible to identify these bacteria based of their released VOCs profile, even when they are in a mixture.

This findings pave the way for a faster detection and identification method for biological contaminations. In the future, this technique may also be applied in other contexts, such as, in a hospital, and with other microorganisms.

Keywords: IMS, bacteria, pattern, VOCs

Resumo

A deteção rápida de contaminações biológicas é de grande importância em várias áreas da saúde humana como sejam, toxicologia, saúde ambiental e medicina. Atualmente, esta é uma das principais preocupações das agências espaciais, pois a duração das missões tripuladas está a aumentar e não é possível recorrer aos métodos convencionais de deteção de contaminações e do tratamento.

Há microrganismos que conseguem prosperar em condições hostis como as que se fazem sentir no espaço. Isto, associado ao sistema imunitário debilitado dos astronautas, pode causar graves problemas de saúde.

Encontrar métodos de recolha de amostras biológicas *in-situ* e técnicas para a sua rápida análise é muito importante, não só para prevenir infeções como para seguir o tratamento adequado. A técnica usada neste trabalho foi a Espectrometria de Mobilidade Iónica associada a Cromatografia Gasosa (GC-IMS). Esta é uma técnica analítica utilizada para identificar compostos no estado gasoso.

De modo a verificar a possibilidade de identificação de bactérias com base nos compostos orgânicos voláteis (VOCs) que libertam, duas bactérias foram estudadas: *Escherichia coli* (ATCC 25922 pCU18) e *Pseudomonas aeruginosa* (ATCC 27853). Foram feitas culturas de ambas as bactérias e da mistura das duas. A amostra foi retirada das culturas com uma cotonete que foi posteriormente colocada num frasco. O ar do frasco foi retirado para análise pelo equipamento de GC-IMS.

O objetivo desta tese era encontrar um padrão composto por VOCs libertados pelas bactérias que permitisse a sua identificação. Foi possível encontrar um padrão para as bactérias que permitiu ainda identificá-las separadamente na mistura das duas.

Estes resultados indicam que com este método é possível identificar contaminações biológicas mais rapidamente. No futuro, pode ser aplicado em outros contextos, como hospitais, e para outros microrganismos.

Palavras-chave: IMS, bactérias, padrão, VOCs

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Abbreviations

API	Analytical Profile Index
DNA	Deoxyribonucleic acid
GC	Gas Chromatography
GC-IMS	Gas Chromatography-Ion Mobility Spectrometry
GC-MS	Gas chromatography Mass Spectrometry
IMS	Ion Mobility Spectrometry
ISS	International Space Station
LAV	Laboratory Analytical Viewer
LB	'Lysogeny' or Luria Broth
LOCAD-PTS	Lab-On-a-Chip Application Development-Portable Test System
MALDI	Matrix Assisted Laser Desorption Ionization
MCC-IMS	Multi-capillary-Ion Mobility Spectrometry
NT	Needle Trap
PAB	Pseudomonas Agar Base
PCR	Polymerase Chain Reaction
RIP	Reactant Ion Peak
SPME	Solid Phase Micro-Extraction
TOF	Time of Flight
TSA	Tryptic Soy Agar
VOC	Volatile Organic Compound

1

Introduction

This thesis is a continuation of a previously developed work for further advancement relating to the methods of *in-situ* sample collection and online analysis of compounds in a collaboration project with NMT, S.A. and Airbus DS GmbH – Space Systems. Even though this work emerged from the need to detect bacterial contamination in a space environment, it can be applied to other settings.

Fast detection of microbiological contamination is at the top of the priority list of space agencies, since astronauts stay for long periods of time in an enclosed environment with limited access to detection methods and without access to specific treatment of infections. The methods for bacterial characterization aboard the International Space Station (ISS) are mostly traditional culture-based methods and molecular biology methods [1].

Even in such a hostile environment as space habitats, bacteria are able to survive and thrive. Astronauts' immune system is weakened in adverse environments and under high working pressure, defined diet, restricted hygienic practices, microgravity and radiation [2]. The basis of such environments contributes to an increased susceptibility and vulnerability to infection.

Consequently, the development of an extremely sensitive portable device able to work online and capable of providing fast results is of extreme importance. There are numerous techniques capable of meeting these requirements, however gas chromatography ion mobility spectrometry (GC-IMS) was the technique chosen.

GC-IMS is an analytical technique for the detection of gaseous compounds. One of its application is the detection of volatile organic compounds (VOC) emanating from

microorganisms. VOCs are chemical substances that contain carbon atoms in its chemical structure that can be volatile at ambient temperature [3]. It is well known that bacteria release a variety of these compounds as a result of their metabolism. Moreover, bacteria may be identified based on the VOCs profiles pushing the necessity to develop databases which will be compared to the obtained profiles. [4]

It has been proven that bacteria are still able to form biofilms during spaceflight or microgravity conditions, including *Escherichia coli* and *Pseudomonas aeruginosa*, which have been found aboard the ISS. A biofilm is a structure formed by bacteria in which they live in communities enclosed in an extracellular matrix mostly composed of exopolysaccharides. This matrix and the bacterial organization confers different types of resistance to bacteria [5], including resistance to antibiotics, cleaning agents and mechanical forces, those characteristics pose serious challenges in the ISS [6]. In addition, bacteria grown in microgravity simulated environments have been shown to possess more resistance and virulence [5].

Bacteria analysed by GC-IMS in this study were *E. coli* and *P. aeruginosa*. The first is still one of the biggest sources of children's infection and one of the main causes of persistent diarrhoea worldwide [7]. The latter is an opportunistic human pathogen that normally, only causes infection when the host's immune system is weakened as is the case of astronauts [8].

The detection of bacteria is of extreme importance, not only in regard to human health but also on safety, since the biofilms formed by microorganisms may induce corrosion of surfaces, electronic devices or life support systems [9].

Objectives

The main aim of this thesis work is to validate a method and experimental protocol previously developed, adding special emphasis in the quantitative analysis of VOCs emitted from bacteria [10].

During the course of this work it is expected to acquire knowledge on the analysis of VOCs with the GC-IMS technology, with attention in calibration, analysis and data interpretation.

This work consists in the study of bacteria profiles from the analysis of GC-IMS spectra and, ultimately, the creation of a database for the direct identification of contamination of biological origin

2

Microbiology

Microorganisms are generally defined as organisms with dimensions close to the micrometre. This definition includes a wide variety of diverse life forms. All are classified into three domains: *Bacteria, Archaea* and *Eukarya* [11].

The aim of this work is the study of bacteria, so a brief introduction to these living forms will be made. Bacteria are single-celled, do not typically possess organelles encased by membrane and are typically on the width of 1 to 2 µm. Most species of bacteria possess a cell wall that provides structural integrity [11][12]. They are very adaptable to different environments and are present in most habitats [12]. Some are even able to thrive in extreme conditions (extremophiles), these being physical extremes, such as temperature, radiation and pressure, and geochemical extremes, for instance, salinity and pH. High temperature variations, vacuums, ionizing radiation and hypervelocity are some of the extreme conditions felt in space that bacteria have been shown to survive [13].

Bacteria are classified according to phylogenetic distinctions, phenotypic characteristics and biochemical and physiological traits. Phenotypic characteristics, being the observable characteristics of a bacteria, are the easiest to describe. In terms of shape, there are three main morphological forms. These are: spherical, rod-shaped and spiral-shaped. Bacteria that possess these shapes are, respectively called, **coccus**, **bacillus** and **spirillum** [11]. These shapes are illustrated in Figure 2.1 (a), (b) and (c). The shape identified by (d), spirochete, is less common.

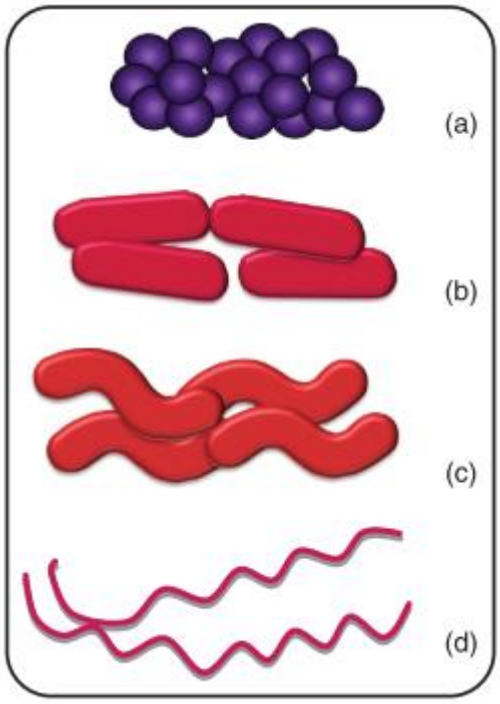


Figure 2.1 – Most common morphological shapes of bacterial cells: (a) spherical shape or coccus, (b) rod shape or bacillus, (c) spiral shape or spirillum and (d) spirochete [11].

Pathogenic bacteria are often spherical, rod-shaped and spiral-shaped [14].

Bacteria may also be classified based on the Gram staining test as Gram-negative or Gram-positive. After following the Gram-stain procedure, the former appear of pink or red coloration, while the latter retain a purple colour. Both may be pathogenic and cause a variety of infections, however Gram-negative bacteria are generally more resistant to antibodies and antibiotics [15].

The most common media in which bacteria can be grown are liquid media, in which bacteria can be maintained, such as the LB broth, and agar plates. The bacterial culture method used in this work was the surface plating made on solid media. This method consists on smearing the bacteria with a loop on a solid media. The loop is then re-sterilized by contact with a flame. More bacteria is smeared in the same way until sufficient smears are made. The plates are then closed and incubated in an inverted position (lid down) [14].

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative rod-shape bacterium responsible for various hospital acquired infections. It is also responsible for much of the morbidity and mortality in patients with the recessive genetic disorder cystic fibrosis and causes bacterial infection in burn patients and immunosuppressed patients [16] [17]. Figure 2.2 corresponds to an image of several bacteria *P. aeruginosa*.

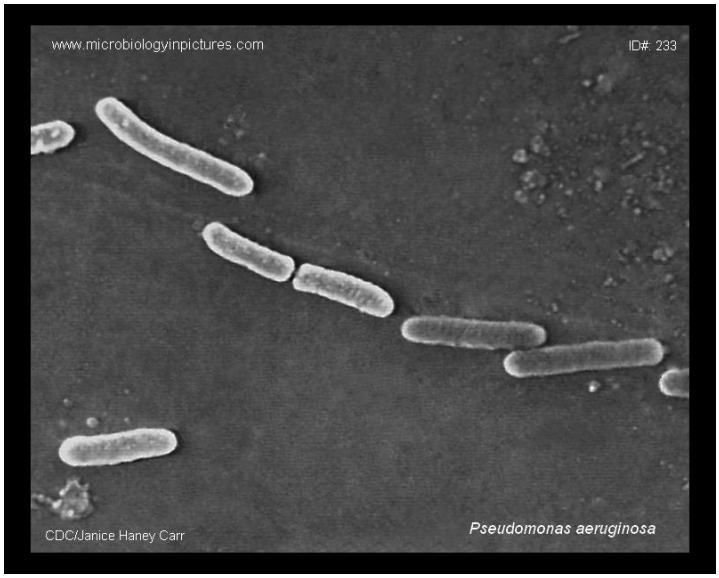


Figure 2.2 – Scanning electron micrograph of *Pseudomonas aeruginosa* [18]**.**

Growth conditions:

The optimum growth temperature is 37 ºC, even though bacteria may survive in the range of 4 ºC to 42 ºC. The choice of the growth temperature may influence the bacteria's virulence. These bacteria are shown to grow better in aeration conditions (strict aerobe – only grows in the presence of significant quantities of oxygen)[16].

Escherichia coli

Escherichia coli is a Gram-Negative rod-shaped bacterium. This is a model organism, meaning it has already been extensively studied. Figure 2.3 shows several *E. coli*.

There are various strains of these bacteria. Some are harmless but others can cause infections or food poisoning [7]. The harmless strains are abundant in human intestines and aid in food digestion [12].

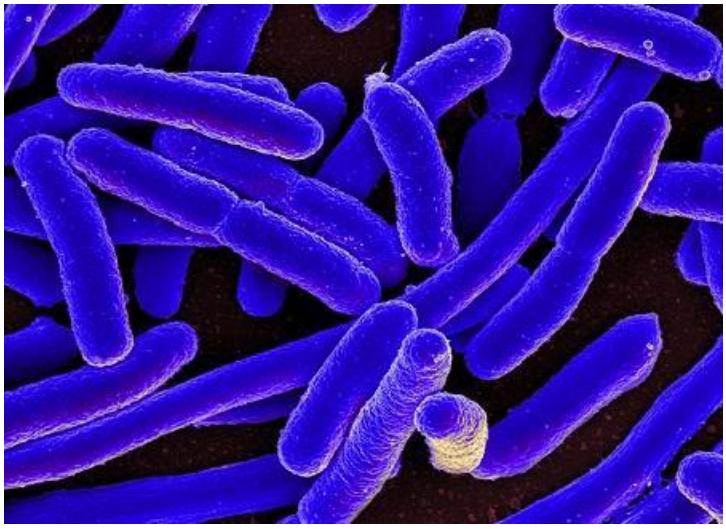


Figure 2.3 – Colorized scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip [19]**.**

Growth conditions:

Presence of carbon in the substrate, constant temperature and water content are important for survival and growth of *E. coli* [20].

Optimum growth of *E. coli* occurs at 37 ºC [21]. The choice of a growth medium is very large since these bacteria grow on a large range of media. Nonetheless *E. coli* require basic nutrient in order to survive, such as, vitamins and minerals and a source of nitrogen. While the first two may be provided by yeast extract, the nitrogen is provided by tryptone [22].

This bacteria is a facultative anaerobe.

VOCs emitted by bacteria

Bacteria release a variety of volatile organic compounds as a result of metabolism and different growth conditions result in different VOCs released [23]. Some of these compounds are ketones, alcohols and hydrocarbons (very difficult to detect in a GC-IMS device), acids, sulphur and nitrogen containing compounds and terpenes [24]. Different strains of the same bacteria may produce different volatile organic compounds and recent studies have shown that different strains of the same bacteria, may also produce different immunologic responses [25].

Ketones

Various ketones have been identified as being released by several strains of *P. aeruginosa*, specially 2-nonanone and 2-undecanone [17]. Other ketones such as 2-pentanone, 2-heptanone, 4-heptanone and 3-octanone were also released [26]. 2-heptanone and 2-nonanone have also been identified as part of the VOCs emitted by *E. coli*. Moreover acetone has been reported to be released by *E. coli* too. Interestingly, acetone and 2-heptanone appear to be correlated with bacterial growth [27].

Alcohols

Ethanol has shown to be produced by *P. aeruginosa* at high concentrations and by *E. coli* [26][27]. 3-methyl-1-butanol and 2-butanol have also been identified in *P. aeruginosa* cultures[26].

Hydrocarbons

1-undecene and isoprene have been identified as compounds significantly released by *P. aeruginosa* [26]. Hexane was recognised as emitted by *E. coli* [27].

Acids

Acids such as acetic, propionic and butyric acids have been identified as being released by bacteria. These substances are by-products of anaerobic metabolism.

P. aeruginosa does not appear to produce any acids [26].

Sulphur containing compounds

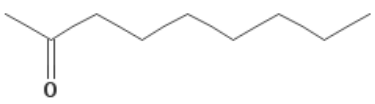
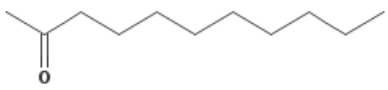
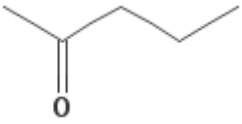
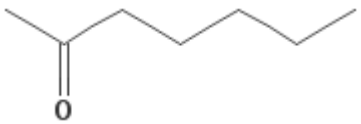
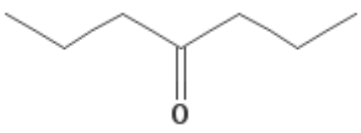
These compounds contribute to fermented foods aroma. Dimethyldisulfide and dimethyltrisulfide were present in various strains of *P. aeruginosa* [17]. In addition, dimethylsulfide, mercaptoacetone, 3-(ethylthio)-propanal and 2-methoxy-5-methylthiophene have also been emitted by the bacteria.

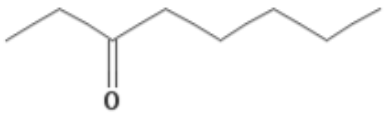
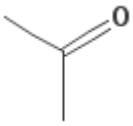
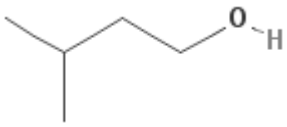
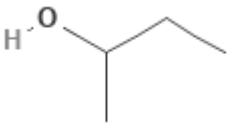
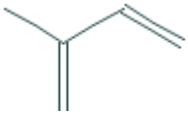
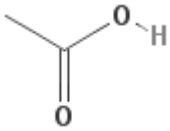
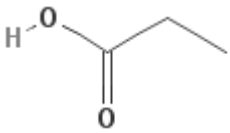
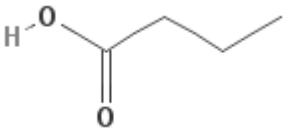
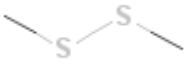
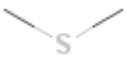
Nitrogen containing compounds

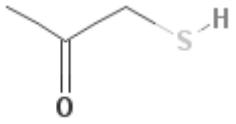
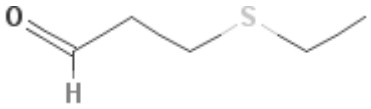
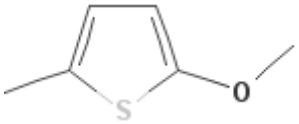
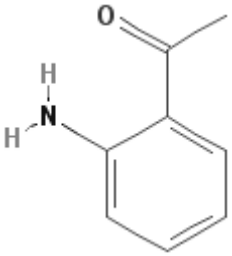
The literature extensively refers 2-amino-acetophenone (2-AA) as a possible biomarker for *P. aeruginosa* infection [26]. This compound is responsible for the grape-like odour characteristic of *P. aeruginosa* cultures [24]. 2-AA has been confirmed to be produced by several strains of this bacteria and absent in other clinically relevant pseudomonads [17].

Table 2.1 contains the chemical structure and the molecular formula of the compounds emitted by the bacteria mentioned in this section.

Table 2.1 – Chemical structure and molecular formula of some compounds known to be released by *E. coli* and *P. aeruginosa.*

Class	Compound name	Chemical structure	Molecular Formula
Ketone	2-nonanone		$C_9H_{18}O$
	2-undecanone		$C_{11}H_{22}O$
	2-pentanone		$C_5H_{10}O$
	2-heptanone		$C_7H_{14}O$
	4-heptanone		$C_7H_{14}O$

	3-octanone		$C_8H_{16}O$
	Acetone		C_3H_6O
Alcohols	Ethanol		CH_3CH_2OH
	3-methyl-1-butanol		$(CH_3)_2CHCH_2CH_2OH$
	2-butanol		$CH_3CHOHCH_2CH_3$
Hydrocarbons	1-undecene		$C_{11}H_{22}$
	Isoprene		C_5H_8
	Hexane		C_6H_{14}
Acids	Acetic acid		CH_3COOH
	Propionic acid		CH_3CH_2COOH
	Butyric acid		$CH_3CH_2CH_2COOH$
Sulphur containing compounds	Dimethyldisulfide		$C_2H_6S_2$
	Dimethyltrisulfide		$C_2H_6S_3$
	Dimethylsulfide		C_2H_6S

	Mercaptoacetone		C_3H_6OS
	3-(ethylthio)-propanal		$C_5H_{10}OS$
	2-methoxy-5-methylthiophene		C_6H_8OS
Nitrogen containing compounds	2-amino-acetophenone		C_8H_9NO

Safety measures

As previously mentioned, even though bacteria are critical for earth's ecology, for example by fertilizing soils and waste treatment, some cause diseases [28]. The bacteria studied in this work are both common and do not represent a paramount threat to health. Although care is always needed when in contact with pathogens, these bacteria do not require great safety measures.

Escherichia coli is a microbe of biosafety level 1 and *Pseudomonas aeruginosa* corresponds to a microorganism in the biosafety level 2 [16]. A biosafety level corresponds to an assortment of precautions necessary to follow when handling biological materials. There are 4 levels, being 1 the lowest level of dangerousness, which were specified by the Centers of Disease and Control Prevention in the United States [29]. The same biosafety levels are defined in the European Union. Biosafety level 1 corresponds to organisms unlikely to cause disease in healthy humans. Biosafety level 2 involves microbes that may cause mild disease to humans.

