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Chemical characterization of human breath

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Abstract

In the present work analysis of exhaled breath has been proposed as a novel way to detect disease, to monitor disease progression, and to monitor clinical intervention.

An analytical method of analysis based upon two stages thermal desorption capillary gas chromatography and mass spectrometry was developed for the analysis of breath samples. All the steps of the analytical procedure were evaluated, trying to identify the critical aspects in order to optimize the entire procedure. A novel breath collection media has been developed that is cheap, disposable and readily available. This material allows breath samples to be collected in a novel manner. After validation the procedure was applied to real samples and preliminary experiments were performed aimed at estimating the variability of the composition of breath as a function of time of day and the inter-subject variability. In particular the trend in time of the two principal compounds in breath, acetone and isoprene was observed. The data showed a wide inter-variability between different people and also confirmed that a meal can influence breath composition. For this reason if possible, it was better to collect a breath sample in the morning before eating. Single substances or sets and patterns of exhaled markers were also investigated in order to establish correlations between the chemical composition of breath and patients' clinical conditions.

Preliminary studies were performed on patients with end-stage renal disease. The results underlined the capacity of the analytical procedure to appreciate small variation in breath composition. In particularly in people under dialysis treatment two compounds were found to show significant differences in breath concentration between patients and healthy people before the dialysis and no important differences after. These two compounds should become an additional and important parameter to determine the end of the dialysis treatment.

In parallel a breath collection system prototype has been designed that enables samples of dead space air to be separated from end tidal breath and be collected independently. This device is also novel and has a great potential in breath analysis field.

CHAPTER 1

State of the Art

1.1 Introduction

How many times do we hear expressions such as: “To sigh with relief, to breathe deeply, to take a breath of fresh air, to feel choked, to take your breath away“. These are all phrases refer to our moods. We often feel tired, lazy, dulled or very commonly we feel stressed. When an emotion is exaggerated respect to an external stimulus, it means that there is not sufficient oxygen in the body and in the mind. Oxygen enables the mind to work and if there is a lack of oxygen, the blood has to flow faster. Meanwhile we lose clarity of thought and our mood changes for the worse. Everybody knows just how important breathing is, but in reality how much do we know about the respiration process? When we breathe many organs are involved, in fact the inspired air passes through the airways to reach the lungs, which represent the real respiration organ[1]. When we breathe, we bring oxygen into the body and the produced substances are eliminated. Besides containing principal gases (nitrogen, oxygen, carbon dioxide, inert gases) and 98% of water, human breath consists of a great variety of organic compounds present at very low concentrations. What is eliminated may come from products of our metabolism or from substances that we have inhaled and adsorbed from an external environment. Breathing is at the base of the life and enables our organism to carry out the principal vital function. Therefore, chemical composition of exhaled air reflects the general metabolic conditions and it may give useful and precious information on the state of health of a person. For these reasons breath has the potential to become a key for reading our organism.

1.2 State of the art

Breath scientific research started in 1784, when Antoine Laurent Lavoisier, father of modern chemistry, and Pierre Simon Laplace, analyzed the breath of a guinea pig and observed that the animal burned oxygen and produced carbon dioxide. This discovery was the first proof of food combustion in human body[2, 3].

In the mid 19th century, colorimetric tests were developed that led to the detection of volatile organic compounds (VOCs) in breath at low concentrations. One of the first colorimetric analyses was developed by the German Nebelthau. He observed a rapid and intense colour variation in an alkaline iodine solution, bubbling in the breath of diabetics. The colour variation was induced by the presence of a high amount of acetone in breath[3, 4].

In 1874 the British scientist Francis Anstie used a colorimetric method to highlight the presence of ethanol in breath. By bubbling breath through an acid chromic solution he observed a colour variation from red to green due to the presence of ethanol. With other experiments the scientist demonstrated that the amount of ethanol eliminated from breath was less than the ethanol ingested. He deduced that most of the ethanol was metabolized[3].

It was only in 1971 that Linus Pauling carried out the first systematic breath characterization, condensing volatile compounds in a cold trap (made from a stainless steel tube cooled with dry ice). An analysis of the condensate using gaschromatography and mass spectrometry highlighted that human breath samples are very complicated as they contain thousand of different compounds[3].