This means some precautionary measures were taken including not eat and drink in the laboratory where these bacteria are present, wash the hands prior and after leaving the laboratory and the use of gloves. Materials that may have been in contact with the

microbes need to be disinfected using alcohol 70 % (v/v). Alcohol is an agent that damages the cell membrane. However, it is only effective if the concentration is between 60 % and 70 % [14]. In addition, special care must be taken when handling sharp contaminated objects [30].

The study of bacteria is of extreme importance not only to understand how a cell works, as the case of model organisms, but also because bacteria have a major impact in our everyday lives. For instance, some bacteria, play vital roles in the maintenance and modification of the environment, while others cause diseases, and some play a role in human metabolism and biology. Finding techniques to rapidly identify bacteria present in surfaces or in the environment may prevent the development of diseases in the human body or aid in the determination of the better treatment to follow.

3

State of the Art

The gold standard for bacterial identification is the DNA sequencing method [31]. The sequence-based identification, relying on the 16S ribosomal DNA genes has become the classification standard [32][33]. However, other methods are routinely used. It is only when conventional methods cannot provide an answer as to the bacteria in question that DNA sequencing is performed [34]. Conventional methods for identification of bacteria include culture-based ones and molecular ones, such as Polymerase Chain Reaction (PCR).

Culture-based methods are considered to be the gold standard for bacterial detection and identification [35]. However, since it relies on incubation, several days are required for the culture to grow and allow identification [32]. The identification of the organisms may be done by the Analytical Profile Index (API) test.

API is a commercially available biochemical identification system of pure cultures [36]. The API test consists of a kit with various test strips that allows the identification of various organisms. An individual test strip is a microtube containing a dehydrated substrate where bacteria are inoculated. Different test strips hold different substrates. By spontaneous reaction or due to the addition of reagents, colour changes are observed due to metabolism. The results are then compared with a database for microbial identification or by means of an identification software. The API 20 E strip permits the identification of Gram-negative bacteria such as the ones used in this work, *Escherichia coli* and *Pseudomonas aeruginosa* [37].

PCR is the most widely used nucleic acid amplification method. In the first step of this method, nucleic acid is extracted from the microorganism, followed by heating of the DNA, which leads to denaturation of the molecule - the DNA double strands are separated into single strands. Next, cooling of the mixture allows the primers to connect to a specific location on the DNA single strand, following by an enzyme that duplicates the target DNA strand [38]. This cycle is repeated several times until sufficient DNA

quantity is obtained. The identification is conducted by DNA comparison with databases, nonetheless, PCR does not provide distinction between viable or non-viable organisms [39].

A relatively recent technology employed aboard the ISS is the Lab-On-a-Chip Application Development-Portable Test System (LOCAD-PTS) developed by NASA [2]. It is a culture-independent portable device for rapid detection of both, alive and dead Gram-negative and Gram-positive bacteria, mould and fungi. This device provides results within minutes [40].

Other devices used that do not require sample preparation are electronic noses, which are part of the chemical sensors.

Chemical sensors

- Electronic nose

Electronic noses are portable devices that work similarly to human noses, meaning that they identify patterns and not the chemical composition or the concentration of the volatiles [41].

An electronic nose consists of a sensitive layer, where the analyte reacts with the sensor, a transducer and a computing system. Different transducers result in different responses. As means of an example, these transducers can be conducting polymers, metal oxides, surface acoustic wave sensors or optical sensors [42].

The most common electronic noses consist of an array of chemical sensors. The signal from these sensors is analysed through pattern recognition algorithms [42]. Since electronic noses detect patterns is necessary to train them first. Each sensor can be developed to detect specific volatile analytes on contact [43]. This makes the e-noses highly selective but poorly sensitive.

Different analytical techniques based on the analysis of VOCs are being used to identify bacteria, most of which resort to mass spectrometry. Recently, mass spectrometry has also been applied to biological molecules such as peptides and proteins [31].

Mass spectrometry

Mass spectrometry is an analytical technique capable of determining the molecular weights and elemental compositions of molecular substances [44].

The working principle of mass spectrometry relies on the ionization and fragmentation of the chemical constituents of the sample and their separation according to the mass-to-charge ratio [45][46]. A mass spectrometer is therefore composed of an ion source, a mass analyser to separate the various ions and a detector.

After the production of gas-phased ions, the molecules are introduced into the mass analyser where, typically, by interaction with electric and/or magnetic fields, they are separated [46].

As means of an example, in the Magnetic Sector Mass Analyser, the type of analyser on which all early work with mass spectrometers was developed [47], the application of an electric field accelerates these particles, which are then deflected by a magnetic field. This deflection depends both on mass and on the charge of the ion. Heavier particles are less deflected than lighter ones, provided their charges are equal [44]. In addition, multiple charged ions suffer a greater deflection.

Once the particles reach the detector, the current generated by these ions is measured, amplified and used to create a spectrum [46].

Mass spectrometers function in vacuum in order to avoid collisions with air molecules to avert trajectory deviation and unwanted reactions as well as loss of ionic charge by interaction with the instrument walls [45].

In order to improve the sensitivity of compound detection a pre-separation technique can be used prior to the sample introduction in the mass spectrometer, such as gas chromatography. The analysis time is normally a few minutes, once the instrument is set up and calibrated [44].

- Gas Chromatography-Mass Spectrometry (GC-MS)

The most common used analytical method for the analysis of volatile organic compounds is gas chromatography allied to mass spectrometry (GC-MS) [41]. This is the Gold Standard for the analysis of VOCs [48].

Gas chromatography performs separation of volatile compounds with great resolution [44]. Mass spectrometry allows the identification of the compound after the pre-separation by ionizing the compound. Here, the most common ionization technique is electron ionization.

The biggest advantage of this method is its sensitivity. The detection is possible at compound concentration of low-parts per billion [41], the limit of detection being in the range of parts per billion and parts per trillion [49]. However, it is impossible for real time analysis, since the separation of compounds by gas chromatography is time consuming, typically between 20 and 100 min [44]. In addition, GC-MS requires off-line analysis and may suffer from sample alteration during storage and transportation, this alteration being due to decomposition, absorption or reaction of VOCs [49].

GC-MS may require sample preparation, being Solid phase micro-extraction (SPME), Purge and trap and Needle trap (NT) the most commonly used [4]. The sample pre-concentration necessary in this technique contributes to the time required to conduct the analysis.

The following techniques perform direct mass spectrometry, meaning that it is not required to perform sampling and separation [4].

- Proton-transfer reaction-mass spectrometry

The novelty of this technique lies in its ion source. The ionization of water vapour is due to a hollow-cathode discharge that generates hydronium ions (H_3O^+) [50]. These ions function as reagent ions and will react with the sample compounds with a proton affinity higher than that of water [50][51]. This is a great advantage since most VOCs have proton affinities larger than water [52][53]. These reactions occur in the drift region where the sample is injected, being then separated by means of the application of an electric field [51].

At the end of the drift tube stands a mass analyser, which separates the compounds according to the mass-to-charge ratio. The most common mass analysers are quadrupole and time-of-flight [48].

This technique permits real-time measurement of analytes as well as online analyses [48][52]. In addition, is a fast and sensitive technology, being able to detect compounds at parts per billion levels [41]. However, it is not capable of resolving different isomers, since it separates compounds exclusively according to mass [52].

- Secondary electrospray ionization mass spectrometry

This method has a different ionization technique. As the previous one, secondary electrospray ionization is due to proton transfer between the electrospray solution and the analytes, both in gaseous phase [4]. Sample volatiles pass through the secondary electrospray reaction chamber where, by interaction with the electrospray cloud, become ionized [54]. The charged products of the reaction can then be identified by mass spectrometry. A typical analysis using this method requires a few minutes [54].

- Selected ion flow tube-mass spectrometry

Different charged particles are formed in an ion source from water and laboratory air. From these ions, usually H_3O^+, NO^+ and O_2^+, the precursor ions, are selected by a quadrupole mass filter [53][55]. The sample and the selected ions are then injected into a flow tube containing an inert carrier gas, normally helium. The interactions between the sample neutral compounds and the selected ions will result in product ions, which

will be analysed quantitatively by the mass spectrometer [53]. This technology allows real-time detection and quantification of analytes in air samples [55].

- Matrix Assisted Laser Desorption Ionization

Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry has been applied to biological molecules. MALDI has been proven to be independent of culture media [56].

An organic compound, the matrix, is mixed or coated with the sample and let to dry. The sample could consist only of a bacteria culture directly smeared onto the MALDI plate [57]. The sample becomes entrapped within the matrix and crystallizes with it. It is then ionized. Laser desorption ionization is a "soft ionization" method, which generates single protonated ions of the analytes. This means that the ionization does not lead to loss of sample integrity [31].

After being accelerated, the ions are introduced into the mass analyser, usually a time-of-flight (TOF) [31]. Identification of the microorganism is typically done by comparison of the spectra obtained with ones from verified bacteria in databases [58].

The technique used in this work, ion mobility spectrometry coupled to gas chromatography (GC-IMS), constitutes an advantage in regard to mass spectrometry because it does not require vacuum and is small in size and portable. Furthermore, it is capable of detecting all kinds of compounds as long as they are ionized. The range of detection is in the parts per billion to parts per trillion depending on the compound.

In a previous cooperation of the Faculty of Sciences and Technology, New University of Lisbon (FCT-UNL), Airbus DS GmbH and NMT, S.A., GC-IMS was tested in a mock-spacecraft habitat conditions during the SIRIUS-17 mission. The experiment, held in November 2017, is part of NASA's SIRIUS (Scientific International Research In a Unique terrestrial Station) Program [59].

The following chapter presents the description of the GC-IMS technique.

4

Ion Mobility Spectrometry

Ion Mobility Spectrometry is an analytical technique that allows the separation and detection of gaseous compounds, such as the VOCs released by bacteria, in a mixture of analytes [60]. The ionization of the sample and subsequent application of a weak electric field permits the ions to travel at atmospheric pressure through a drift tube. The application of the electric field accelerates the ions towards a detector located at the end of the drift region. The time it takes for an ionized compound to transverse a drift tube of a fixed length is what will allow the identification of said compound [61]. This time is specific for each substance and is called drift time [62].

The separation is performed by collisions with molecules from a drift gas moving against the direction of the ions, which slows their initial velocity, imposed by the electric field [63]. Ions may be distinguished depending on their mass, charge and collision cross-section [64]. The ions are collected in a detection area, usually, the Faraday plate [62]. The polarity of the applied electric field is also important in determining what kind of ionized compounds are detected. In the positive polarity mode, positive ions are detected. On the contrary, if in the negative mode of detection, negative ionized compounds are collected in the Faraday plate. Figure 4.1 illustrates the working principle of the IMS device.

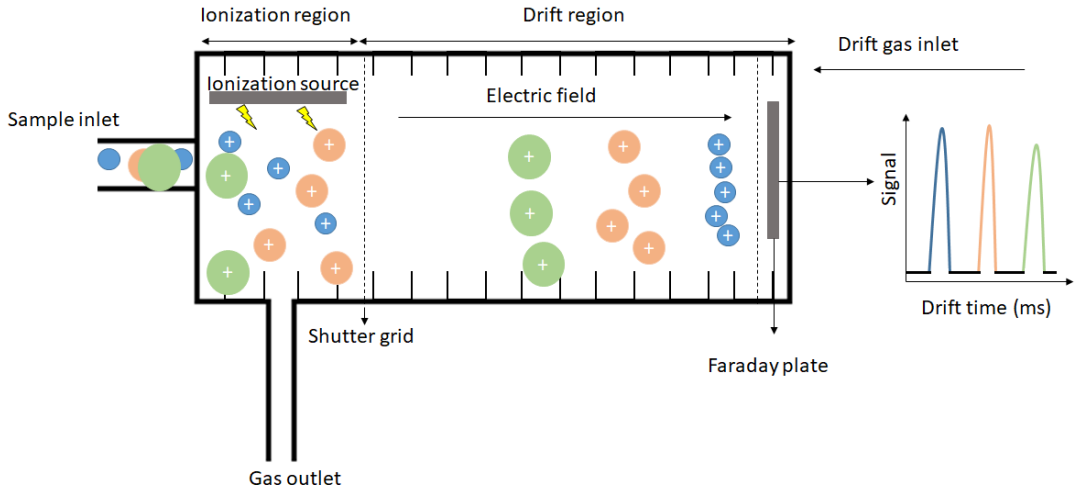


Figure 4.1 – Schematic design of the IMS part of the device. The pre-separated sample enters the ionization region where it is ionized. The ionized compounds are transported to the drift region by the carrier gas through a shutter grid. By the influence of the electric field applied in the drift region and collisions with the molecules from the drift gas, the ionized compounds are separated.

Ion mobility represents how quickly an ion transverses a drift region, of a specific length, when a specific electric field is applied. This being said, the ion mobility (K) is inversely dependent on the electric field (E) applied and directly on ion velocity (v_d) in the drift region (equation 4.1).

$$K = \frac{v_d}{E} = \frac{l}{t_d E} = \frac{l^2}{t_d U} \tag{4.1}$$

Where t_d represents de drift time, l, the length of the drift region and U the voltage applied across the drift region.

Even though ion mobility is different for each substance, it is dependent on the temperature and pressure [62]. In order to be able to compare ion mobility measurements made in different conditions and different devices, the reduced mobility K_0 is introduced (equation 4.2) [65]. This value is characteristic for each ion and the influence of temperature and pressure were removed

$$K_0 = K\left(\frac{P}{P_0}\right)\left(\frac{T_0}{T}\right) \tag{4.2}$$

P, is the gas pressure, T, the gas temperature and P_0 and T_0 the standard conditions (P_0 = 760 Torr, T_0 = 273.16 K) [66].

Measurements made with this technique have been shown to be extremely reproducible [65].

Production of Reactant ions

The collision of fast electrons originated from the decay of a β-radiation source with the molecules from the device's gaseous atmosphere, instigates a serious of reactions that will produce the reactant ions. In the case of this device, the atmosphere was air. The nitrogen and oxygen present in the air play a very important role on the formation of the reactant ions.

If the electrons possess an energy higher than that of the ionization energy of nitrogen, which is approximately 15.6 eV in the latest data, nitrogen ionizes (equation 4.3) [67][68]. The electrons emitted from the tritium source used in the device have an average energy of 5.86 keV [60].

$$N_2 + \beta \rightarrow {N_2}^+ + \beta' + e^- \qquad 4.3$$

Where β′ represent an electron with less energy than the initial. By interaction with water molecules, the ${N^+}_2$ will produce the positive reactant ion species (equations 4.4 to 4.8), $H^+(H_2O)_n$, where n represents the number of molecules formed [63].

$${N_2}^+ + 2N_2 \rightarrow {N_4}^+ + N_2 \qquad 4.4$$

$${N_4}^+ + H_2O \rightarrow 2N_2 + {H_2O}^+ \qquad 4.5$$

$${H_2O}^+ + H_2O \rightarrow {H_3O}^+ + OH \qquad 4.6$$

$${H_3O}^+ + H_2O + N_2 \rightarrow {(H_2O)_2H}^+ + N_2 \qquad 4.7$$

$$(H_2O)_2H^+ + H_2O + N_2 \rightarrow (H_2O)_3H^+ + N_2 \quad 4.8$$

…

The production of the negative reactant ions species is due to the interaction between the electrons and oxygen (equations 4.9 to 4.11). Electrons attach to oxygen forming a negative ion, which will later react with water molecules to form $(H_2O)_nO_2^-$.

$$O_2 + e^- \rightarrow O_2^- \quad 4.9$$

$$O_2^- + H_2O \rightarrow (H_2O)O_2^- \quad 4.10$$

$$H_2O + (H_2O)O_2^- \rightarrow (H_2O)_2O_2^- \quad 4.11$$

These ions, $(H_2O)_nO_2^-$ and $(H_2O)_n H^+$, form the Reaction Ion Peak (RIP), which represents the amount of ions available to react with the analyte [64].

The number of water molecules, n, in the cluster depends on the temperature and water content of the drift gas [63].

Production of product ions

Product ions are mostly formed through proton transfer from the reactant ions to the analyte neutral molecules. This only occurs if the molecules of the sample have a greater proton affinity than that of water (the molecule responsible for most of the reactant ions), which is the case of most VOCs, such as, ketones, alcohols, amines, esters, alkanes, among many others[64][61].

There are a lot of possible ways to form positive ions [63]. Equations 4.12 and 4.13 illustrate the reactions that take place at low concentrations of the substance A.

$$(H_2O)_nH^+ + A \rightarrow A(H_2O)_nH^+ \rightarrow AH^+ + nH_2O \quad 4.12$$

$$(H_2O)_nH^+ + A \rightarrow A(H_2O)_nH^+ \rightarrow A(H_2O)_mH^+ + (n-m)H_2O \quad 4.13$$

If the concentration of a substance is high, the formation of dimers and even trimers may occur (equation 4.14).

$$A(H_2O)_mH^+ + A \rightarrow A_2(H_2O)_xH^+ + (m-x)H_2O \quad 4.14$$

The formation of negative ions is also possible (equation 4.15 to 4.17). It is made by the formation of adducts [63][68]. AB represents a substance.

$$[(H_2O)_3O_2]^- + AB \rightarrow [AB]^- + 3H_2O + O_2 \quad 4.15$$

$$[(H_2O)_3O_2]^- + AB \rightarrow [(AB)O_2]^- + 3H_2O \quad 4.16$$

$$[(H_2O)_3O_2]^- + AB \rightarrow A^- + B + 3H_2O + O_2 \quad 4.17$$

Depending on the mode of detection chosen for the device, positive or negative polarization, different molecules will be measured. Bear in mind that the amount of compounds detected in the positive mode is much more significant than that the amount obtained in the negative mode because of the proton affinity of water being smaller than most VOCs, which means these compounds will mostly react with the positive reactant species [63].

After ionization, the product ions are injected into the drift region through an ion-molecule injection shutter. This injection shutter opens repetitively every 100 μs, which contributes to decrease the interactions between the ionized compounds. The drift region has several metal rings in order to maintain a homogeneous electric field gradient. This maintenance is important since any inhomogeneity leads to a larger drift time [69].

Once the ions reach the end of the drift region, they are collected in a Faraday plate which produces a plot of the electric current generated from the ions against their drift time [70].

Gas Chromatography

Ion mobility spectrometry has the big disadvantage of having poor selectivity, which can become a problem when analysing complex mixtures, such as the VOCs released by bacteria. This problem may be diminished by adding a pre-separation step. This step is made by using gas chromatography [62]. Chromatographic columns minimize the interaction of analytes before entering the ionization region, which contributes to reduce the complexity of the obtained signal [27].

Gas chromatography is a separation technique for gaseous phase compounds. It depends on the interaction between the mobile and stationary phases. A carrier gas transports the sample through the system. The most commonly used one is helium. Once in the chromatographic column, compounds are separated in time. The fastest to elude are the ones that do not interact with the column's coating. The less interactions and the more volatile a compound, the sooner it leaves the column. Different coatings allow the identification of different compounds[44].

When coupled with Ion Mobility Spectrometry, gas chromatography can enhance compound separation by adding a pre-separation step. A multi-capillary column or a single gas chromatographic column are two examples of systems that pre-separate the analytes in the sample prior to introduction in the ion mobility spectrometer. A multi-capillary column contains of a series of capillaries. Figure 4.2 shows a cross section of a multi-capillary column. The MCC-IMS device previously used had approximately 1200 capillaries, with a diameter of 40 μm and a length of 20 cm.

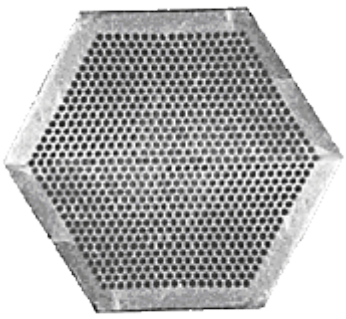


Figure 4.2 - Cross section of a multi-capillary column [71].

The carrier gas used in the MCC-IMS device is nitrogen. In this case, the inner walls of the capillary are coated with a viscous liquid material [60]. This coating is indicated

to find alkaloids, aromatics, drugs of abuse, fatty acid methyl esters, herbicides, hydrocarbons, halogenated compounds, and pesticides [72]. A solid porous material may also be used as coating. The capillary columns are made of fused silica quartz [44]. Figure 4.3 illustrates how a sample is separated in a multi-capillary column.

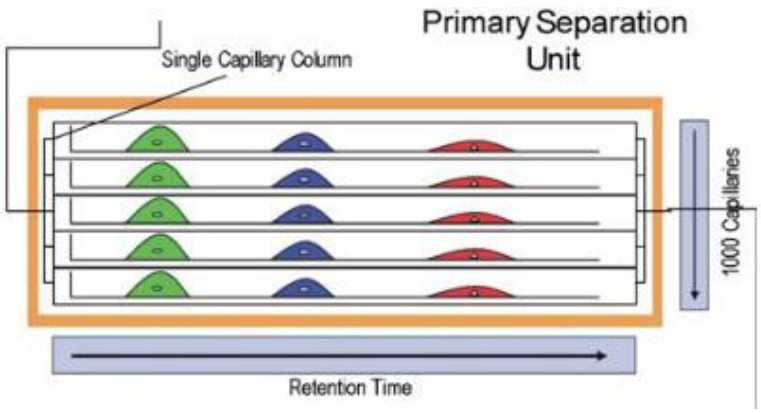


Figure 4.3 – Schematic view of sample separation performed by a multi-capillary column [27].