The most significant results on the studies of the correlation between metabolism and the presence of organic substances in human breath, and between pathologies and markers are reported below. However, in most cases the metabolic pathways that lead to their formation are not well known yet[5, 6, 7].

In the breath of patients with hepatic cirrhosis, sulfur compounds such as ethylmercaptan, dimethylsulphide and dimethyldisulphide were identified. These

compounds were produced in the human organism from an incomplete metabolism of methionine, intermediate of the transaminase process[2, 6, 8, 9, 10].

In the breath of patients suffering from lung cancer, alkanes with low molecular weights, such as hexane, heptane, pentane, methylpentane and benzene derivatives were identified. They are produced from an alteration in the lipid peroxidation process (oxidative stress), probably due to an increase in oxygen free radical activities in the cancer cells. Active species of oxygen are continuously produced in the mitochondrion and exit in the cytoplasm where they cause peroxidative alterations in proteins, polyunsaturated fatty acids and in DNA. Oxygen free radicals degrade the cell membranes and convert polyunsaturated fatty acids into volatile alkanes, which are eliminated in breath. These alkanes could be converted into alkyl alcohol by cytochrome P450 (CYP) mixed with oxidase enzymes, which are active in lung cancer. The activation of CYP enzymes in patients affected by lung cancer can accelerate the degradation of alkanes and methylalkanes produced by oxidative stress[4, 7, 11, 12, 13, 14].

Increases in 8-isoprostane, hydrogen peroxide and 3-nitrotyrosine concentration have been found in the breath condensate of people suffering from lung inflammation [15].

In patients undergoing dialysis a decrease of some compounds in blood was highlighted. Some of these include nitrogenous compounds such as urea, ammonia, methylamine, ethylamine, dimethylamine and trimethylamine[16], other are aromatics compounds such as m–cresol and other uremic toxins such as malondialdehyde or oxalic acid[16]. In the literature there are few papers on breath variation after dialysis treatment and only some studies concerning pentane[17] and ammonia[2, 6, 18].

1.3 Tests used in the clinical diagnosis

Many tests on breath analysis have been approved by the U.S. *Food and Drug Administration (FDA)* and are now used for the diagnosis and monitoring of some diseases:

- A nitric oxide breath test is used for monitoring asthma therapy. During tidal respiration, the nitric oxide levels in asthmatic people can reach higher values than in healthy people. It has been demonstrated that the breath concentration of nitric oxide is proportional to the degree of eosinophilic inflammation of the airways and decreases if the subject starts a corticosteroid therapy[19]. It is known that nitric oxide forms almost exclusively in the superior and inferior tract of the airways and diffuses towards the lumen. The alveolar level of nitric oxide is very low due to the fact that it easily linked with hemoglobin in capillary blood. In the inflamed airways of asthmatic people the production of nitric oxide is higher than in healthy people[20].

Other tests (listed below) determine the concentration of a volatile substance directly taken (ethanol test) or derived from the metabolism of a specific precursor, given to the patient some hours before the test.

- The ethanol screening test is used to determine alcohol levels in blood. The subject has to breathe inside a plastic tube; 1 cm^3 of breath is collected through a piston. The sample is sent inside a combustible cell where an electron current is generated. The electric current measured is proportional to the alcohol concentration in blood[21, 22].

- The hydrogen breath test is used to diagnose intestinal diseases, such as malabsorption syndrome[3] or pancreas diseases. Usually this test is used with children with cystic fibrosis. A quantity of rice starch is given to the child; children with cystic fibrosis are not able to secrete a sufficient amount of amylase enzyme to digest the flour in the small intestine. The bacterial present in the intestine are the final part of the digestion process and produce high amounts of hydrogen. The hydrogen is eliminated by the breath, thus we can observe a significantly increase in the hydrogen breath content[3, 23].

- The $^{14}CO_2$ breath test is used to diagnose other pancreas diseases. A quantity of ^{14}C labeled triglycerides is given to the patient; the triglycerides are metabolized from pancreas enzymes, producing a certain amount of labeled $^{14}CO_2$. If the amount of $^{14}CO_2$ is lower than a specific value, it means that the pancreas functionality is reduced[3, 23].