The working principle of both devices is the same. The difference between the two lies in the separation of the sample. In the MCC-IMS, the sample is lead through a multi capillary column, while in the GC-IMS, the sample goes through a single column. This column has a length of 30 m and a diameter of 0.53 mm. Figure 4.4 shows a schematic representation of an IMS equipment coupled with a pre-separation technique.

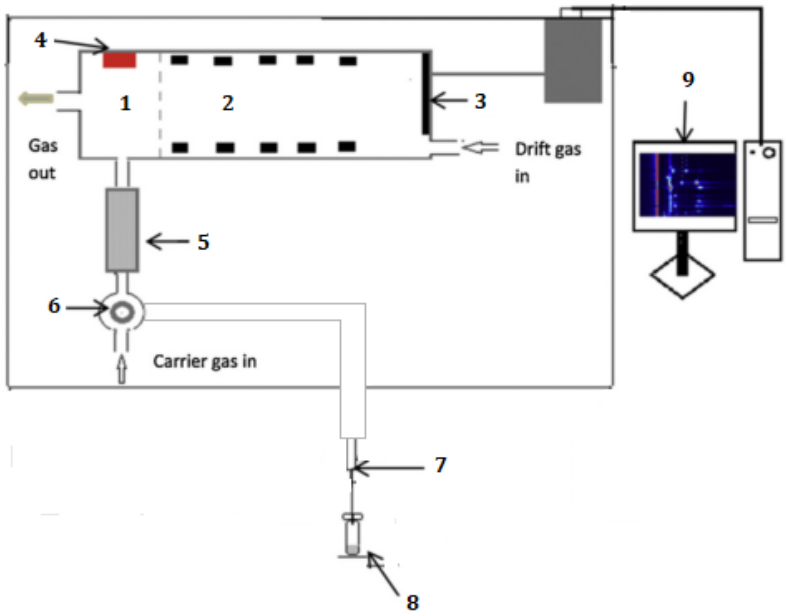


Figure 4.4 - Schematic diagram of an IMS equipment associated with a pre-separation technique. (1) Ionization region; (2) Drift region; (3) Detector (Faraday plate); (4) Radiation source; (5) Gas-chromatography column; (6) Loop and valve system; (7) Syringe; (8) Sample vial; (9) PC. Adapted from [73]

The multi-capillary column, as the GC column, adds pre-separation, which brings a dimension to the obtained spectra (Figure 4.5). The pre-separation step matches the retention time of the compounds travelling through the multi-capillary column or the gas chromatography column.

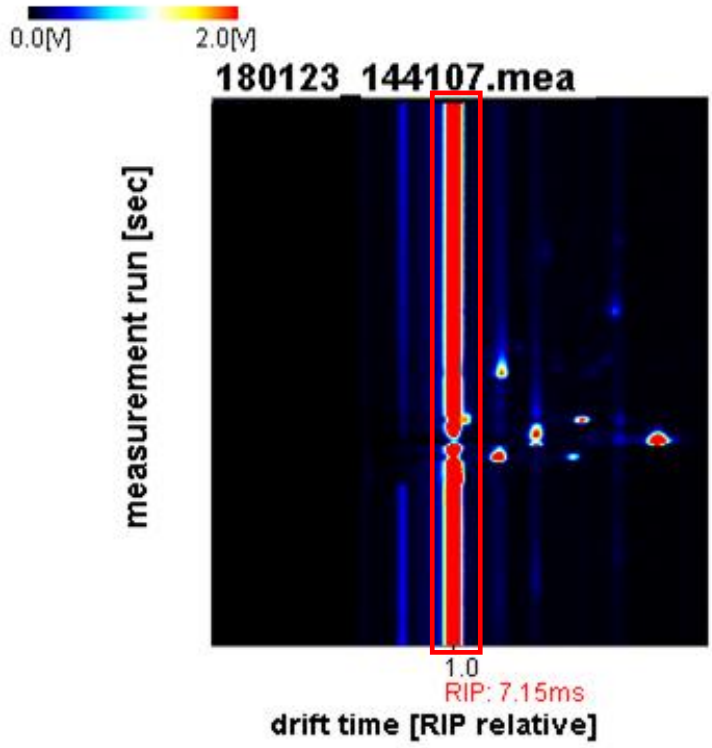


Figure 4.5 – Spectra of a room air sample obtained using the LAV software. The x-axis represents the drift time in milliseconds, the y-axis the retention time of the elements in the gas-chromatography column in seconds. The z-axis represents the intensity of the signal measured in Volts. The RIP is an intense peak, seen has a wider line from top to bottom in the spectra (red rectangle). Other elements present in the air sample are also represented in red.

After the pre-separation step, the compounds are led to the ionization and drift region. In this region, they are again separated. The separation is made by collisions with the drift gas, nitrogen in the MCC-IMS device and air in the GC-IMS, which will introduce another dimension to the spectra, the drift time.

The third dimension of the spectra is the intensity of the current generated by the collection of the ions in the Faraday-plate, which is displayed using colour.

5

Development and application of a method for detection and identification of bacterial VOCs

Experimental setup

Figure 5.1 shows the experimental setup used in this thesis. It can be divided into two parts: the GC-IMS and the preparation of the sample to be injected into the device.

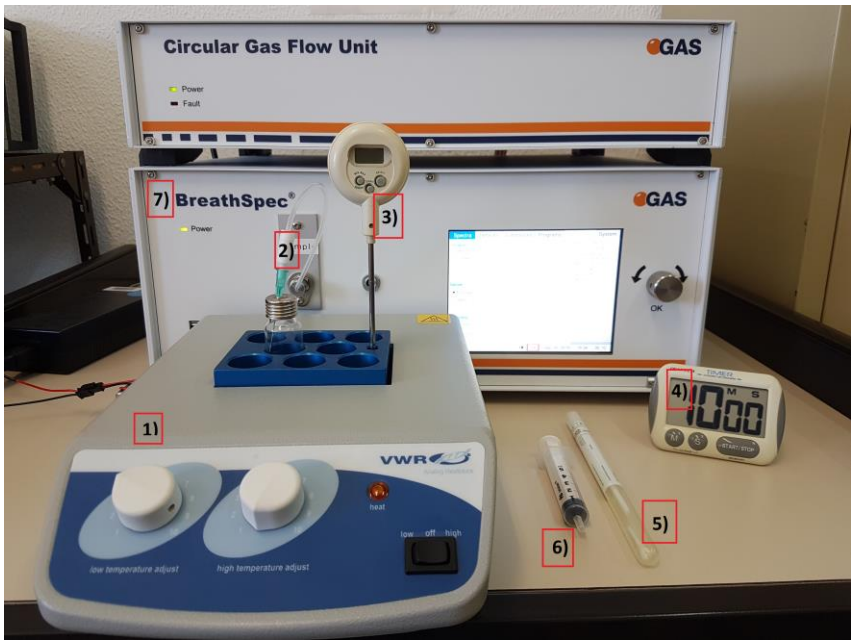


Figure 5.1 –Experimental set-up: 1) heatblock; 2) vial; 3) thermometer; 4) chronometer; 5) cotton swab; 6) syringe; 7) GC-IMS device.

GC-IMS device

The spectrometer consists of a sample introduction system, a GC column, an ionization area, a drift tube and a detection area. The detector is an electrometer that measures the ion current as a function of time. Figure 5.2, illustrates the principal setup and internal gasflow of the equipment.

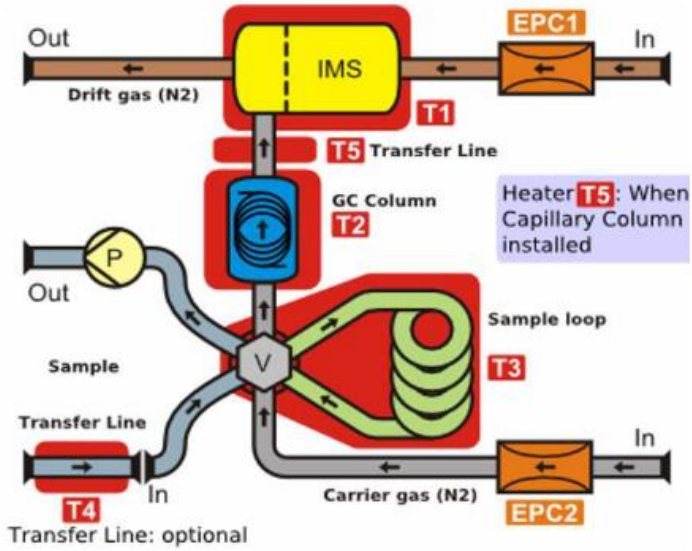


Figure 5.2 - Schematic of the internal gasflow and the composing elements of the GC-IMS device [64].

The sample is introduced into the device at the sample port. If the 6-Port-Valve (V) is at the default position, the gas sample will move through the loop by the action of the pump P and be redirected to the sample out socket. In addition, the carrier gas will be flushing the GC column and, both the carrier and the drift gases will leave the device at the gas out socket. At the second position of the valve, the carrier gas transports the sample into the GC column. The sample is then directed to the ionization region of the device. Here the sample molecules are ionized by an ionization source, which in the case of this device is a radioactive tritium source, with β-emission.

After ionization, the product ions, resulting from the ionization of the molecules in the sample are injected into the drift region through an ion-molecule injection shutter. The drift region, whose length is 9.8 cm, has several metal rings in order to maintain a homogeneous electric field gradient. The maintenance of a homogeneous field is important since any irregularity in the field leads to a larger drift time or non-repeatable reads [69]. The potential difference between the ends of the drift region is of 5000 V.

Certain regions of the device, IMS region (T1) and the GC column (T2), the sample loop (T3) and the transfer lines (T4 and T5), are heated and can be changed for different application, such as for analysing different substances, and for cleaning processes.

The drift and carrier gases are controlled by electronic pressure control units, EPC1 and EPC2, respectively. These are the parameters, as well as the opening time of the valve and the percentage of the pump to fill or clean the loop, passible of being altered by the user.

In Table 5.1 the characteristics of all the materials used in this experiment are registered.

Table 5.1 - List of the materials used during the course of this work, as well as their dimension and their manufacturer

Materials	Manufacturer
Ion Mobility Spectrometer coupled to a Gas Chromatography column (GC-IMS): 450 x 500 x 295 (WxDxH); 20 kg	G.A.S.® GmbH, Dortmund, Germany
GC column: MXT-200, 30 m X 0,53 mm ID	-------
Power Supply	Adjustable DC power supply PS23023 0-30V/0-3A x 2Way 5V Fixed
Digital Thermometer and hygrometer	------
Digital Chronometer	------
Analogic Heatblock	VWR®, Radnor, PA
Vials (20mL), with magnetic caps and rubber seals	VWR®, Radnor, PA
Teflon tube with female thread	Bohlender GmbH, Grünsfeld, Germany Swagelok, Solon Ohio
Sterile swab / cotton swab	FL medical/------
5 mL syringes	PIC Solution®, Pikdare S.r.l., Italy
21 G needles	BD™, Becton, Dickinson and Company, Franklin Lakes, New Jersey
Petri dishes	-------

Spectra

A GC-IMS chromatogram or spectrum is represented by the x-axis, the drift time, a y-axis, retention time and a colour scheme representing the signal intensity from the detector, z-axis. The x-axis has information regarding the drift time in milliseconds (ms) or relative to the RIP. The z-axis corresponds to the intensity obtained by the faraday plate as a voltage (V). Gas chromatography provides the y-axis information with the retention time given in seconds (s). As the separated compounds elude from the GC

column into the ionization region, a spectrum is plotted for a specific retention time. Each spectrum only requires 30 ms to be recorded [64]. The spectrometer may work in a positive or negative ionization mode of detection.

The chromatograms allow both a qualitative and a quantitative analyses where analyte concentration is correlated with peak height and area [64].

To study the resulting data, the Laboratory Analytical Viewer (LAV) software is required.

LAV software

The LAV software was developed by GAS and is used to visualize, both in 2D and 3D, and analyse the spectra produced by the GC-IMS equipment. Although the software has lots of functionalities, the most used ones were the standard and comparison Green/Red/Blue views, the Reporter and Gallery plugins and the Analytics functions. Figure 5.3 illustrates the main window of the LAV software. The standard view allows the visualization of the measured data in 2D, with the x-axis being the drift time, in milliseconds, and the y-axis the retention time, in seconds (Figure 5.3). The peaks' intensity is displayed resorting to colour. The higher the intensity, the redder the peaks.

The comparison Green/Red/Blue view allows the comparison between a maximum of three spectra. One of the three colours is attributed to a selected spectrum. If a new peak is present, it will be displayed by the colour attributed to the respective spectrum. Figure 5.4 illustrates the comparison Green/Red/Blue view.

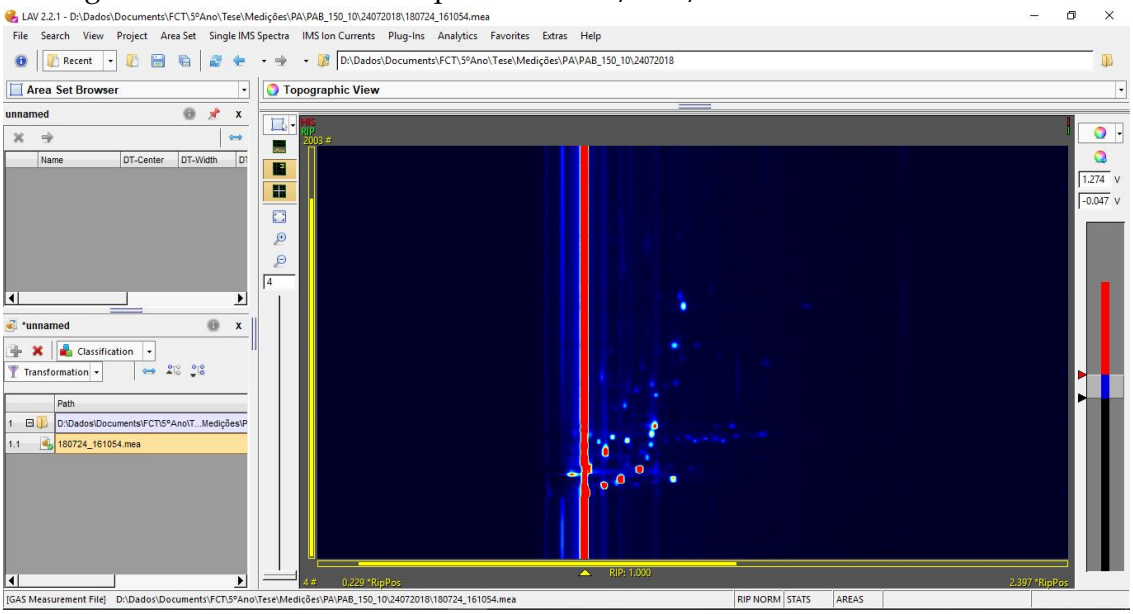


Figure 5.3 – Typical view of the LAV software. A spectrum of room air is shown in the standard view.

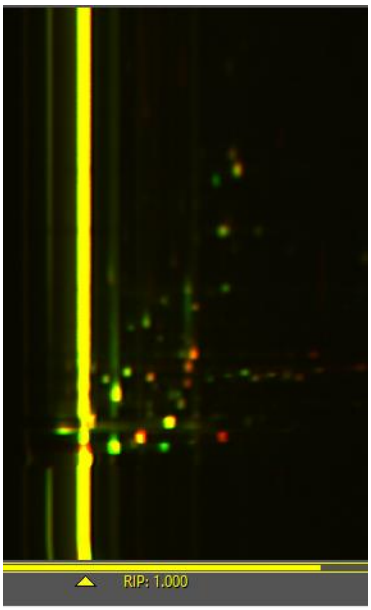


Figure 5.4 – Spectra with the Comparison Green/Red/Blue view. In this case, only two spectra were analysed. These are two spectra from room air. As can be observed, the peaks shared in both spectra present a yellow colour, while the non-shared ones present the colour attributed to their spectrum.

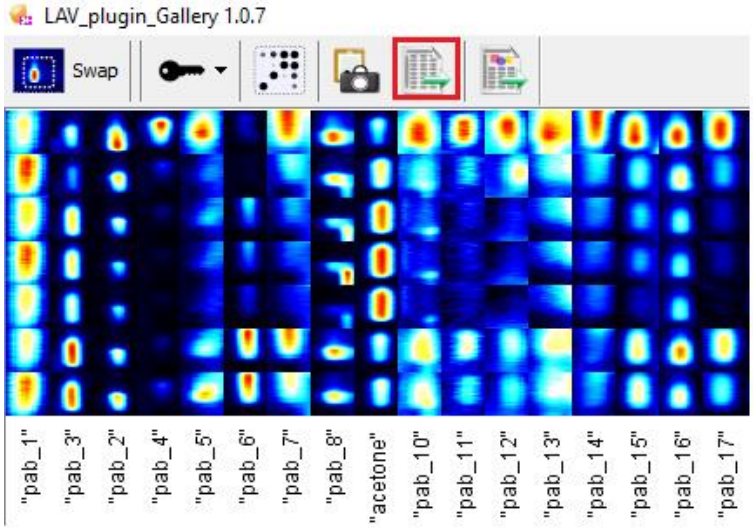


Figure 5.5 – Part of the window form the Gallery plugin. In this case, there are seven spectra being compared for the identified area sets. The name of the area set is placed bellow each column. The red rectangle identifies the functionality that provides the relative intensity of the area sets.

The first step in the spectra analysis is the definition of area sets. The area sets are defined by the user and enclose the peaks in the spectra. These area sets can then be compared throughout multiple spectra in the Gallery plugin of LAV to characterize and identify peaks. This feature provides the relative intensity of the peaks, which allows a much easier analysis. By exporting this data to an Excel sheet is possible to, automatically, obtain the peaks that show an intensity increase or decrease, from one spectra to the other. Figure 5.5 illustrates a part of the window of this plugin.

The characteristics of the peaks, such as retention time, drift time and intensity, RIP position and intensity can also be obtained in the Analytics tab, among other values. About the Reporter plugin, it places the chosen spectra side by side which allows a much easier comparison between spectra (Figure 5.6). In this plugin, spectra zoom in and zoom out is possible and retention times, drift times, intensity values and area sets can also be displayed.

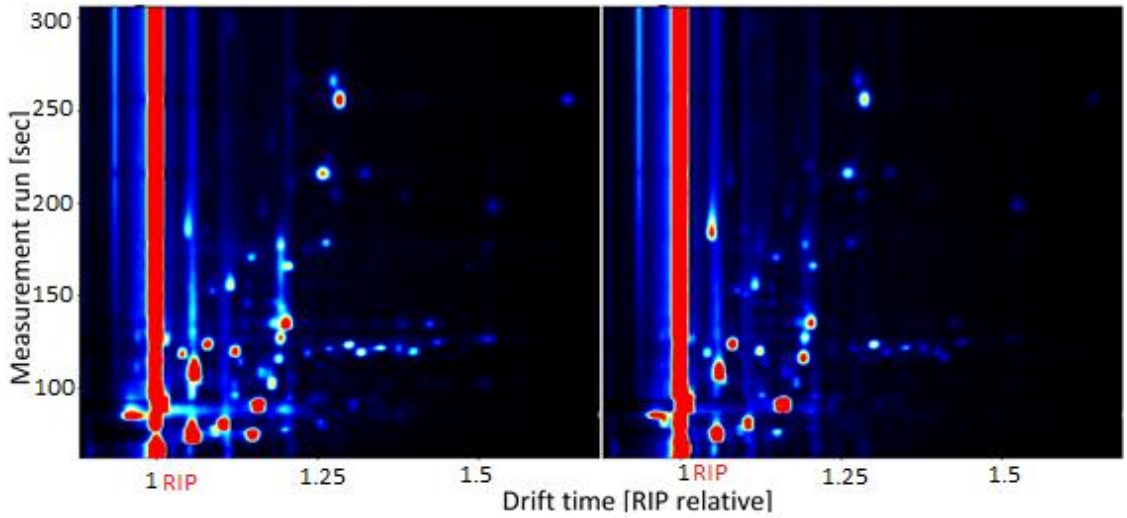


Figure 5.6 – Window for the Reporter plugin. Two or more spectra can be compared in this view. Here, it is possible to obtain the drift and retention times of the peaks and see if they coincide in the various spectra.

Preparation of the sample

In order to retrieve a bacterial sample from the culture, cotton swabs were used. The swabs were then placed into a 20 mL glass vial. The vial's septum was perforated with two needles in order to make retrieving of the headspace possible. The sample was introduced into the device by means of a syringe or a Teflon tube depending on the experimental procedure followed.

In addition, a heat block, a chronometer, thermometers and a hygrometer to measure room humidity in order to control the parameters of the measurement were used.

Previous protocol using MCC-IMS

Previous work has already been developed in the MCC-IMS equipment to determine the measurement parameters that contribute to attain the best results [10]. These parameters are both related to the equipment and the treatment of the sample.

Parameters relating to the device include the drift and carrier gases flows, 500 mL/min and 25 mL/min, respectively, and the running time of the program, 10 minutes.

To obtain the sample, it was concluded that heating the swabbed bacteria at 40 ºC during 10 minutes, would provide the best results. To achieve sample headspace equilibrium 10 minutes are required, meaning that the released VOCs have reached a uniform distribution inside the vial.

The pump of the device can be used to suck the headspace from the vial into the equipment.

The protocol used was as follows:

1. Gently roll the cotton swipe's head back and forth on the bacterial culture present in the Petri dishes.
2. Cut the swipe's head into a vial.
3. Close the vial and place it in the heat block.
4. Attach the Teflon tube from the sample port of the device to the vial.
5. Heat the vial at to 40 ºC during 10 minutes.
6. Run the measurement program.

Table 2 below contains the program previously developed.

Table 5.2 – Previously developed program used to perform the measurements.

Time (ms)	V	P	R	Drift gas (mL/min)	Carrier gas(mL/min)
0	|	12%	|	500	25
240	|	|	Rec	|	|
4160	Open	0%	|	|	|
16200	Closed	|	|	|	|
600000	|	|	Stop	|	|

Optimization of the parameters

The previous developed protocol was followed in order to obtain the first measurements. Despite providing good results, the program could be improved for a better performance.

Since the chromatographic columns of the GC-IMS and the MCC-IMS are different, the drift and carrier gases flow had to be adjusted. For the drift gas flow the value chosen was 150 mL/min, not only because it was already used in other works of the group but also because previous calibrations were performed with the same value. The calibration is necessary to identify signals in the spectra.

The carrier gas flow was also altered considering pre-separation would not be compared between the two GC columns. After preliminary measurements, it was clear

that the majority of the compounds were present at the bottom of the spectra. A low flow was chosen at the start of the program to allow a better separation of these substances. The carrier flow started at 10 mL/min and was increased to 40 mL/min by the end of the program, allowing a short running time for each measurement.

Finally, the opening time of the valve was studied. As the valve controls the quantity of injected sample to be analysed, it is important that the majority of the sample enters the drift region for analysis. The sample loop has 1 mL. If the sample introduced at the sample port is less than the capacity of the loop, the rest will be filled with air. If, however, it is more, only 1ml will go through.

The valve was open for 10 seconds, which is equivalent to approximately 1.7 mL of headspace with the initial flow of 10 mL/min.

Figure 5.7 illustrates the effect of the carrier gas flow and the opening of the valve on the spectra. In the program of the Figure 5.7 a), the valve was opened for 4 seconds at an initial flow of 10 mL/min for the first minute. In Figure 5.7 b), the valve was opened for 10 seconds at the initial flow (10 mL) for the first 1 min 30 s.

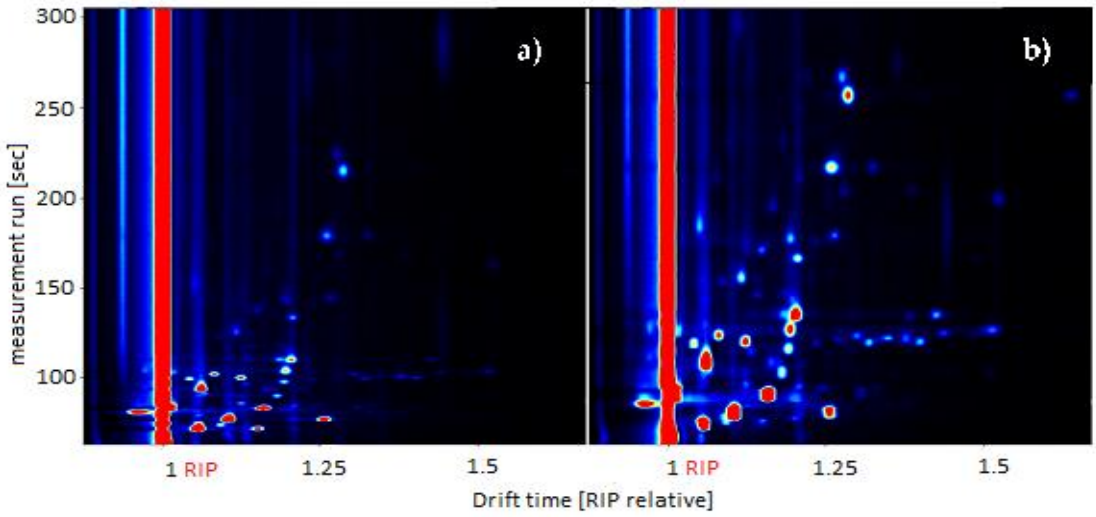


Figure 5.7 – Comparison between the spectra obtained in the GC-IMS equipment with different carrier gas flows and valve opening time: a) carrier gas flow of 10 mL/min during 1 min and valve opening time of 4 seconds; b) carrier gas flow of 10 mL/min during the first 1min30s and valve opening time of 10 seconds.

As can be observed from the previous figure, the flow changes allow a much better resolution of the peaks.

Figure 5.8 shows the final program used to obtain the spectra.

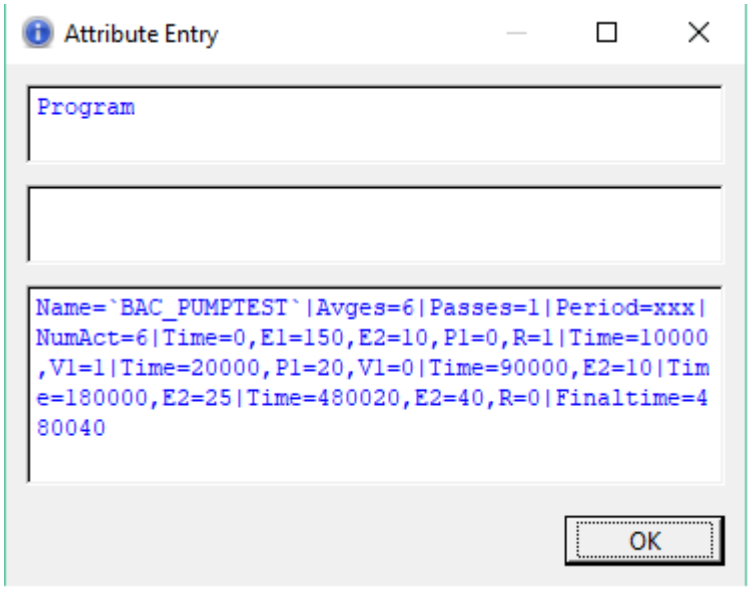


Figure 5.8 – Program used in the GC-IMS to perform the measurements (BAC_PUMPTEST). The different parameters are time (in milliseconds), drift and carrier gases flows (E1 and E2, respectively), pump (P), valve (V) and record (R).

The time is in milliseconds. R represents the recording of the data and V1 is the valve. Once these variables are set to 1, the recording is on and the valve opened. If set to 0, the data is not being recorded and the valve is closed. The E1 and E2 are equivalent to EPC1 and EPC2, which control the flow of the drift and carrier gases, respectively. P1 represents the percentage activated of the pump. In this case 20 %. Once again, if P1 is 0, the pump is turned off. Turning the pump on after the valve is turned off does not affect the data in any way since the connection with the sample inlet is cut off once the valve is closed. This is very useful since it helps to clean the sample loop during the running of the program.

Experimental procedure

As mentioned before, the first spectra were obtained using a previously developed protocol. However, since the GC-IMS is more sensible to VOCs' than the MCC-IMS, it was hypothesised that there would be enough VOCs in the headspace at room temperature retrieved from the vial to obtain good results of bacterial samples. A measurement for one vial was made at room temperature, followed by a measurement after the same vial heated to 40 ºC.

The experimental procedure used was as follows:

1. Gently roll the cotton swipe's head back and forth on the bacterial culture.
2. Cut the swipe's head into a vial.
3. Close the vial and place it in the heat block.

4. Attach the Teflon tube from the sample port of the device to the vial.
5. Heat the vial at 40 ºC during 10 minutes/wait for 10 minutes at room temperature for the headspace to reach an equilibrium.
6. Run the program.

The only difference from this protocol to the one with the optimized values is at point 4. Instead of attaching the Teflon tube to the vial, a syringe was used to retrieve 2 mL of headspace, which was later injected into the device. The means of introducing the sample into the device was adjusted because it was noticed that the pump was not working at the best conditions. Despite this set back, it was confirmed that, even after several measurements, the spectra did not show signs of substance accumulation.

During the course of this work two types of swabs were used: sterile and normal swabs (bought in the local market). Both were used because the sterile swabs ran out. Nonetheless, the measurements were not affected since the spectra of both swabs did not show significant changes.

Figure 5.9 contains an illustration of the experimental procedure followed to obtain the measurements.

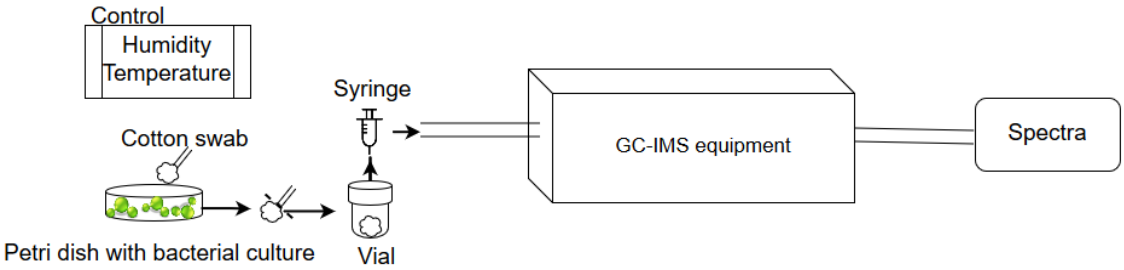


Figure 5.9 - Experimental procedure of the used method to obtain VOCs profile of bacteria. The cotton swab used to swab the bacterial culture is placed in a vial. Headspace air is extracted from the vial and introduced in the GC-IMS equipment. The data is displayed in the form of a spectra

Bacteria and cultures measured

The bacteria species analysed were *Escherichia coli* and *Pseudomonas aeruginosa*, each grown in three different media: one specific for each of the bacteria and one where both can grow under the same conditions. The Tryptic Soy Agar (TSA) and Pseudomonas Agar Base (PAB) media are specific to *E. coli* and *P. aeruginosa*, respectively, while both can grow in LB agar ('Lisogeny' or Luria Broth). A mixture of both bacteria grown in LB agar was also studied. The *E. coli* strained used in this work was ATCC 25922 pCU18. The *P. aeruginosa* strained used was ATCC 27853. Bacteria were maintained in LB broth, which is a liquid medium.

The growth media were prepared by following the instructions provided by the manufacturer. TSA is composed of pancreatic digest of casein, papaic digest of soya bean, sodium chloride and agar. PAB contains gelatin peptone, casein hydrolysate, potassium sulfate anhydrous, magnesium chloride anhydrous and agar. LB agar is composed of agar, tryptone, yeast extract and sodium chloride. LB broth consists of tryptone, yeast extract and sodium chloride. Detailed information is given in Appendix A. The preparation of the bacterial samples was made by surface plating. This method is described in detail in Appendix B.

Bacteria were incubated at 37ºC during a 24 and 48-hour period.

In order to know if the bacteria were indeed producing VOCs and the peaks obtained were not due to the agar in which the bacteria grew, measurements of the culture growth medium, called blank, were performed too. The blank consists of the headspace contained in the vial from the swabbing of the growth medium, which suffered the same conditions as the culture. With the purpose of obtaining enough data, 5 swabs were made from each culture and, at least, 3 from the blank.

Figure 5.10 shows a schematic representation of the cultures made, the number of collected swabs and the number of samples analysed.

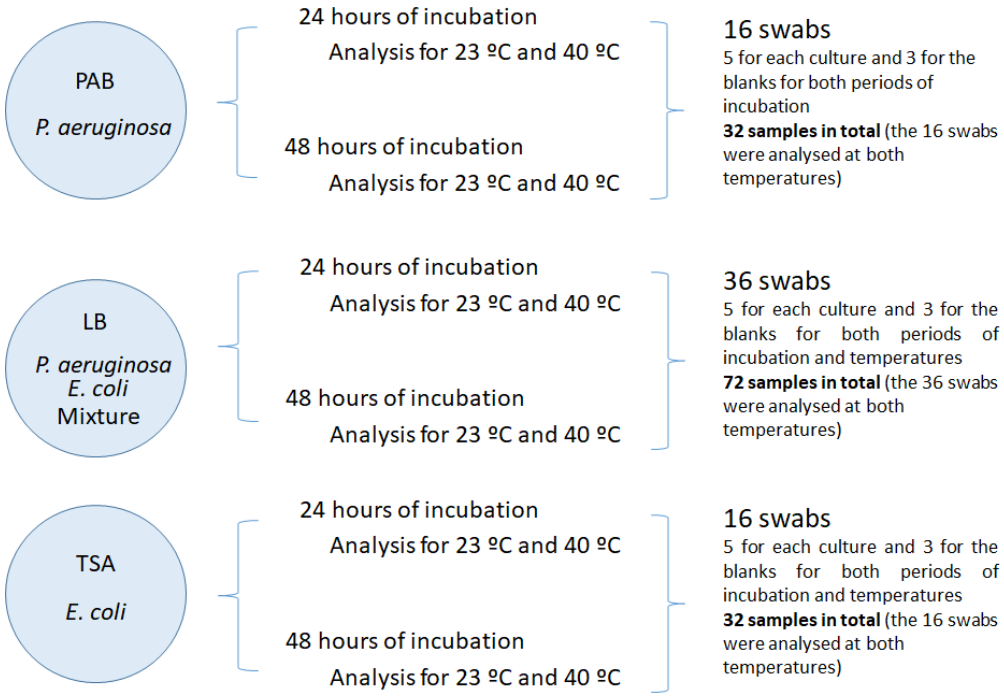


Figure 5.10 – Schematic of the cultures made in each media and their incubation periods. In addition, there is information regarding the temperatures in which the bacterial sample was analysed, the number of swabs taken from each media and the number of samples analysed.

The cultures were disposed of in an appropriate container for biological waste.

6

Results and Discussion

In this chapter, the data from LB growth medium for both bacteria and the mixture are analysed first. Then, the data obtained for the specific media are studied.

Since several parameters were studied in order to obtain the most representative spectra, flows, media and temperature, it is necessary to choose the best ones. Bearing in mind the optimization of the protocol, only the results obtained from the optimized protocol will be analysed in this chapter.

In this analysis, special attention was given to the growth medium LB because this is the medium in which both bacteria grow and seems more interesting for the purpose of bacterial identification when in the presence of a mixture.

The effect of the temperature will be studied for each bacteria. As a hypothesis, the increase in temperature may have an effect on the volatility of the compounds or, since it is higher than the temperature in which the bacteria grew, on their growth. The common peaks for both temperatures will be analysed in terms of their intensity.

Throughout the analysis of all spectra, the relevant peaks are either the ones that changed the intensity from the blank to the culture or new peaks only present in the culture. The intensity increase and the new peaks are represented in all of the graphs below by red dots. The intensity decrease is illustrated by green dots. The drift position present in the x-axis of all graphs shown below is RIP relative and, therefore, is dimensionless.

LB growth media

P. aeruginosa

After a period of 24 hours of incubation in LB growth medium

In this section, the spectra from *P. aeruginosa* in the LB medium after a 24 h-period incubation are analysed. Figure 6.1 illustrates the result of this analysis.

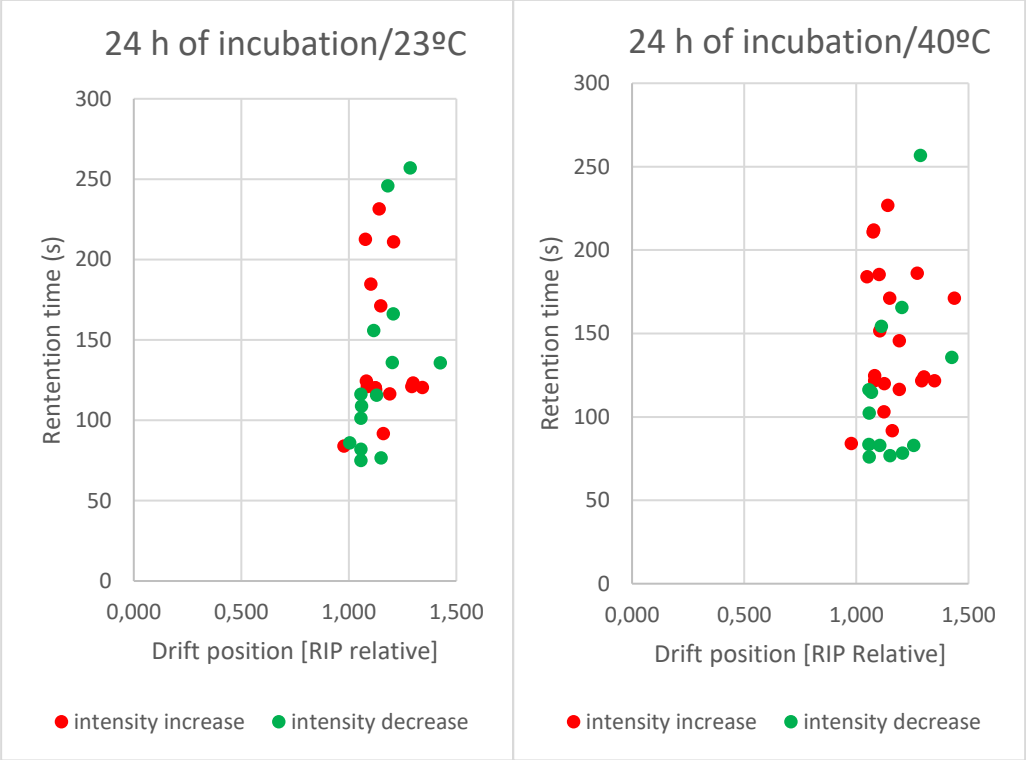


Figure 6.1 – Relevant peaks found after 24 hours of incubation for *P. aeruginosa* at 23 ºC (left) and 40 ºC (right) in the nutrient medium LB. The red dots indicate intensity increase from the blank to the culture. The green dots illustrate intensity decrease from the blank to the culture. The x-axis represents a drift position relative to the RIP and therefore has no units. The y-axis represents the retention time and is expressed in seconds.

The amount of relevant peaks obtained is approximately the same for both temperatures: 30 for room temperature (23 ºC) and 33 for 40 ºC. From these peaks, the amount of points refering to emission (red dots) and consumption (green dots) is also quite similar: 16 red and 14 green for room temperature and 20 red and 13 green for the higher temperature.

For a more in depth analysis, it is essential to analyse the common peaks from both temperatures. These common compounds are illustrated in Figure 6.2.

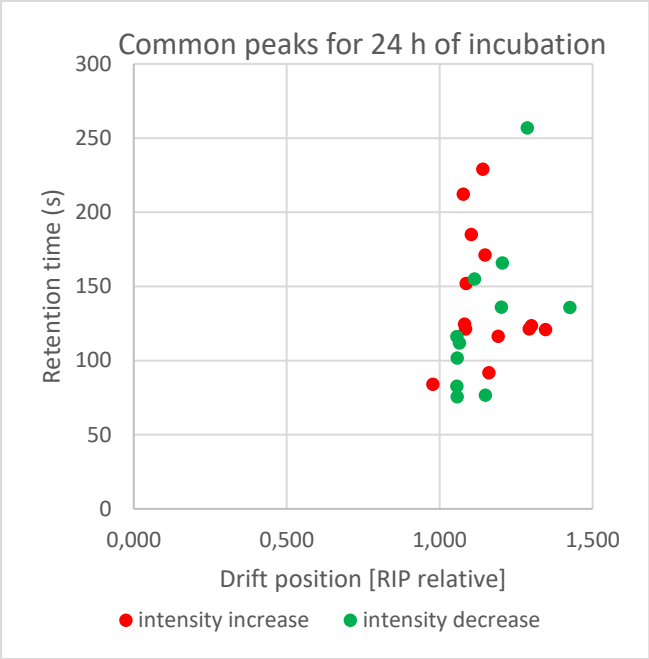


Figure 6.2 - Design created by the common peaks for *P. aeruginosa* after 24 hours of incubation in LB growth medium at both temperatures. The y-axis represents the retention time, expressed in seconds, and the x-axis, the drift position relative to the RIP. The red dots identify peaks with an intensity increase when compared to the blank. The green dots represent the opposite.