- The infection caused by Helicobacter pylori is diagnosed by an urea breath test. Helicobacter have the urease enzyme, which is not present in human organism. It enables to urea to be metabolized thus producing CO_2. If a quantity of labeled ^{14}C urea is given to the infected subject, the bacteria metabolizes it in the stomach, producing $^{14}CO_2$. This gas is eliminated by breath, thus a breath analysis is performed at a certain time after the administration, revealing the infection[3, 23].

In addition to these tests, many studies on breath, analysis and the characterization of healthy people and subjects suffering from different pathologies have been reported in the literature. However, going from what is reported in the literature to real clinical practice is not straightforward, since the correlation between breath composition and a subject's state of the health is difficult to understand[24, 25].

In terms of the medical application of breath analysis, the differences between the chemical breath composition of healthy people and people with diseases are not great. Only in a few cases were found compounds in the samples of people with a disease and not in healthy people. Usually small differences in compound concentrations are observed, thus very accurate analytical techniques are needed.

1.4 Exposure assessment

In human breath there are thousands of VOCs. These compounds can be generated inside the body (endogenous substances) or can be adsorbed from exposure to the environment (exogenous substances). In fact breath VOCs content can show whether a subject has been exposed to a polluting environment[26, 27]. The VOCs used in productive activities and industrial processes (laundry, the shoe industry, the production of paints or inks, etc.), can be adsorbed from the skin or through inhalation and are eliminated with breath[28, 29, 30, 31]. Most of these compounds are toxic and can cause several diseases, such as cancer (benzene and toluene) or pulmonary inflammation (acrolein and an α,β unsaturated aldehydes). In most cases it is very difficult to find a correlation between the chemical agents and the disease, due to the complexity of interactions between human organisms and the environment, particularly when a lot of time has passed between exposure and the onset of the disease[32].

For some solvents biological monitoring involves sampling and the analysis of blood or urine. These are invasive methods, there are operative difficulties during sampling and it is possible to perform only a limited number of analyses. For this reason, breath analysis has been proposed as an alternative. Inhaled air during exposure stays inside the alveoli for sufficient time to permit to the VOCs to reach equilibrium with the blood of the pulmonary arteries. This equilibrium is regulated by partition coefficients, which determine the relative blood/breath concentration of each compound[33]. Thus the analysis of solvents in expired air may be an indication of their levels in blood[28]. Breath measurements principally reflect recent exposures. This is due to the fact that after a subject has stayed in a place for a few hours, equilibrium is reached with the ambient air; at this point breath principally reflects the actual exposure of the subject[34].

Breath analysis is also used to determine occupational exposure to industrial solvents and many studies have demonstrated the correlation between expired VOCs and chemical substances in work places[32]. Guidotti et al.[35] assessed the amount of chlorinated solvents in the breath of exposed subjects in the work place. Chloroform, trichloroethylene, tetrachloroethylene, tetrachloroethane and carbon tetrachloride were assessed. Detection limits of 0.5 and 5 ppb were found useful to analyze these compounds in the breath of subjects exposed to very low concentration levels.

Chen et al.[36] evaluated the correlation between the exposure of normal people to toluene, xylenes and ethylbenzene and the concentrations in the breath of petrol station attendants. Attendants provided breath samples after their shift at the petrol station and ambient air samples were simultaneously collected. It was found that the levels of toluene and xylenes correlated with those of ambient air, whereas the ethylbenzene levels were too low to show a correlation with the exposure that a particular subject experienced. The concentrations of toluene, xylenes and ethylbenzene in breath were in the ranges 16 to 200, 5 to 66, 1 to 33 ng/l respectively.

Sweet et al.[37] developed a method to determine perchloroethylene in breath. The measurements taken from exposed subjects gave values ranging from 7 to 44 μg/l, with variability coefficient of about 6 %.

Perbellini e al.[38] demonstrated the correlation between the concentrations of 1,3-butadiene, 2,5-dimethylfurane and benzene in breath, together with urine and blood samples taken at the same time. The median concentrations of 1,3-butadiene, 2,5-dimetilfurano and benzene in breath were 1 ng/l, 0.5 ng/l, and 6 ng/l, respectively.

The *American Conference of Governmental Industrial Hygienists* (ACGIH)[39] proposed indexes of biological exposure in breath for some compounds, which represent the probable concentration values for healthy workers exposed to these compounds in the air at a concentration equal to the TLV (Threshold Limit Value).

The biological exposure indexes for some compounds, as defined according to ACGIH, are reported as follows:

- Tetrachloroethylene is the solvent used in dry washing. Within the industry it is also used to skim the fat from metals and in the preparation of glues and adhesives. It is measured before the last shift of the working week and has a limit of 5 ppm.

- Ethylbenzene is an intermediate product in styrene production. It is used to obtain resins and synthetic rubbers, or as a solvent for paint or motor cleaning. It is measured at the end of the working week.

- Methylchloroform is used for degreasing and cleaning. It has fallen into disuse due to the damaged ozone. It is measured before the end of the daily shift; the TLV is 40 ppm.

- Trichloroethylene was banned in 1977 in the USA; it was used as an anesthetic and as a solvent for corn sterilization. Now it is used for metal degreasing, as a painting solvent and for dry washing.

Breath analysis is a potential biomarker for occupational and environmental exposure since its major advantage is that it involves a noninvasive procedure. However, more research is necessary for it to be used routinely. It is especially important to establish relationships between exposures (dose) and level of substances in the various breath samples[32].

1.5 Physiology of respiration

The major function of the respiratory system is the gas exchange between the external environment and an organism's circulatory system. In humans, this exchange facilitates the oxygenation of the blood with a concomitant removal of carbon dioxide and other gaseous metabolic wastes from the circulation.[40] The simultaneous activity of the respiratory apparatus and the cardio-circulatory system guarantees that oxygen is brought to the tissues and that carbon dioxide is removed. Oxygen has to reach every cell, to be used for metabolic oxidation and the production of energy, while carbon dioxide, which is an end-product of cell metabolism, has to be removed from venous blood and transferred to the lung to be eliminated[41].

The respiratory system can be divided in two sections (Figure 1.1): dead space which mainly acts as a conducting airway (nose, pharynx, larynx, and other airways without alveoli), and a section whose main function is gas exchange (alveoli and alveolar sacs)[42].

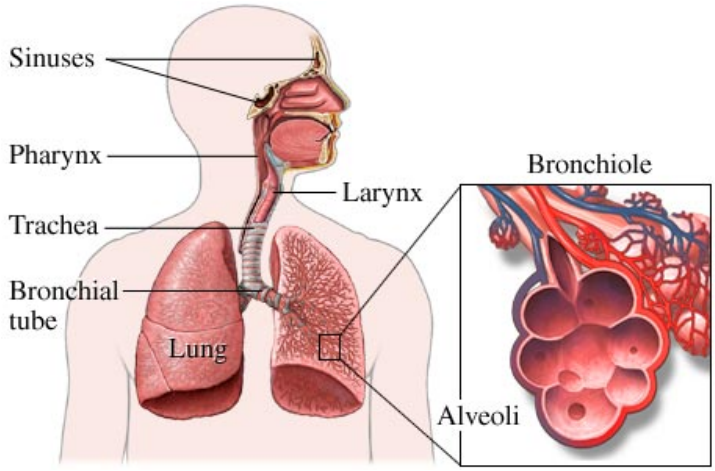

Figure 1.1 *Respiratory system*

Upon inhalation, a gas exchange occurs in the alveoli, the tiny sacs which are the basic functional component of the lungs. The alveolar walls are extremely thin (approx. 0.2 micrometers). These walls consist of single layers of epithelial cells in close proximity to the pulmonary capillaries which consist of single layers of endothelial cells. The close proximity of these two cell types allows permeability to gases and, hence, a gas exchange.