From the common data for the 24 hour period incubation, 11 peaks show reduction in intensity and 13 show increase. In order to check if the increase of temperature had any effect on the VOCs' volatility, the intensity of each of these peaks was analysed. Table 6.1 contains the values of intensity of each peak and their variation. The peaks' intensity was taken as relative to the RIP's intensity so it is dimensionless. The variation of the intensity was then calculated. It was considered that any difference smaller than 0.01 was insignificant. The blue lines identify the substances with a significant change in intensity.

Table 6.1 - Relative intensities of the mutual peaks found for *P. aeruginosa* after 24 hours of incubation in the LB growth media at 23 ºC and at 40 ºC and the absolute difference (variation) between the two. The intensity variation column indicates whether the peaks' intensity increased (+) or decreased (-).

Relative Intensity at 23 ºC	Relative Intensity at 40 ºC	Variation	Intensity variation
0.566	0.450	0.117	-
0.122	0.039	0.083	-
0.050	0.031	0.019	-
0.753	0.823	0.070	+
0.270	0.266	0.004	-
0.094	0.102	0.008	+

0.222	0.271	0.049	+
0.053	0.043	0.010	-
0.010	0.010	0.000	-
0.043	0.052	0.009	-
0.028	0.032	0.003	-
0.032	0.036	0.003	-
0.039	0.130	0.091	+
0.117	0.153	0.036	-
0.135	0.274	0.140	+
0.027	0.030	0.003	+
0.073	0.101	0.028	+
0.103	0.186	0.083	+
0.036	0.198	0.163	+
0.085	0.088	0.003	+
0.043	0.063	0.019	+
0.167	0.187	0.020	+
0.028	0.054	0.026	+
0.270	0.116	0.154	-

The number of compounds that had a significant difference was 15 out of 24. From those, all except one accentuated their behaviour, meaning that if at room temperature the bacteria were consuming a compound, at 40 ºC its intensity was reduced. If, however, at room temperature a compound was being produced, at 40 ºC its intensity was greater. The temperature appears to be having an effect on the bacteria's metabolism.

In addition, there are also peaks only present in culture. Table 6.2 shows the retention time, in seconds, and drift position relative to the RIP of these peaks.

Table 6.2 - Retention time, in seconds, and drift position relative to the RIP of the peaks only present in the culture for *P. aeruginosa* in LB growth medium after 24 hours of incubation.

Retention time (s)	Drift position [RIP relative]	Retention time (s)	Drift position [RIP relative]
84	0.978	171	1.149
102	1.058	184	1.049
121	1.085	185	1.103
121	1.293	212	1.078
121	1.346	229	1.141
124	1.301		

Figure 6.3 is a series of typical spectra of the blank, the culture at 23 ºC and at 40ºC, respectively. Identified by green rectangles are the substances only present in the culture enumerated in Table 6.2.

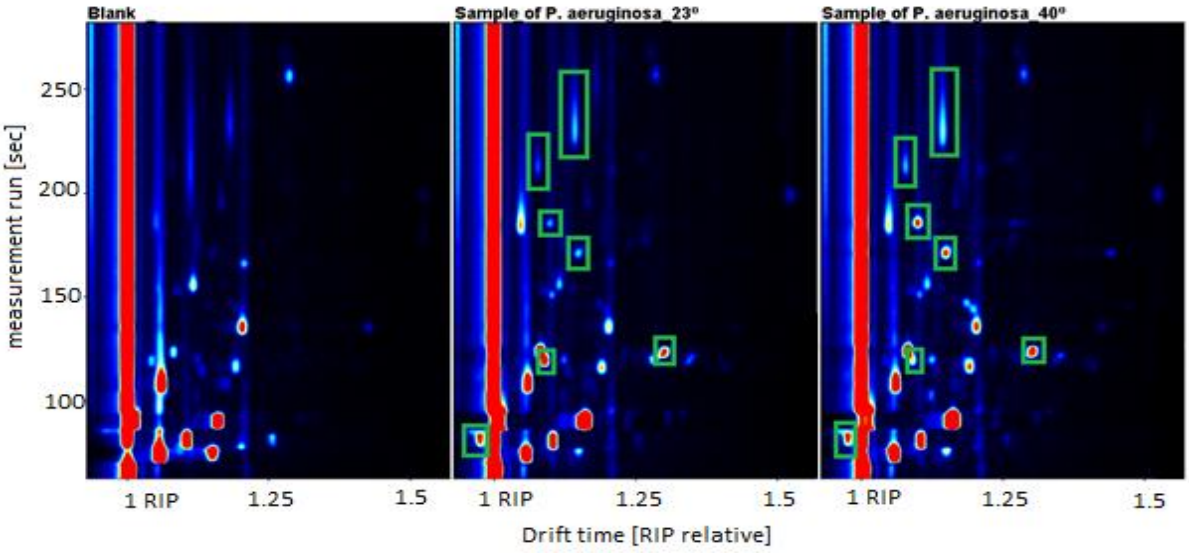


Figure 6.3 – Characteristic spectra of the blank and the culture of *P. aeruginosa* at 23 ºC and 40 ºC after 24 hours of incubation. Identified in green rectangles are the peaks only found in the culture.

After a period of 48 hours of incubation in LB growth medium

In this section, the spectra from *P. aeruginosa* in the LB medium after a 48 hour period incubation are analysed. Figure 6.4 illustrates the result of this analysis.

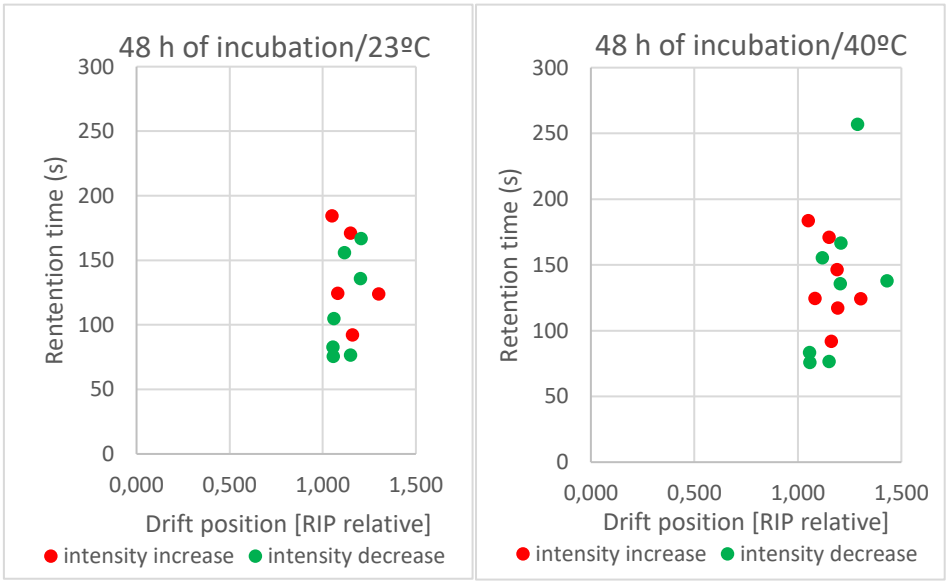


Figure 6.4 - Relevant peaks found after 48 hours of incubation at 23 º C (left) and at 40 ºC (right) for *P. aeruginosa* in the nutrient medium LB. The red dots indicate intensity increase from the blank to the culture. The green dots illustrate intensity decrease from the blank to the culture. The x-axis represents a position relative to the RIP and therefore has no units. The y-axis represents the retention time and is expressed in seconds.

The most obvious difference from the two periods of incubation is the amount of relevant peaks. After a 48 hour period incubation, the bacteria seem less active than after 24 hours of incubation.

From the 12 peaks found relevant at room temperature and the 15 found at 40 ºC, 11 are shared. There are 5 compounds which suffered an intensity increase from the blank to the culture and 6 that suffered an intensity decrease.

Figure 6.5 illustrates the design found for 48 hours of incubation, with the common compounds produced at both temperatures.

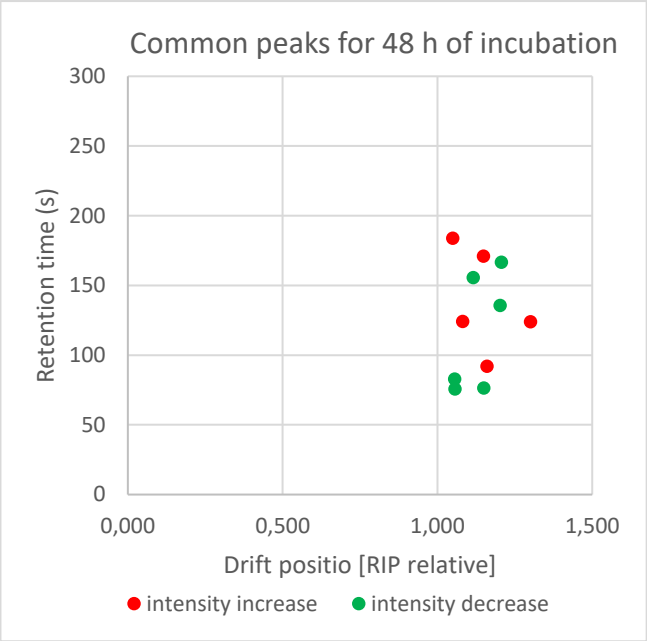


Figure 6.5 - Design created by the common peaks for *P. aeruginosa* after 48 hours of incubation in LB growth medium. The y-axis represents the retention time, expressed in seconds, and the x-axis, the drift position relative to the RIP. The red dots identify peaks with an intensity increase when compared to the blank. The green dots represent the opposite.

Once more, the effect of the temperature on the compounds' intensity was studied. Table 6.3 contains the peaks' relative intensity at both 23 ºC and 40 ºC and the absolute difference between the two. The intensity variation indicates whether the compound increased (+) or decreased in comparison to the blank. The blue shading identifies the substances with a significant change in intensity.

Table 6.3 - Relative intensities of the mutual peaks found for *P. aeruginosa* after 48 hours of incubation in the LB growth media at 23 ºC and at 40 ºC and the absolute difference (variation) between the two. The label indicates whether the peaks' intensity increase when compared to the blank (+) or decreased (-).

Relative Intensity at 23 ºC	Relative Intensity at 40 ºC	Variation	Intensity variation
0.584	0.495	0.089	-
0.095	0.045	0.049	-
0.038	0.029	0.009	-
0.710	0.771	0.061	+
0.120	0.116	0.004	-
0.187	0.263	0.076	+
0.036	0.032	0.004	-
0.033	0.071	0.038	+
0.051	0.139	0.088	+
0.092	0.110	0.018	+
0.047	0.043	0.004	-

Most of the peaks were affected by the increase in temperature (7 out of 11). Here, it appears that the emission of compounds ordinarily released by the culture (red dots) at room temperature is improved by the increase in temperature. On the contrary, the increase in temperature, seems to have augmented the consumption of compounds, since the decrease in intensity is accentuated.

All of the relevant compounds found at room temperature after 48 hours of incubation are also present at 24 hours of incubation. The same is true for 40 ºC, except for one. At 48 hours of incubation there are two compounds only present in the culture. Table 6.4 contains the characteristics relating to retention time, in seconds, and drift position, relative to the RIP, of these compounds.

Table 6.4 - Retention time, in seconds, and drift position relative to the RIP of the peaks only present in the culture for *P. aeruginosa* in LB growth medium after 48 hours of incubation.

Retention time (s)	Drift position [RIP relative]
124	1.302
184	1.049

Figure 6.6 illustrates the position of the compounds previously mentioned in the previous in Table 6.4.

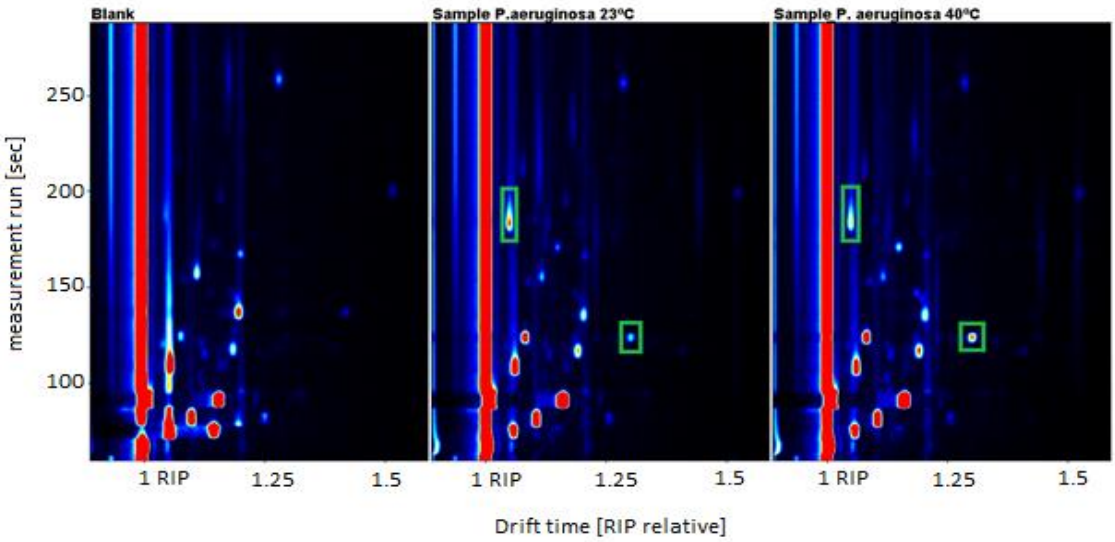


Figure 6.6 - Characteristic spectra of the blank and the culture of *P. aeruginosa* at 23 ºC and 40 ºC after 48 hours of incubation. Identified in green rectangles are the peaks only found in the culture.

For this bacteria species the best option for identification appears to be a pattern instead of just compounds only produced by the bacteria. Since all of the relevant peaks found after 48 hours of incubation are present after 24 hours of incubation (except one at 40 ºC) and in less quantity, these should be the peaks used to find the pattern.

Therefore, there are two possibilities for the pattern to identify *P. aeruginosa*. The one with the peaks present at both periods of incubation at room temperature (23 ºC) or the one with the common peaks for both periods of incubation at 40 ºC. Both patterns will be tested when analysing the relevant peaks of the bacterial mixture. The one that provides the best results will be chosen.

Table 6.5 shows the retention time, in seconds, and drift position, RIP relative, of the peaks from the possible pattern. In addition, the intensity variation identifies the peaks that suffered an intensity increase from the blank to the culture (+) and the ones that decrease their intensity (-).

Table 6.5 - Retention time, in seconds, and drift position (RIP relative) of the peaks found for the possible patterns for *P. aeruginosa* grown in LB growth medium. The patterns were obtained for 23 ºC (room temperature) and 40 ºC. Intensity variation indicates whether the peaks have increased their intensity from the blank to the culture (+) or decreased (-).

Pattern for 23 ºC			**Pattern for 40 ºC**		
Retention time (s)	Drift position [RIP relative]	Intensity variation	Retention time (s)	Drift position [RIP relative]	Intensity variation
76	1.056	-	76	1.058	-
77	1.150	-	77	1.151	-
83	1.055	-	83	1.056	-
92	1.160	+	92	1.161	+
105	1.059	-	117	1.192	+
124	1.081	+	124	1.082	+
124	1.300	+	124	1.303	+
136	1.202	-	138	1.431	-
156	1.115	-	146	1.189	+
167	1.205	-	155	1.117	-
171	1.148	+	167	1.208	-
184	1.048	+	171	1.150	+
			184	1.050	+
			257	1.288	-

E. coli

After a period of 24 hours of incubation in LB growth medium

In this section, the spectra from *E. coli* in the LB medium after a 24 hour period incubation are studied. Figure 6.7 illustrates the result of this analysis.

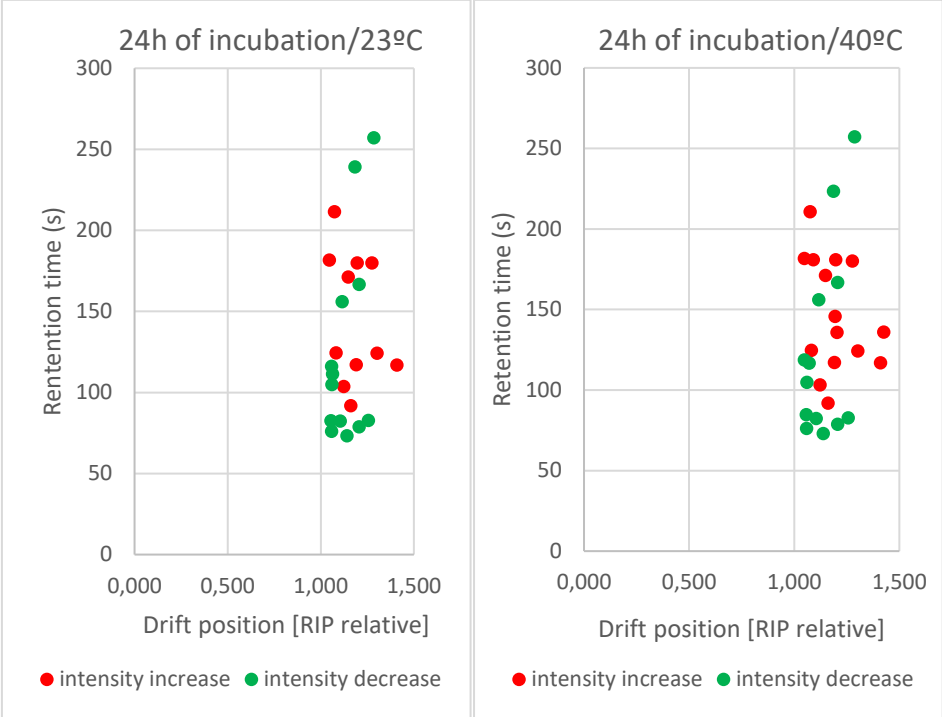


Figure 6.7 - Results of the analysis for *E. coli* in the LB growth medium, after 24 hours of incubation. The red dots identify peaks with an intensity increase in the culture when compared to the blank. The green dots illustrate the peaks with an intensity decrease in the culture when compared to the blank. The y-axis represents the retention time, in seconds, and the x-axis represents the drift position taken relative to the RIP.

The number of relevant peaks for both temperatures is quite similar. At room temperature, 13 peaks suffered an intensity decrease from the blank to the culture (green dots) and 11 peaks, an intensity increase (red dots). At 40 ºC, 15 peaks are green and 15 are red.

Figure 6.8 illustrates the mutual peaks found at both temperatures.

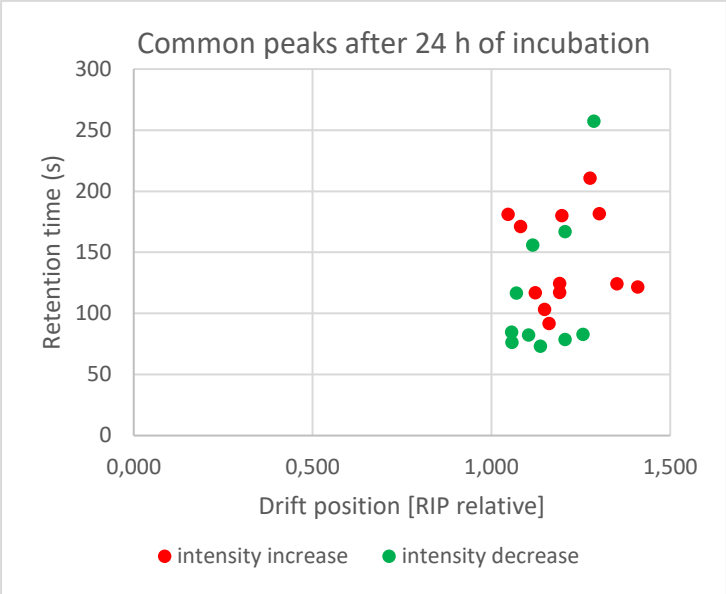


Figure 6.8 - Design created by the common peaks for *E. coli* after 24 hours of incubation in LB growth medium. The y-axis represents the retention time, expressed in seconds, and the x-axis, the drift position relative to the RIP. The red dots identify peaks with an intensity increase when compared to the blank. The green dots represent the opposite.

Table 6.6 contains the relative intensity of the common peaks at both 23 ºC and 40 ºC, as well as, the absolute difference between the two. Again, the blue lines represent the peaks which suffered a significant variation in intensity when the sample temperature was increased. The intensity variation identifies the peaks that suffered an intensity increase from the blank to the culture (+) and the ones that decrease their intensity (-).