The blood supplying the alveoli is pumped to the lung from the right ventricle of the heart and then flows into the entire body. Thus it is poor in oxygen (used from the cells) and rich in carbon dioxide (produced from cells). (Figure 1.2)

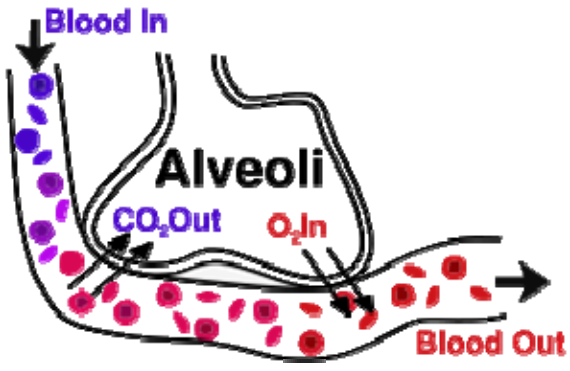

Figure 1.2 *Gas exchange in the alveoli*

The chemical process of gas exchange is diffusion: a substance always diffuses from A to B if the concentration or pressure is higher in A rather in B. Thus in the alveoli the oxygen pressure (100-110 mmHg) is lower than in the inspired air and higher than in

blood capillaries (40 mmHg). Carbon dioxide pressure in the alveoli, on the other hand, is lower (40 mmHg) than in capillary blood (46 mmHg) and the CO_2 moves in the opposite direction of the O_2. For the CO_2 the difference in pressure is small, but it is enough to eliminate the carbon dioxide produced by the organism as a result a good diffusivity of this gas. It is also important to consider that gas exchanges are also regulated by gas solubility in the liquid solution[43].

A typical respiratory cycle in a time span of about 5 seconds involves the exchange of half a litre of air with the lungs (tidal volume), so that the total ventilation, i.e. the volume of air moved in and out of the lungs per unit of time, is about 6 l/min. Not all the air we breathe is useful for the renewal of respiratory gases.

Before inspiration, dead space is filled with end-tidal air remaining from the previous respiratory cycle. End-tidal air is the last fraction of expired air, whose composition resembles alveolar air. During inspiration, half a litre of fresh ambient air is then inhaled into the body, but only the first 350 ml reach the alveoli together with 150 ml of end-tidal air contained in the dead space, where they are diluted and mixed with alveolar air. During expiration, 150 ml of ambient air, which filled the dead space, and 350 ml of air coming from the alveolar region are exhaled through the nose and/or mouth in sequence. By analyzing a respiration cycle, it can be seen that dead space is alternately filled with ambient and end-tidal air, and that only 350 ml of ambient air actually ventilates the lungs. Since the volume of air contained in the lungs during normal breathing is approximately 3 l, it follows that the composition of alveolar air is pretty stable during respiration (the cyclic variations of oxygen and carbon dioxide are about 2% and 5%, respectively).

During expiration the breath composition changes in order to empty the airways and the lungs. Such a process can be followed by measuring the CO_2 in breath, see Figure 1.3[6,55].
Three phases can be identified: during Phase I the air in dead space is eliminated and the composition of this fraction is low in CO_2, being similar to inspired air; during Phase II the partial pressure of CO_2 increases quickly until it reaches a maximum value of 35 mmHg; only in Phase III is the alveolar air eliminated, containing the volatile compounds released from blood.

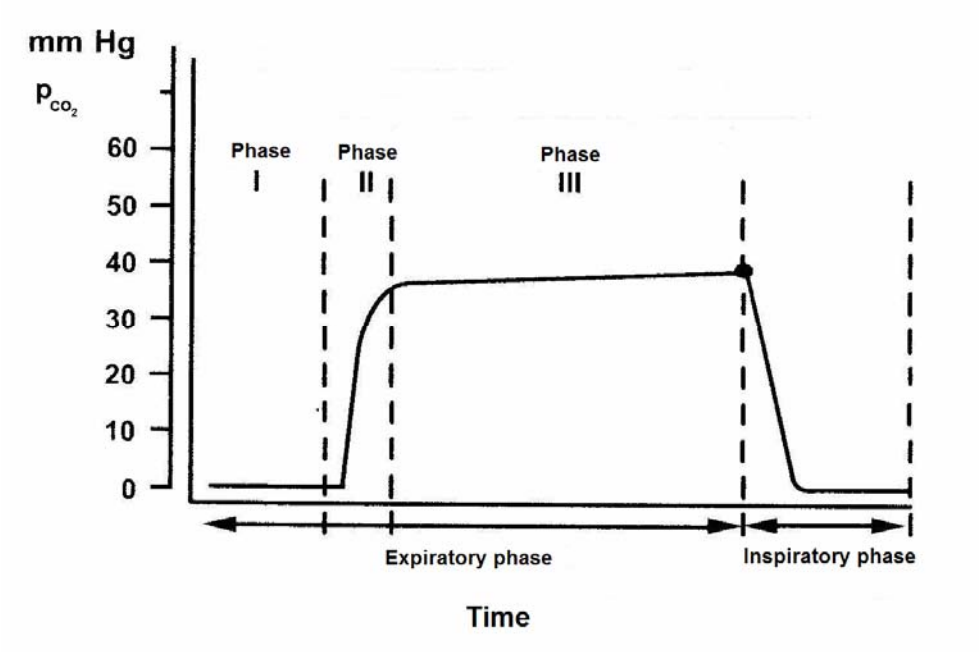

Figure 1.3 *CO_2 breath profile during the respiration process*

Ambient air is the main source of xenobiotic contaminants and oxygen. Its content of oxygen and carbon dioxide is fairly constant, while the concentrations of xenobiotics may be highly variable.

Dead space air has a composition close to ambient air (but a higher water content). Differences may arise due to the chemicals originating and/or released in the conducting airways or to gas exchanges with the mucus layer (mainly for water soluble compounds).

The composition of alveolar air is due to the interaction of ambient air with blood through the alveolar membrane. There are considerable non-homogeneities in the composition of alveolar air in different lung regions even in healthy subjects, since posture and gravity alter both local ventilation (exchange of the air in the lungs) and perfusion (blood circulation). Pulmonary ventilation and perfusion are mainly regulated by respiratory and cardiac frequency[44,55].

When the composition of ambient air changes, the time needed for each breath fraction to reach a new equilibrium ranges from a few seconds for dead space air to minutes or hours for alveolar air.

In most cases, blood is the main source of markers and for this reason alveolar air should be considered the most representative of an individual's condition. If markers are released from conducting airways, dead space air should be sampled.

Dead space and end-tidal air can be distinguished on the basis of their composition. Unfortunately, a net separation cannot be made between the two due to the absence of a sharp anatomical boundary. A boundary region has been identified at the terminal bronchioles with a 2 mm diameter, where the convective flow gives way to diffusion[43]. The diffusion and mixing of gases in this region depend on their physical–chemical properties, on the rate of respiration and on the anatomy. The concentration of expiratory gases is also greatly influenced by the ratio of ventilation-to-volume, perfusion-to-volume and ventilation-to-perfusion inequalities[45], as well as lung volume[46], posture[47] and flow rate[48].

Thanks to the presence of different path lengths of dead space between the mouth and alveoli, the lack of a uniform velocity across the cross section of the tracheobronchial tree, and due to convective and diffusive mixing of gases at the boundary region, the transition in expired air is not so sharp, so that the concentration profile is rather S-shaped. (Figure 1.4)

In 1948 Fowler defined dead space as the volume of the conducting airway until the point where a large change in gas composition occurs, and he proposed a method for determining its value[49].

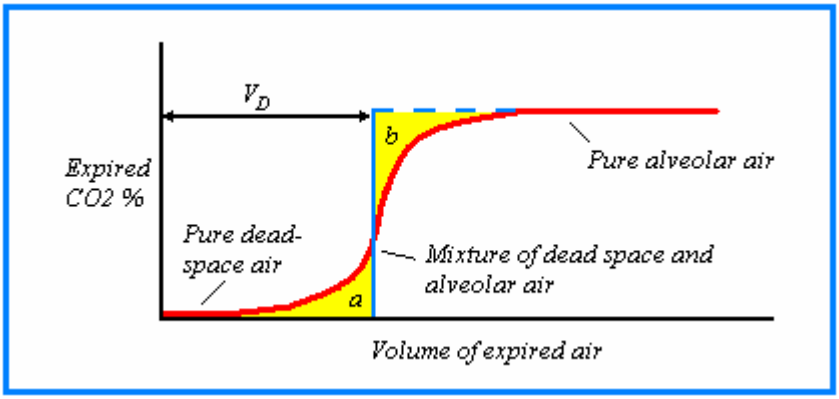

Figure 1.4 *CO_2 concentration profile as a function of expired volume Fowler's graphic method is also shown: dead volume corresponds to the point where (a) and (b) regions have the same area.*