Table 6.6 - Relative intensities of the mutual peaks found for *E. coli* after 24 hours of incubation in the LB growth media at 23 ºC and at 40 ºC and the absolute difference (variation) between the two. The label indicates whether the peaks' intensity increase when compared to the blank (+) or decreased (-).

Relative intensity at 23 ºC	Relative intensity at 40 ºC	Variation	Intensity variation
0.234	0.176	0.058	-
0.257	0.248	0.009	-
0.014	0.015	0.001	-
0.011	0.010	0.000	-
0.018	0.016	0.002	-
0.020	0.019	0.000	-
0.757	0.775	0.018	+
0.266	0.268	0.003	-
0.148	0.150	0.002	+
0.265	0.283	0.018	+
0.031	0.035	0.004	-
0.032	0.040	0.007	-

0.024	0.026	0.003	+
0.044	0.043	0.001	-
0.023	0.042	0.018	+
0.019	0.019	0.000	+
0.013	0.014	0.001	+
0.141	0.205	0.064	+
0.318	0.312	0.006	+
0.067	0.059	0.008	+
0.046	0.039	0.006	+
0.052	0.042	0.010	+

From the 22 mutual peaks, 6 show a variation of 0.01 or bigger. From these, the behaviour is similar to the previously described: the consumed compounds suffered a decrease in intensity for 40 ºC and the produced ones, an increase. Nonetheless, the intensity of the great majority of the compounds was not altered.

Table 6.7 contains the retention time, in seconds, and drift position, RIP relative, of the peaks only present in the culture.

Table 6.7 - Retention time, in seconds, and drift position relative to the RIP of the peaks only present in the culture for *E. coli* in LB growth medium after 24 hours of incubation.

Retention time (s)	Drift position [RIP relative]
124	1.302
180	1.276
181	1.197
182	1.047
211	1.075

Figure 6.9, identifies the position of these peaks in the spectra.

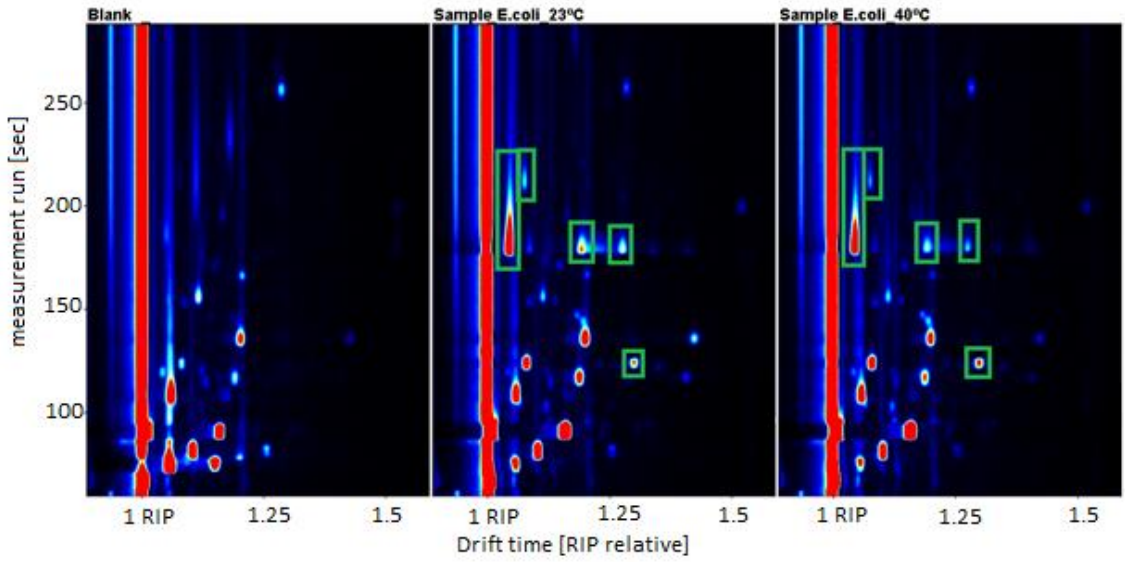


Figure 6.9 - Characteristic spectra of the blank and the culture of *E. coli* at 23 ºC and 40 ºC after 24 hours of incubation. Identified in green rectangles are the peaks only found in the culture.

After a period of 48 hours of incubation in LB growth medium

In this section, the spectra from *E. coli* in the LB medium after a 48 hour period incubation are analysed. Figure 6.10 illustrates the results of this analysis.

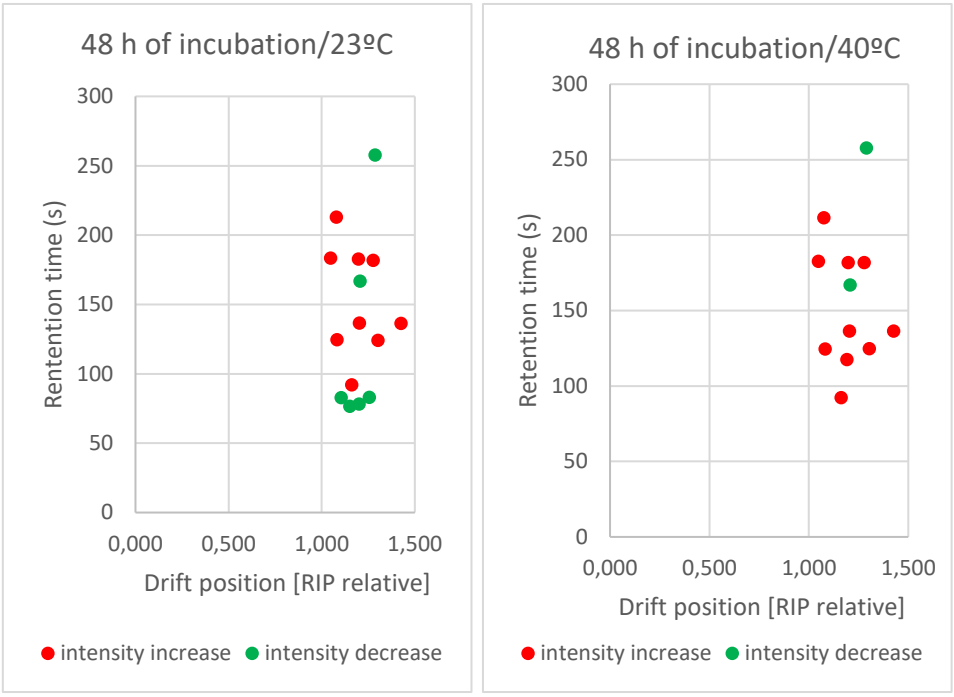


Figure 6.10 – Results of the analysis for *E. coli* in the LB growth medium, after 48 hours of incubation. The red dots identify peaks with an intensity increase in the culture when compared to the blank. The green dots illustrate the peaks with an intensity decrease in the culture when compared to the blank. The y-axis represents the retention time, in seconds, and the x-axis represents the drift position taken relative to the RIP.

The graphs for both temperatures at 48 hours of incubation are slightly different. The majority of peaks referring to consumption of substances has disappeared from room temperature to the higher temperature. At the higher temperature, the bacteria do not appear to be consuming as much as when at room temperature. On the contrary, the bacteria seem to be producing approximately the same compounds, since the amount of peaks with increased intensity is quite similar.

Further analysis will be done referring to the common peaks from both temperatures. The pattern formed by the common peaks is illustrated in Figure 6.11.

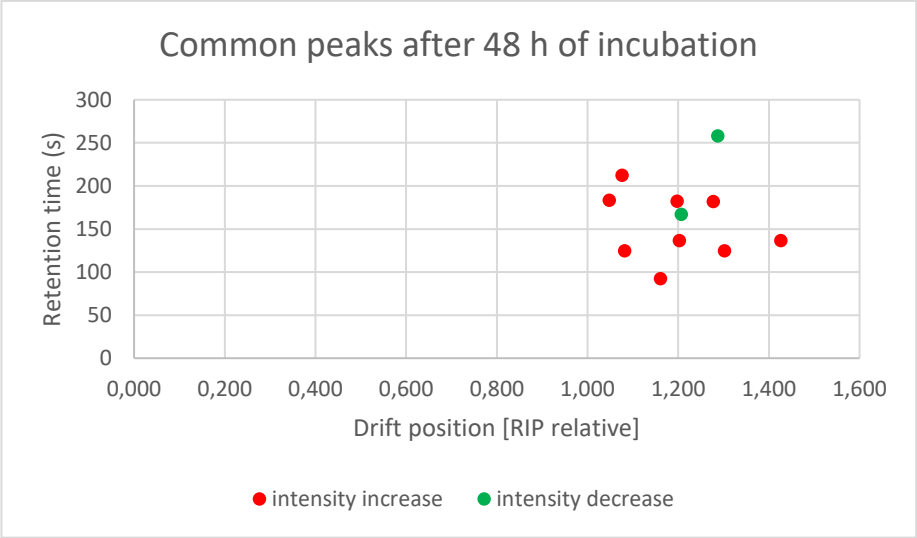


Figure 6.11 – Design created by the common peaks for *E. coli* after 48 hours of incubation in LB growth medium. The y-axis represents the retention time, expressed in seconds, and the x-axis, the drift position relative to the RIP. The red dots identify peaks with an intensity increase when compared to the blank. The green dots represent the opposite.

Table 6.8 shows the relative intensity of the shared peaks and their variation. Once more, the blue lines identify the compounds that presented a significant variation in intensity. The intensity variation column indicates a growth (+) or decline (-) in intensity when comparing the culture and the blank.

Table 6.8 – Relative intensities of the mutual peaks found for *E. coli* after 48 hours of incubation in the LB growth media at 23 ºC and at 40 ºC and the absolute difference (variation) between the two. The intensity variation column indicates whether the peaks' intensity increased when compared to the blank (+) or decreased (-).

Relative intensity at 23 ºC	Relative intensity at 40 ºC	Variation	Intensity variation
0.668	0.760	0.092	+
0.187	0.204	0.017	+
0.138	0.216	0.078	+
0.028	0.035	0.006	+
0.025	0.025	0.000	-
0.032	0.033	0.000	-
0.026	0.077	0.051	+
0.250	0.274	0.024	+
0.033	0.040	0.007	+
0.021	0.025	0.004	+
0.032	0.029	0.003	+

Only 5 of the 11 peaks show a variation bigger than 0.01 with the increase in temperature. The increase in temperature does not appear to affect the intensity of the common peaks in a significant way since less than half of them were affected by this variation.

Table 6.9 shows the peaks only present in the culture after 48 hours of incubation.

Table 6.9 – Retention time, in seconds, and drift position relative to the RIP of the peaks only present in the culture for *E. coli* in LB growth medium after 48 hours of incubation.

Retention time (s)	Drift position [RIP relative]
124	1.302
182	1.198
182	1.277
183	1.048
212	1.076

The characteristics of the peaks only found in the culture for both temperatures are the same as the ones in Table 6.9.

Figure 6.12 contains the spectra of the blank, the sample of *E. coli* at 23 ºC and 40 ºC. The peaks identified by green rectangles are the ones shown in Table 6.9.

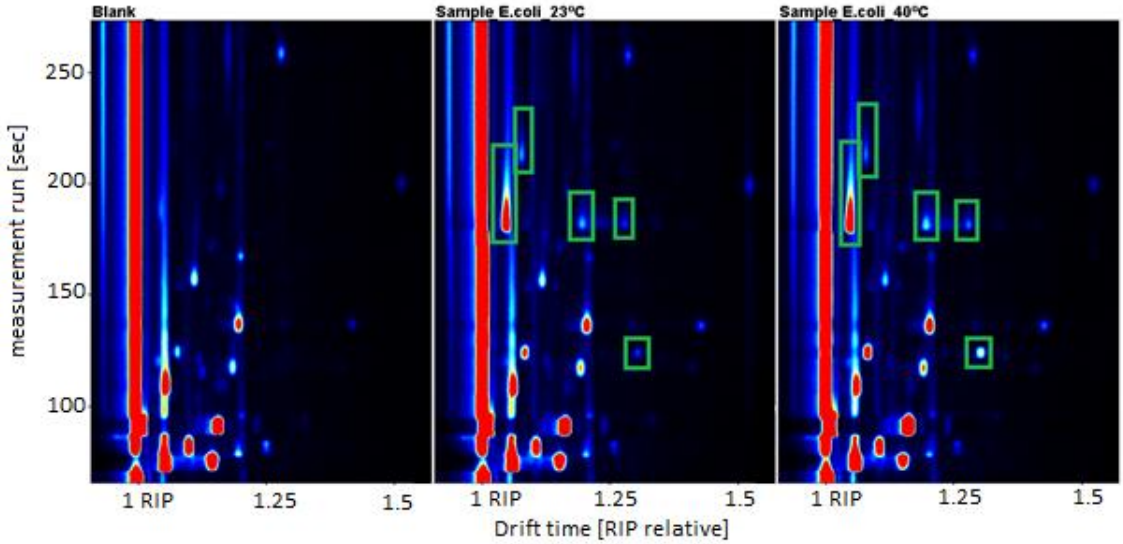


Figure 6.12 - Characteristic spectra of the blank and the culture of *E. coli* at 23 ºC and 40 ºC after 48 hours of incubation. Identified in green rectangles are the peaks only found in the culture. The x-axis represents the drift time relative to the RIP. The y-axis indicates the retention time/measurement run, in seconds.

When comparing both periods of incubation, the amount of relevant peaks found after 48 hours of incubation suffers an accentuated drop. It seems that there are two options for the pattern: either the pattern to identify *E. coli* arises from the peaks found at room temperature or from the peaks found at 40 ºC. If the former option is chosen, the

pattern for 23ºC includes 13 peaks. If the latter, this number cuts to 12. Both will be put to test in the analysis of the bacterial mixture to see which one provides the best results.

Table 6.10 shows the retention times (in seconds) and RIP relative drift positions for the possible patterns.

Table 6.10 – Retention time, in seconds, and drift position (RIP relative) of the peaks found for the possible patterns for *E. coli* grown in LB growth medium. The patterns were obtained for 23 ºC (room temperature) and 40 ºC. Intensity variation indicates whether the peaks have increased their intensity from the blank to the culture (+) or decreased (-).

Pattern for 23 ºC			**Pattern for 40 ºC**		
Retention time (s)	Drift position [RIP relative]	Intensity variation	Retention time (s)	Drift position [RIP relative]	Intensity variation
77	1.150	-	92	1.162	-
78	1.201	-	117	1.191	-
83	1.104	-	125	1.082	+
83	1.256	-	125	1.303	+
92	1.161	+	136	1.203	-
124	1.301	+	137	1.427	-
125	1.082	+	167	1.207	+
167	1.206	-	182	1.198	+
182	1.277	+	182	1.278	-
183	1.197	+	183	1.048	+
184	1.048	+	212	1.075	-
213	1.078	+	258	1.288	-
258	1.286	-			

Mixture

After the individual analysis of each bacteria, it is necessary to verify the possibility of identification of both bacteria in a mixture. One way to do this is to analyse the mixture and see if the relevant peaks that arise are matching any of the peaks found to be relevant in the analysis of the bacteria individually.

There were two options for the usable patterns of both bacteria: room temperature (23 ºC) and 40 ºC. Both were tested. The one that allowed a better identification of both bacteria was the one for room temperature, which also brings the big advantage of taking less time to obtain the measurements. Therefore, the results to be presented bellow refer to room temperature. The results for 40 ºC are present in Appendix C.

After 24 hours of incubation in LB growth medium

Figure 6.13 illustrates the design created by the relevant peaks found at room temperature for 24 hours of incubation for the mixture.

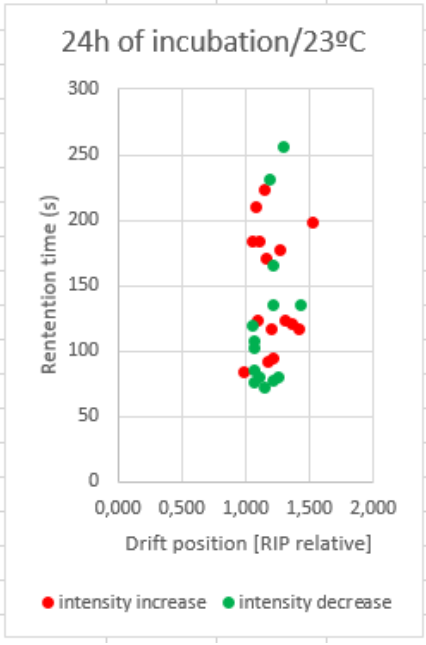


Figure 6.13 – Relevant peaks for the bacterial mixture in the LB growth medium after 24 hours of incubation and at 23 ºC. Intensity increase from the blank to the culture is represented by red dots. Intensity decrease is represented by green dots. The y-axis represents the retention time, in seconds and the x-axis, a drift position relative to the RIP.

Table 6.11 contains the retention times, in seconds, and the drift positions, RIP relative, of the peaks present in the previous graph (Figure 6.13). The peaks identified with the label PA are characteristic of *P. aeruginosa*. The ones identified with EC are characteristic of *E. coli*. When both bacteria produce the same compound, both labels are present.

Table 6.11 - Retention time and drift position of the relevant peaks found for the bacterial mixture after 24 hours of incubation. The label EC is for compounds belonging to the pattern for *E. coli*. The label PA identifies compounds belonging to the pattern for *P. aeruginosa*. The label PA24h identify peaks not present in the chosen pattern for *P. aeruginosa* but that can be found in the spectra of this bacteria after 24h of incubation. The intensity variation indicates whether the peaks' intensity increased (+) or decreased (-) from the blank to the culture.

Retention time (s)	Drift position [RIP relative]	Label/Intensity variation	Retention time (s)	Drift position [RIP Relative]	Label/Intensity variation
73	1.142	EC (-)	124	1.083	EC+PA (+)
76	1.059	PA (-)	124	1.304	EC+PA (+)
78	1.209	EC (-)	135	1.428	-
80	1.106	EC (-)	136	1.204	PA
80	1.250	EC+PA (-)	166	1.206	EC+PA
84	0.984	PA24h	171	1.150	PA (+)
85	1.057	PA (-)	178	1.269	+
92	1.163	EC+PA (+)	184	1.049	EC (+)
95	1.209	+	185	1.103	PA24h
102	1.062	PA (-)	199	1.524	+
109	1.062	-	211	1.076	EC (+)
117	1.192	+	224	1.141	+
117	1.412	+	231	1.186	-
120	1.044	-	257	1.286	EC (-)
122	1.353	PA24h			

The patterns chosen are enough to identify the presence of both bacteria in the mixture. However, some of the remaining peaks can also be identified as belonging to *P. aeruginosa*. After 24 hours of incubation, this bacteria produces more peaks than those in the chosen pattern. This peaks are identified in Table 6.11 with the label PA24h.

What's more, when the bacteria are incubated together, there are peaks that show the interaction of the two. Figure 6.14 shows the spectra of the blank, *E. coli*, *P. aeruginosa* and the mixture of the two with some of these points, identified by yellow rectangles. From Figure 6.14 it is possible to observe three different behaviours. Point 1 is only produced when both bacteria are together. Point 2 shows that perhaps *P. aeruginosa* is more prevalent at 24 hours of incubation since *E. coli* produces the compound and *P. aeruginosa* consumes it. In the mixture, the compound is almost absent which may mean that it was consumed. Point 3 shows increase in intensity in the mixture when both bacteria individually produce the same compound.

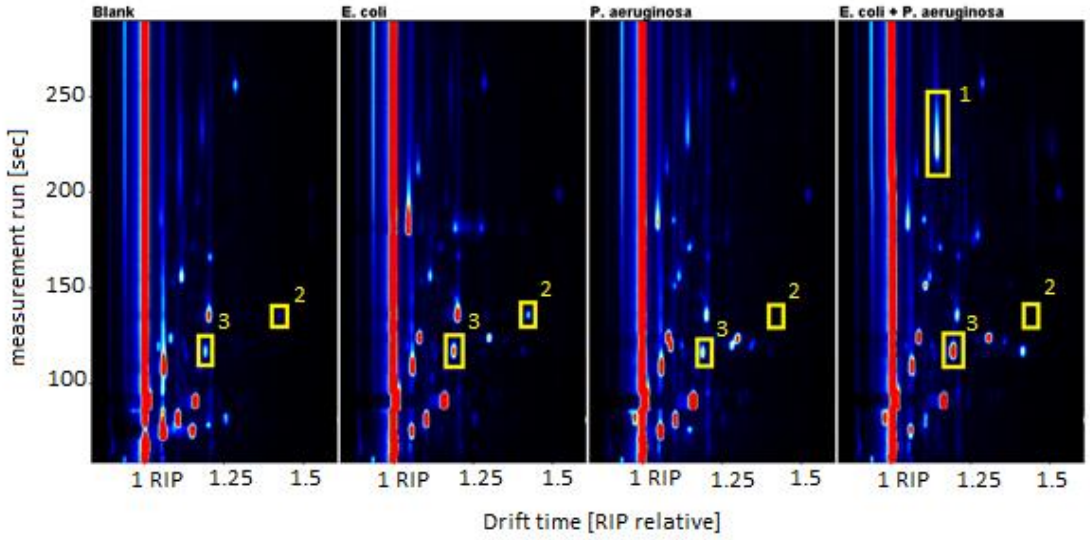


Figure 6.14 - Characteristic spectra of the blank, *E. coli*, *P. aeruginosa* and the mixture of both at room temperature after 24 hours of incubation. Peak 1 is only found in the mixture. Peak 2 shows that possibly *P. aeruginosa* is more active than *E. coli*, since the compound produced by the latter seems to have been consumed. Peak 3 is much more intense in the mixture than in the individual spectra of the bacteria, which may imply a contribution from both bacteria.