This consisted of giving the subject a single breath of pure oxygen and monitoring the nitrogen concentration in the exhaled air. The nitrogen concentration profile in exhaled air resembles the carbon dioxide profile reported in Figure 1.4. The volumes i.e.,

shaded areas *a* and *b* are equivalent and represent the ideal transition point between dead space and alveolar air. Fowler used nitrogen as a tracer gas as he was able to use a fast sensor to measure its concentration levels in the expired air. In cases where the diffusivities were comparable, other gases, such as oxygen, carbon dioxide or helium, provided similar results[50, 51].

Bohr using carbon dioxide as a tracer gas proposed a different method based on mass balance at the end of the 19th century[52]. His formula was the expression of a simplified model that considered the expired breath as the sum of two unmixed homogeneous fractions, the inspired air contained in the dead space and the alveolar air from the lungs:

$$V_E \times C_E = (V_E - V_{DS})\, C_A + V_{DS} \times C_{DS} \qquad (1)$$

Where V_E and V_{DS} are the volumes of expired and dead space air, C_E is the average concentration of carbon dioxide in expired air (the value that would be measured in a sampling bag), and C_{DS} and C_A are the concentrations of the same gas in dead space and alveolar air respectively. The contribution $V_{DS} \cdot C_{DS}$ of dead space air to the carbon dioxide balance can be neglected as the concentration of this gas in ambient air is close to zero. If the carbon dioxide concentration C_A in the alveolar air is known (it is reasonably close to the end-tidal CO_2 concentration level), Bohr's formula can be rearranged and used to estimate either the dilution factor *D* of the alveolar air due to dead space air:

$$D = \frac{V_E}{V_A} = \frac{V_E}{V_E - V_{DS}} = \frac{C_A}{C_E} \qquad (2)$$

or the dead space volume:

$$V_{DS} = V_E \left(1 - \frac{C_E}{C_A}\right) \qquad (3)$$

Even though they provide about the same estimate of dead space volume in healthy individuals, there is an important conceptual difference between Bohr's and Fowler's

approaches, originating from the use of tracer gases such as carbon dioxide and nitrogen which are involved or not involved in the respiration process. Fowler's method estimates the volume of the conducting airways to the point where the pure oxygen delivered to the subject mixes with the nitrogen in alveolar air (anatomic dead space). Bohr's method on the other hand, measures the volume of air not involved in the gas exchange with blood (physiological dead space).

The difference is due to the alveolar dead space, i.e. the volumes of the lung constituted by alveoli that are ventilated but insufficiently perfused (or perfused but poorly ventilated) for a gas exchange to be effective. Such volumes are normally very small (less than 5 ml) in healthy individuals, but can increase dramatically in patients affected by lung diseases characterized by ventilation or perfusion impairments such as bronchitis, emphysema or pulmonary embolism. Thus, if Bohr's method is important to clinicians in order to assess the alveolar dead space, at the same time the estimate of the dilution factor may be used by researchers in breath analysis to normalize data and increase reproducibility[42].

1.6 Breath sampling techniques

Despite its great potential, breath analysis is still far from being used in clinical practice. A list of argued reasons has been proposed by Risby et al[53]. From a technical point of view, one of the main problems is the lack of a standard sampling procedure and sufficiently accurate measurements for the trace compound analyses. To carry out a reliable quantitative analysis, breath samples need collecting in a standard and reproducible way.

In the absence of a standardization of the respiratory process and the breath collection procedure, the ratio between alveolar air and dead volume can vary a lot from breath to breath, leading to a large variability in the data.

Breath collection can be performed in two principal ways: mixed sampling and end-tidal sampling. In mixed breath sampling all the expired air is collected, including the air in the superior airways, which are not involved in a gas exchange (dead volume). In end-tidal sampling, only end-tidal air (principally alveolar air) is collected, which contains most of the information regarding compound composition in blood.
Mixed sampling is generally preferred for its simplicity, although dilution by dead volume air (15-20%), may depends on the general condition of a person, such as pulmonary capacity, pulmonary ventilation and blood flow[6, 15, 55, 54]. Pulmonary capacity varies according to age, gender, build. Thus in the case of mixed breath sampling it is important to normalize the data on CO_2 concentrations. The amount of CO_2 in alveolar air during respiration, together with that present in mixed breath sample, enables us to evaluate the dilution factor due to the dead anatomical volume[6, 55, 56].