After 48 hours of incubation in LB growth medium

Figure 6.15 illustrates the design made by the relevant peaks found at room temperature for the period of incubation of 48 hours.

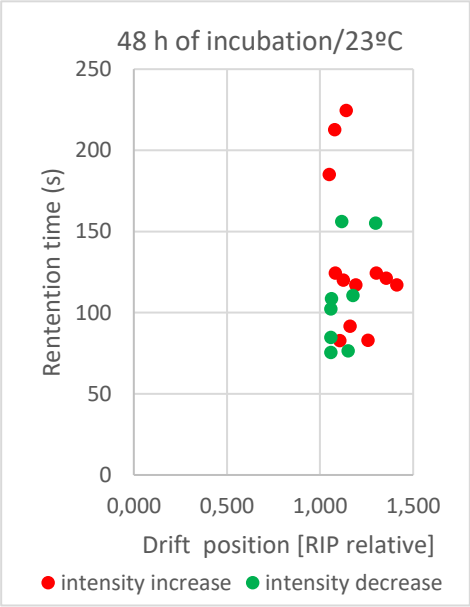


Figure 6.15 – Relevant peaks found after 48 hours of incubation of the bacterial mixture in LB growth medium, at room temperature. While the ones that are more intense in the culture than in the blank are represented by red dots, the ones less intense are represented by green dots. The y-axis represents retention time, in seconds and the x-axis represents a drift position relative to the RIP.

Table 6.12 contains the retention times, in seconds, and drift position relative to the RIP of the peaks present in the previous graph (Figure 6.15). The labels are the same as for Table 6.11.

Table 6.12 – Retention time and drift position [RIP relative] of the relevant peaks found for the bacterial mixture after 48 hours of incubation. The label PA identifies compounds belonging to the pattern for *P. aeruginosa*. The label EC is for compounds belonging to the pattern for *E. coli*. The intensity variation indicates whether the peaks' intensity increased (+) or decreased (-) from the blank to the culture.

Retention time (s)	Drift position [RIP relative]	Label/Intensity variation	Retention time (s)	Drift position [RIP relative]	Label/Intensity variation
75	1.058	PA (-)	117	1.411	+
76	1.151	EC+PA (-)	120	1.124	+
83	1.257	EC	121	1.355	+
83	1.105	EC	124	1.082	EC+PA (+)
85	1.057	PA (-)	124	1.302	EC+PA (+)
92	1.161	EC+PA (+)	155	1.298	-
102	1.058	PA (-)	156	1.116	PA (-)
109	1.061	-	185	1.049	EC+PA (+)
110	1.177	-	213	1.078	EC
117	1.191	+	224	1.141	+

Only two peaks did not show the same intensity behaviour on the mixture and the pattern. These are the ones highlighted in blue shown in Table 6.12.

Once again it is possible to identify the presence of both bacteria in the mixture (Figure 6.16). In addition, the new compound found to be released in the mixture (Point 1 in the previous analysis), is also emitted after 48 hours of incubation. This compound is at 224 s and the relative position to the RIP of 1.141. The point 2 in Figure 6.16 is much more intense in the mixture than in the individual spectra of the bacteria, which may imply a contribution from both bacteria. Point 3, in the same figure, shows that perhaps *P. aeruginosa* is still more prevalent after 48 hours of incubation since *E. coli* produces the compound and *P. aeruginosa* consumes it.

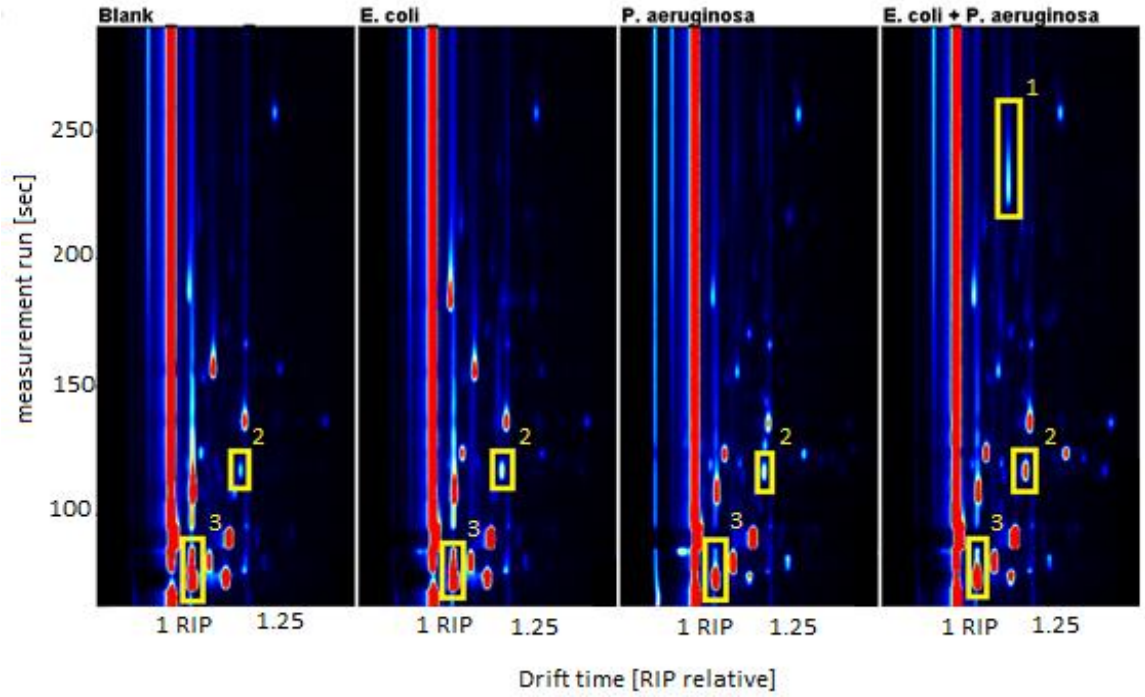


Figure 6.16 - Characteristic spectra of the blank, *E. coli*, *P. aeruginosa* and the mixture of both at room temperature after 48 hours of incubation. Peak 1 is only found in the mixture. Peak 2 is much more intense in the mixture than in the individual spectra of the bacteria, which may imply a contribution from both bacteria. Peak 3 shows that possibly *P. aeruginosa* is more active than *E. coli*, since the compound produced by the latter seems to have been consumed.

Specific media

Bacteria were also grown in specific growth media: PAB (Pseudomonas Agar Base) for *P. aeruginosa* and TSA (Tryptic Soy Agar) for *E. coli*.

The aim of this section is to study the effect of the growth medium on the production of VOCs and to, ultimately find VOCs produced by each bacteria independently of culture media.

Here, the search for relevant peaks is made in the same way as the analysis for the other medium (LB). Since room temperature was shown to produce better results, the following analysis will be conducted at this temperature.

P. aeruginosa in PAB

The relevant peaks found for *P. aeruginosa* in the LB growth medium are compared against the ones relevant for the PAB growth medium.

After 24 hours of incubation

Figure 6.17 illustrates the arrangement of the relevant peaks for PAB growth medium after 24 hours of incubation.

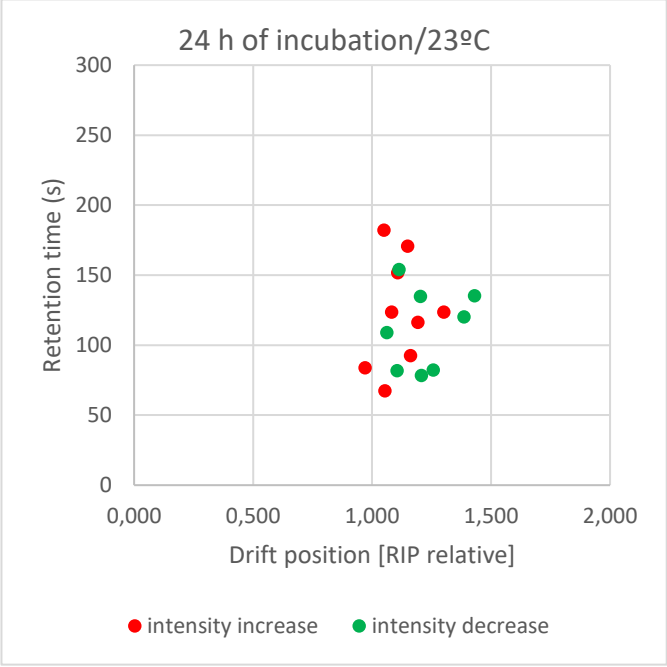


Figure 6.17 – Relevant peaks found for *P. aeruginosa* in the PAB growth medium after 24 hours of incubation and for sample temperature of 23 ºC. The red dots represent peaks with increased intensity in the culture when compared to the blank. On the contrary, the green dots represent peaks with decreased intensity in the culture. The y-axis represents the retention time, in seconds, and the x-axis the drift position, relative to the RIP.

There are 9 peaks with increased intensity in the culture when compared to the blank and 8 with the opposite behaviour.

The retention time, in seconds, and the drift position, RIP relative, of the compounds only produced by the bacteria in this medium are shown in the Table below (Table 6.13).

Table 6.13 – Retention time, in seconds, and RIP relative drift position of the peaks only found in the spectra from *the P. aeruginosa* in PAB growth medium after 24 hours of incubation.

Retention time (s)	Drift position [RIP relative]
84	0.970
124	1.302
152	1.107
182	1.049

The compounds found at both growth media, LB and PAB, after 24 hours of incubation are shown in Table 6.14.

Table 6.14 – Retention time, in seconds, and drift position relative to the RIP of the mutual peaks found for 24 hours of incubation and for both growth media, LB and PAB. The intensity variation of the peaks from the blank to the culture is represented by "+" when it increases and a "-" when it decreases.

Retention time (s)	Drift position [RIP relative]	Intensity variation	Retention time (s)	Drift position [RIP relative]	Intensity variation
84	0.97	+	135	1.203	-
93	1.161	+	135	1.430	-
109	1.061	-	154	1.112	-
116	1.192	+	171	1.149	+
124	1.082	+	182	1.049	+
124	1.302	+			

As for the compounds only present in the culture, three are matching in the two media for this period of incubation. The characteristics of these compounds (retention time and drift position taken relative to the RIP) are in Table 6.15.

Table 6.15 – Retention time and drift position of the shared peaks of the two growth media (LB and PAB) after 24 hours of incubation.

Retention time (s)	Drift position [RIP relative)
84	0.970
124	1.302
182	1.049

After 48 hours of incubation

Next the relevant peaks found for *P. aeruginosa* are illustrated in Figure 6.18.

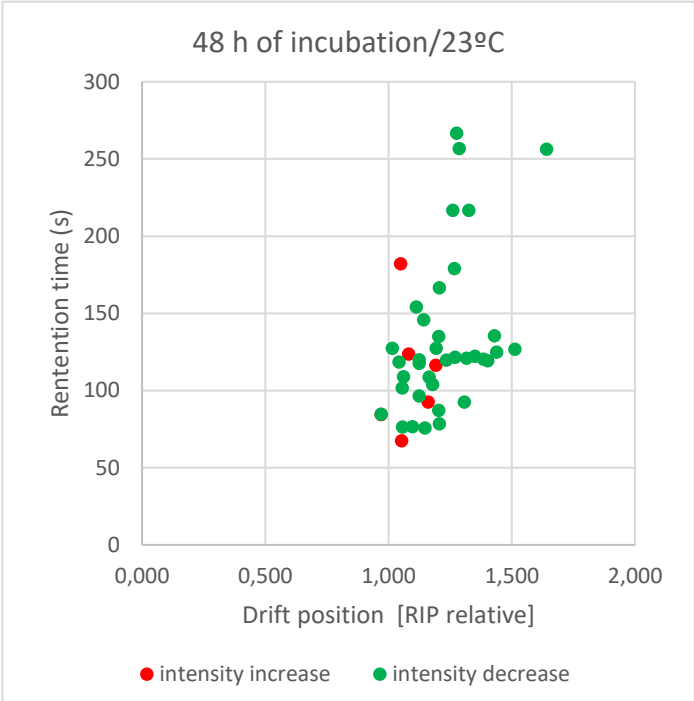


Figure 6.18 – Relevant peaks found for *P. aeruginosa* after 48 hours of incubation. The y-axis represents the retention time in seconds. The x-axis represents the drift position calculated in relation to the RIP. The red dots represent intensity increase from the blank to the culture and the green ones represent intensity decrease.

As can be observed, the amount of peaks suffering an intensity decrease is much more significant when compared to 5 peaks with an intensity increase. Here, there is one peak only present in the culture. Its retention time is 84 ms and the drift position relative to the RIP is 0.970.

The characteristics of the mutual substances found after 48 hours of incubation for both growth media (LB and PAB), retention time, in seconds, and drift position (RIP relative) are shown in Table 6.16.

Table 6.16 - Retention time, in seconds, and drift position (relative to the RIP) of the peaks found to be present in the culture of *P. aeruginosa* after 48 hours of incubation and for both growth media (LB and PAB). The peaks' intensity variation from the blank to the culture, increase (+) or decrease (-), is also shown.

Retention time (s)	Drift position [RIP relative]	Intensity variation
76	1.057	-
76	1.148	-
93	1.161	+
102	1.056	-

124	1.082	+
135	1.203	-
154	1.112	-
166	1.207	-
182	1.049	+

From both tables it is possible to identify substances mutual to both periods of incubation. Table 6.17 contains the retention times, in seconds, and RIP relative drift positions of these compounds, as well as their intensity variation from the blank to the culture (increased intensity: +; decreased intensity: -). These peaks are identified in Figure 6.19 by green rectangles.

Table 6.17 – Retention time, in seconds, and drift position (relative to the RIP) of the peaks found to be present in the culture of *P. aeruginosa* for both periods of incubation and for both growth media (LB and PAB). The peaks' intensity variation from the blank to the culture, increase (+) or decrease (-), is also shown.

Retention time (s)	Drift position [RIP relative]	Intensity variation
93	1.161	+
124	1.082	+
135	1.203	-
182	1.049	+

It is likely that the compounds with intensity variation + are produced by the bacteria independently of the growth medium in which they are in. Nonetheless, *P. aeruginosa* should be grown in other media to confirm these findings.

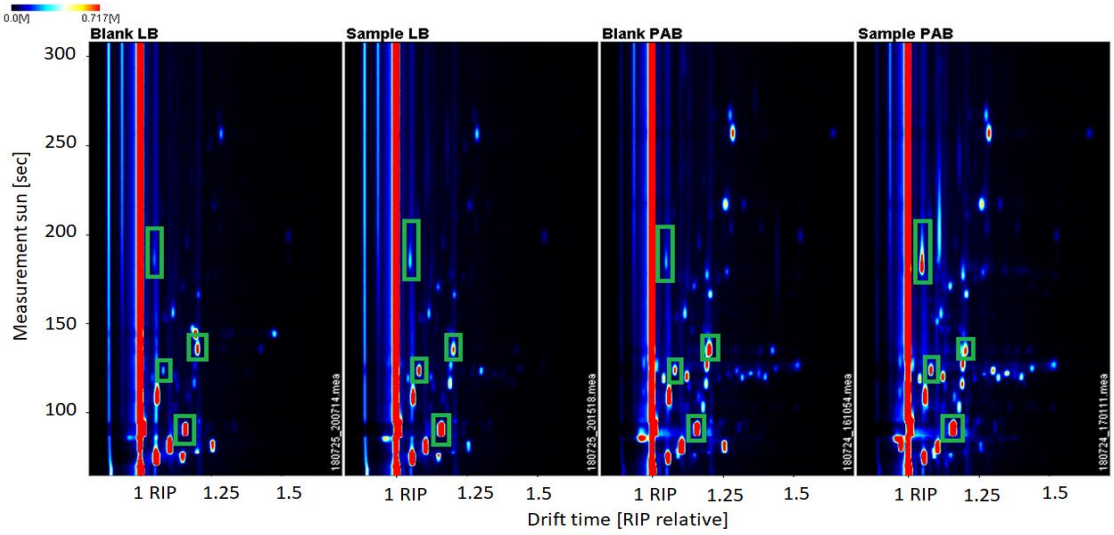


Figure 6.19 - Example of spectra of *P. aeruginosa*. From left to right: blank of the LB agar, sample of *P. aeruginosa* grown in LB agar, blank of PAB and sample of *P. aeruginosa* grown in PAB. The peaks identified in green represent the ones present in both growth media. The x-axis represents the drift time, relative to the RIP. The y-axis represents the retention time, in seconds.

E. coli in TSA

The relevant peaks found for *E. coli* in the LB growth medium are compared against the ones relevant for the TSA growth medium.

After 24 hours of incubation

The relevant peaks for *E. coli* in TSA growth medium for room temperature are shown in the Figure 6.20.

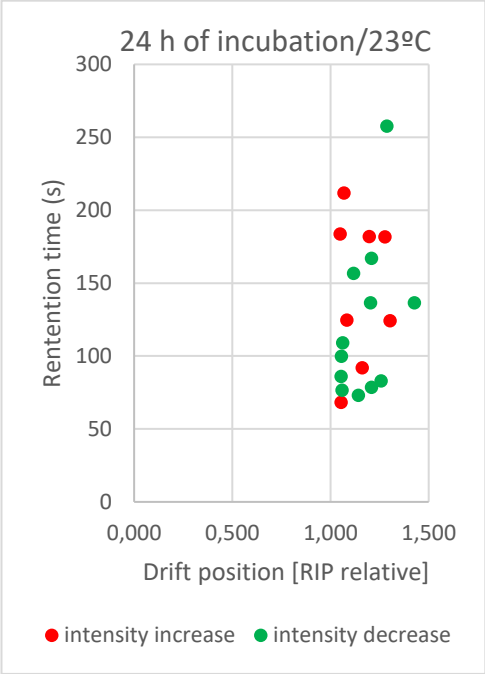


Figure 6.20 - Relevant peaks found after 24 hours of incubation for *E. coli* in the nutrient medium TSA. The red dots indicate intensity increase from the blank to the culture and the green dots illustrate intensity decrease from the blank to the culture. The x-axis represents a drift position, since the drift time is relative to the RIP and therefore has no units. The y-axis represents the retention time and is expressed in seconds.

At room temperature there are a total of 20 peaks, 8 of which representing production of substances (red dots) and 12 representing consumption (green dots). There are shared relevant compounds when comparing both growth media. Retention time, in seconds, and RIP relative drift positions of these compounds are present in Table 6.18. In addition, the behaviour of the peak, whether it increased (+) or decreased (-) its intensity from the blank to the culture is under Intensity variation.

Table 6.18 - Mutual peaks after a 24 hour period incubation of *E. coli* for both growth media: LB and TSA. Retention time is in seconds and the drift position of each peak is relative to the RIP. The intensity variation indicates increase (+) in intensity from the blank to the culture and intensity decrease (-) from the blank to the culture.

Retention time (s)	Drift position [RIP relative]	Intensity variation	Retention time (s)	Drift position [RIP relative]	Intensity variation
73	1.142	-	125	1.083	+
77	1.058	-	157	1.117	-
79	1.209	-	167	1.208	-
83	1.258	-	182	1.198	+
86	1.054	-	182	1.278	+
92	1.162	+	184	1.049	+
109	1.062	-	212	1.068	+
124	1.303	+	258	1.288	-

After 48 hours of incubation

The relevant peaks for *E. coli* in TSA growth medium for both temperatures are in the Figure 6.21.

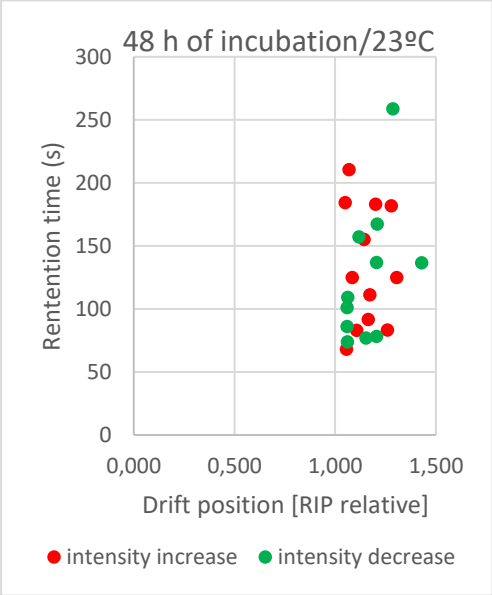


Figure 6.21 – Relevant peaks found after 48 hours of incubation for *E. coli* in the nutrient medium TSA. The red dots indicate intensity increase from the blank to the culture and the green dots illustrate intensity decrease from the blank o the culture. The x-axis represents a position, since the drift time is relative to the RIP and therefore has no units. The y-axis represents the retention time and is expressed in seconds.

For TSA after 48 hours of incubation, there are 12 peaks representing production (red dots) and 11 representing consumption (green dots). The same previously used procedure of finding shared peaks between the two growth media was followed. The results are present in Table 6.19.

Table 6.19 - Mutual peaks after a 48 hour period incubation for *E. coli* in both growth media: LB and TSA. Retention time is in seconds and the drift position of each peak is relative to the RIP. The intensity variation indicates increase in intensity (+) in intensity and intensity decrease (-) from the blank to the culture.

Retention time (s)	Drift position [RIP relative]	Intensity variation	Retention time (s)	Drift position [RIP relative]	Intensity variation
77	1.154	-	137	1,430	
78	1.206	-	167	1.209	-
83	1.108		182	1.278	+
83	1.261		183	1.201	+
92	1.165	+	184	1.051	+
125	1.084	+	211	1.070	+
125	1.306	+	259	1.288	-
137	1.206				

The peaks with no identification in the column intensity variation, do not show the same behaviour (in one medium their intensity increased and in the other it decreased). The final step was to identify peaks present at both periods of incubation. The retention time, in seconds, and the drift position relative to the RIP of each of these peaks are shown in Table 6.20.

Table 6.20 – Mutual peaks to both periods of incubation (24 hours and 48 hours) and to both growth media: LB and TSA for *E. coli*. Retention time is in seconds and the drift position is taken relative to the RIP. The intensity variation indicates increase (+) in intensity and intensity decrease (-) from the blank to the culture.

Retention time (s)	Drift position [RIP relative]	Intensity variation	Retention time (s)	Drift position [RIP relative]	Intensity variation
77	1.154	-	259	1.288	-
78	1.206	-	125	1.306	+
83	1.261		182	1.278	+
92	1.165	+	183	1.201	+
125	1.084	+	184	1.051	+
167	1.209	-	211	1.070	+

The peak with no identification in the column intensity variation, does not show the same behaviour (in one medium its intensity increase and in the other it decreases).

When comparing both periods of incubation at room temperature, some peaks were present in the culture and were highlighted in blue in Table 6.20. Since these compounds are found in both growth media, it is possible that the bacteria produce them independently of the nutrient media. These peaks are identified in Figure 6.22 by green rectangles.

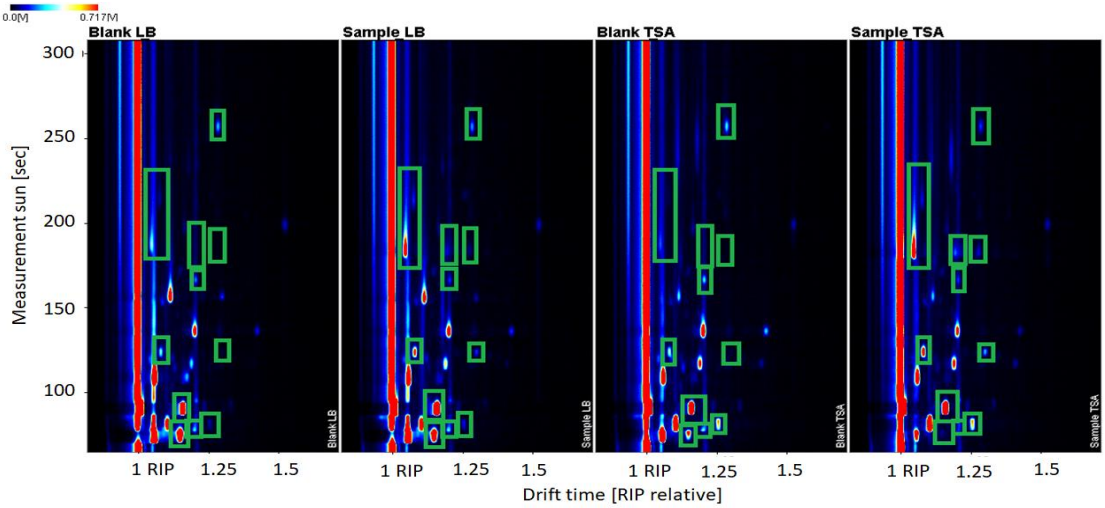


Figure 6.22 – Example of spectra of *E. coli*. From left to right: blank of the LB agar, sample of *E. coli* grown in LB agar, blank of TSA and sample of *E. coli* grown in TSA. The peaks identified in green represent the ones present in both growth media. The x-axis represents the drift time, relative to the RIP. The y-axis represents the retention time, in seconds.

Relevant VOC characterization

After identifying the pattern for both bacteria, the reduced mobility constant was calculated for each one. The equations used to perform these calculations were the ones present in Chapter 4.

Ion Mobility:

$$K = \frac{v_d}{E} = \frac{l}{t_d E} = \frac{l^2}{t_d U} \qquad 4.1$$

l – Drift the length = 9.8 cm

U – Potential difference between the ends of the drift tube = 5000 V

t_d – Drift time

Reduced Ion Mobility:

$$K_0 = K\left(\frac{P}{P_0}\right)\left(\frac{T_0}{T}\right) \qquad 4.2$$

P – Pressure in the drift tube = 0.752 kPa = 5.64 Torr

P_0 – Ambient pressure = 101.325 kPa = 760.00 Torr

T_0 – Standard temperature = 273.16 K

T – Temperature in the drift tube = 343.16 K

Tables 6.21 and 6.22 show the calculated values for the mobility constant and the reduced mobility constant for the pattern of *P. aeruginosa* and *E. coli*, respectively. The drift time, in milliseconds, was calculated by multiplying the drift position by the average drift time of the RIP (7.280 ms).

Table 6.21 – Calculated values of ion mobility K ($cm^2V^{-1}s^{-1}$) and reduced ion mobility K_0 ($cm^{-2}V^{-1}s^{-1}$) for each of the peaks in the pattern for *P. aeruginosa*, as well as, its retention time, in seconds, and drift position relative to the RIP and the corresponding drift time, in milliseconds.

Pattern for *P. aeruginosa*

Retention time (s)	Drift position [RIP relative]	Drift time (ms)	K ($cm^2\,V^{-1}\,s^{-1}$)	K_0 ($cm^2\,V^{-1}\,s^{-1}$)	$1/K_0$ ($cm^{-2}\,V\,s$)
76	1.056	7.688	2.499	0.015	67.750
77	1.150	8.372	2.294	0.014	73.780
83	1.055	7.680	2.501	0.015	67.686
105	1.059	7.710	2.491	0.015	67.942
124	1.300	9.464	2.030	0.012	83.404
156	1.115	8.117	2.366	0.014	71.535
167	1.205	8.772	2.190	0.013	77.309
171	1.148	8.357	2.298	0.014	73.652
92	1.160	8.445	2.275	0.013	74.422

124	1.081	7.870	2.441	0.014	69.354
136	1.202	8.751	2.195	0.013	77.117
184	1.048	7.629	2.518	0.015	67.236

Table 6.22 - Calculated values of ion mobility K ($cm^2\ V^{-1}\ s^{-1}$) and reduced ion mobility K_0 ($cm^{-2}\ V^{-1}\ s^{-1}$) for each of the peaks in the pattern for *E. coli*, as well as, its retention time, in seconds, and drift position relative to the RIP and the corresponding drift time, in milliseconds.

Pattern for *E. coli*

Retention time (s)	Drift position [RIP relative]	Drift time (ms)	K ($cm^2\ V^{-1}\ s^{-1}$)	K_0 ($cm^2\ V^{-1}\ s^{-1}$)	$1/K_0$ ($cm^{-2}\ V\ s$)
83	1.104	8.037	2.390	0.014	70.829
77	1.150	8.372	2.294	0.014	73.780
78	1.201	8.743	2.197	0.013	77.052
83	1.256	9.144	2.101	0.012	80.581
92	1.161	8.452	2.273	0.013	74.486
124	1.301	9.471	2.028	0.012	83.468
125	1.082	7.877	2.439	0.014	69.418
167	1.206	8.78.	2.188	0.013	77.373
182	1.277	9.297	2.066	0.012	81.928
183	1.197	8.714	2.204	0.013	76.796
184	1.048	7.629	2.518	0.015	67.236
213	1.078	7.848	2.448	0.014	69.161
258	1.286	9.362	2.052	0.012	82.506

The compounds found to be present in both media for both periods of incubation are also present in the patterns for each bacteria species. For *P. aeruginosa* these are the last four compounds of Table 6.21. For *E. coli*, all but the first one, are common to both growth media.

The reduced mobility constant is a characteristic of the compound. It was possible to identify one substances that is present in both bacteria, which is acetone (92 s and 8.445 ms for *P. aeruginosa* and 92 s and 8.452 ms for *E. coli*). Calibrations still need to be performed in order to identify the remaining substances. In order to do so, a group of pure ketones present in the NIST library was to be analysed to establish the retention times of said substances in the GC-IMS device used and then extrapolate the retention times of the remaining substances in previously existing software. These ketones were ordered, however, for reasons beyond control, they did not arrive in time. Nonetheless, calibrations will be performed and the results presented in a future article.

The quantitative analysis of the peaks was not possible since the calibrations were not performed and, without this, it is not possible to establish calibration curves.

7

Conclusions and Future Work

During this work bacteria profiles of *E. coli* and *P. aeruginosa* obtained with the GC-IMS device were analysed at different conditions. The cultures were swabbed and the cotton swab's head was cut into a vial. The volatile organic compounds released by the bacteria were then analysed by the GC-IMS device, either at room temperature or after heating the vial at 40ºC during 10 minutes. Measurements made in this device have been shown to be very repeatable: even though the peaks may vary in terms of intensity, which may be due to the amount of sample collected, in general, all of them show the same behaviour (more intense or less intense when comparing to the blank).

From the analysis conducted in this work it was possible to conclude that room temperature provided the best results. With the pattern obtained for both bacteria in the LB growth medium at room temperature more peaks from both bacteria were identified. In addition, with the data from different growth media, it was possible to identify peaks present in both. This may imply that there are some compounds released by the bacteria independently of the substrate in which they are grown and may be used to identify the bacteria in any medium or surface.

The main objective of finding a pattern that allowed the identification of bacteria was achieved. Furthermore, the patterns found for both bacteria individually were enough to identify both microorganisms when they were grown together in the same medium.

The identification of a pattern possibly independent of substrate is very important for the creation of a database that permits the direct identification of bacteria. The peaks found in this thesis may be entries on this database.

The quantitative analysis of the peaks was not possible because of the delay in the delivery of the pure ketones needed to perform calibrations.

Future Work

This worked proved the possibility of identifying a bacteria strain based on the VOCs pattern released by them. Pursuing the work on these bacteria, more should be conducted especially by experimenting with other growth media. Measurements in the field, such as, swabbing surfaces of medical devices and of even the skin and wounds, are also a very interesting step to take.

The identification of the substances that originated the peaks present in the pattern that was found is very important. Some of these may even be a biomarker of the contamination, especially in *E. coli*, since there were a few peaks found to be present throughout the measurements made.

It would be very interesting to study other strains of the same bacteria analysed in this work, in order to check if it is possible to differentiate the strains.

In the future, more effort should be put into studying the profiles of other bacteria species or other microorganisms, such as fungi, in order to add data to the database. This database will allow the direct identification of volatile organic compounds released by the microorganisms based on their retention and drift times and even the direct detection of microbes if a biomarker is found.

The creation of a compound library is of the utmost importance to be able to identify which compound belongs to which peak. Additionally, a calibration curve is also very important to be able to determine the compounds' concentration based on the respective peak's intensity.

An article with the results of the quantitative analysis of the patterns found in this work will be prepared.

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Appendix A – Culture medium preparation

The culture media used in this work were prepared by following the instructions present in the product.

LB Agar

The LB Agar powder is composed of agar (15 g/L), tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L).

To prepare one litre of LB Agar, it is necessary to dissolve 40 g of the powder in one litre of distilled water. Sterilize the mixture in the autoclave during 15 minutes at 121ºC.

TSA

Tryptic Soy Agar (TSA) is composed of pancreatic digest of casein (15 g/L), papaic digest of soya bean (5 g/L), sodium chloride (5 g/L) and agar (15 g/L).

To prepare one litre of TSA, 40g of powder in one litre of distilled water. Heat the mixture to obtain complete dissolution. Sterilize in the autoclave for 15 minutes at 121 ºC.

PAB

Pseudomonas Agar Base (PAB) is composed of gelatin peptone (16 g/L), casein hydrolysate (10 g/L), potassium sulfate anhydrous (10 g/L), magnesium chloride anhydrous (1.4 g/L) and agar (15 g/L).

To prepare one litre of PAB medium, suspend 52.4 g of powder in one litre of deionized or distilled water. Add 10 mL of Glycerol Supplement. Boil and shake the mixture until completely dissolved. Sterilize in the autoclave at 121 ºC for 15 minutes.

LB Broth

This medium was used to store bacteria.

LB Broth (Luria Broth) is composed of tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L).

To prepare one litre of LB Broth, suspend 25 g in one litre of distilled water. Sterilize by autoclaving at 121 ºC for 15 minutes.

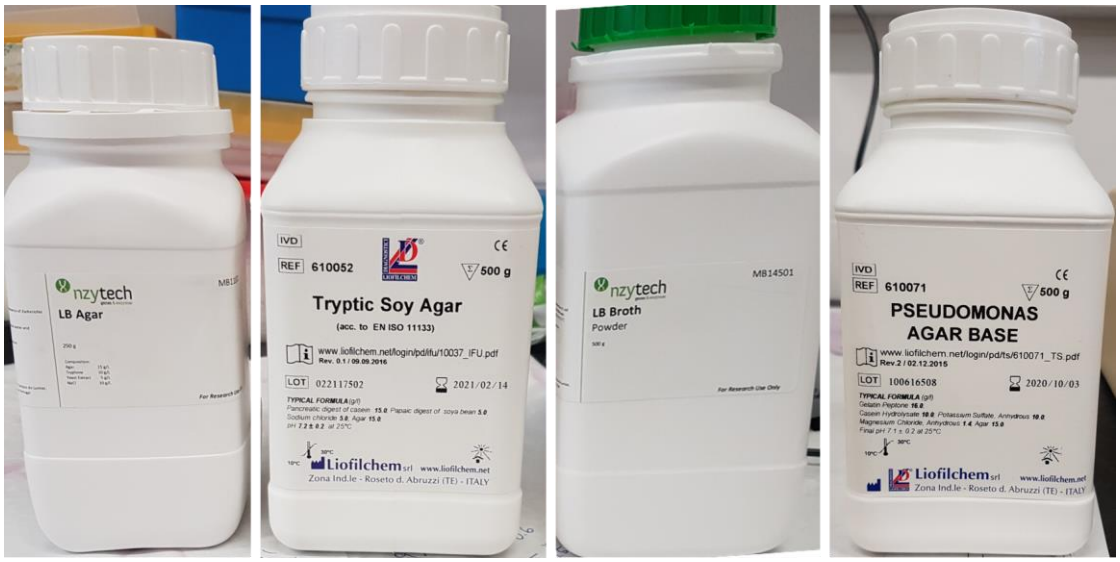

Figure 1 – Growth media used during the course of this work. From left to right: LB Agar, Tryptic Soy Agar, LB Broth and Pseudomonas Agar Base.

Appendix B – Bacterial sample preparation

The growth media were poured into petri dishes and left to solidify at room temperature for a few minutes. The materials required for the plating, as well as the growth media, were sterilized in an airflow chamber using UV radiation.

The Eppendorf tube containing the LB broth and the bacteria was placed in a vortex mixer to homogenize the bacterial sample. The bacterial sample was removed from the Eppendorf using a sterilized inoculation loop. The loop was used to smear the bacterial sample on the agar plates. The process of transferring the sample from the Eppendorf tube to the agar plates is made near an alcohol burner to avoid contaminations.

After plating, the agar plates were placed with the top down in an incubator at 37 ^{o}C, during either 24 hours or 48 hours.

The plating method is illustrated in the following schematic (Figure 2).

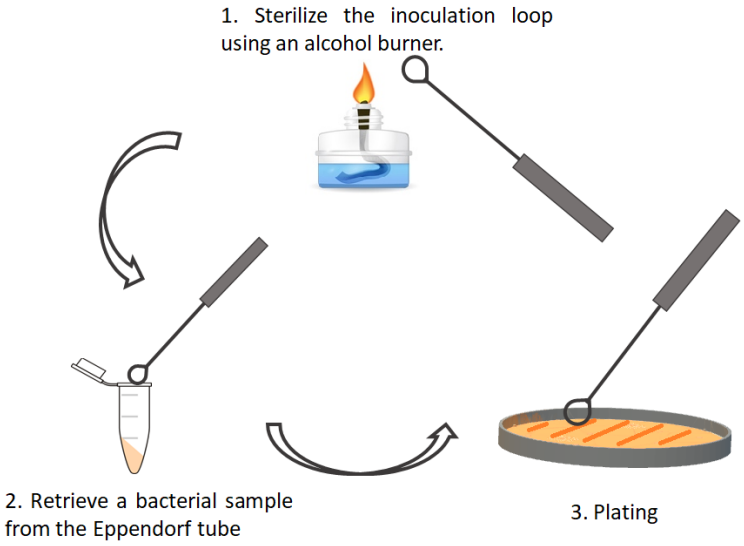

Figure 2 – Schematic of the steps taken to obtain the bacterial cultures.

Figure 3 contains examples of the bacterial cultures obtained.

Figure 3 – Examples of the bacterial cultures obtained. From left to right: *E. coli* in LB Agar, *E. coli* in TSA, *P. aeruginosa* in LB Agar and *P. aeruginosa* in PAB.

Appendix C

The relevant peaks from the mixture found at 40 ºC are present in the Tables below. Table 1 refers to 24 hours of incubation, while Table 2 refers to 48 hours. The compounds that are found in the patterns of *E. coli* and P. aeruginosa are identified with EC and PA, respectively.

Table 1 – Relevant peaks found for the mixture after 24 hours of incubation and for 40 ºC.

Retention time (s)	Drift position	Identification	Retention time (s)	Drift position	Identification
76	1.059	PA	84	0.984	
73	1.142	PA	117	1.412	
78	1.209		124	1.304	PA+EC
80	1.250		122	1.353	
80	1.106		152	1.108	
85	1.057	PA	151	1.374	
92	1.163	EC+PA	145	1.197	PA
102	1.062		162	1.084	
109	1.062		163	1.025	
117	1.192	EC+PA	185	1.103	
120	1.044		178	1.269	EC
124	1.083	EC+PA	185	1.271	
136	1.204	EC	211	1.076	EC
166	1.206	EC+PA	224	1.141	
171	1.150	PA	104	1.124	
184	1.049	EC+PA	171	1.438	
257	1.286	EC+PA	215	1.142	
199	1.524		135	1.428	PA+EC

Table 2 - Relevant peaks found for the mixture after 48 hours of incubation and for 40 ºC.

Retention time (s)	Drift position	Identification	Retention time (s)	Drift time	Identification
75	1.058	PA	199	1.524	
76	1.151	PA	117	1.411	
78	1.202		124	1.302	PA+EC
85	1.057	PA	121	1.355	
92	1.161	PA+EC	144	1.200	
102	1.058		185	1.103	
109	1.061		178	1.268	EC
117	1.191	PA+EC	213	1.078	EC
119	1.045		224	1.141	

124	1.082	PA+EC	96	1.208	
115	1.132		103	1.123	
120	1.124		177	1.196	EC
156	1.116	PA	176	1.260	
167	1.206	PA+EC	166	1.177	
171	1.150	PA	217	1.262	
257	1.287	PA+EC	85	0.975	

As can be observed, there are 3 peaks that individually identify *E. coli* and 5 for *P. aeruginosa* for both periods of incubation. When comparing this data with the information regarding 23 ºC, the latter has more peaks that allow a separate identification of both bacteria (6 and 3 for *E. coli* at 24 hours and 48 hours of incubation, respectively, and 5 and 4 for *P. aeruginosa* at the same periods of incubation).