It would be useful to be able to collect dead volume and end-tidal during the expiratory phase separately, because it would enable us to identify the origin of the compounds in breath. The presence of exogenous (substances coming from ambient air) compounds, in fact, is one of the main sources of pollution. It would therefore be useful to distinguish them from endogenous compounds (substances produced from physiologic or pathologic metabolism).

The solution to this problem is dealt with in different ways: Phillips asserts that every compound needs to be quantified on the basis of the concentration gradient between breath and ambient air[57]; instead Schubert et al., assert that those compounds whose concentration in ambient air is comparable or higher than in breath, do not have to be considered in the characterization of the subject's breath[58]; and finally, to get round the problem, other scientists, give pure air[59, 60] to the subject. In reality this latter solution is also questionable, in fact, after exogenous compounds have reached pulmonary alveoli dissolved in blood on the basis of their blood/air repartition coefficients, the compounds are then transferred through the body and released in the different tissues depend on the chemical affinity. The concentration of each compound in alveolar air is the result of a dynamic equilibrium that involves several compartments, each with its specific time constant. It has been demonstrated that different compounds have different elimination

times ranging from minutes to hours[61], and this limits the use of pure air only to a few cases.

Further complications depend on the water solubility of some exogenous compounds, which dissolve in the mucus layer of the upper airways, and are thus present in breath in unpredictable amounts[62]. At the same time, it is known that some endogenous compounds of clinical interest, such as nitric oxide, are produced in the superior airways and do not come from alveolar air[63].
All these considerations lead to the conclusion that separating the different breath fractions could be an effective for future research in this field.

There are many different sampling techniques for breath analysis. Several containers are used to sample exhaled air, such as glass tubes and plastic bags, from which a sub-sample is transferred directly to the analysis system by means of syringes, or solid adsorbents, from which the components are thermally desorbed[32].

Several plastic materials were used in gaseous sampling field; among these the most used are Tedlar, Nalophan and the Cali-5-Bond, a multilayer material.
Tedlar is a polyvinylfluoride film with excellent chemical, electrical properties and a high mechanics resistance. Furthermore Tedlar material has the property to be a barrier at UV rays.

Nalophan (polyethylenterepthalate, PET) is a waterproof material and it is principally used in foods storage. It resists against most of the organic solvent, oils, weak acids and corrosive solutions; it also resists to temperature between -60°C and 220°C and it can not be welded.
The Cali-5-bond is composed by an internal layer of high density polyethylene, followed by polyamide, an aluminum sheet, which avoids diffusion processes, a polyvinylidenchloride layer and the last external layer a polyester film. This material was specifically projected for the sampling, the transport and the storage of ambient air or gaseous mixtures. This material guarantees a lower permeability and a high mechanics resistance, together with high chemical inertia.

Several types of sampling devices[54, 64, 31, 35, 37] have been proposed to guarantee efficient sampling that will truly represent the content of exhaled air during the exhalation time, some of which are commercially available.
Pleil et al. collected breath in preconditioned stainless steel canisters[56, 65]. The subject controls the sampling, manually opening the valve through which the breath enters. The subject breathes ambient air and mixed samples are collected.

There are two commercially available samplers that permit end-tidal breath to be collected. The Quintron system which consists of a tee connector, connected to two one way valves. The heart of each valve is a silicon disc that seals the exit until the threshold pressure is reached. The valves are regulated to open at different pressures; a 250 ml bag is connected to the valve that opens at the lowest pressure, whereas a 750 ml bag is connected to the other valves. There is a mouthpiece at the entrance of the tee connector. When the subject breathes in the system, the pressure of the connector increases until the first valve opening and the bag are filled with dead volume air. The pressure increases again until the second valve opening and the second bag are filled with end-tidal breath.

The other collection system, BioVOC™, is produced by Markes International. In this case the subject is asked to breathe through a mouthpiece into an open cylinder. Only the last portion of end-tidal breath (150 ml) remains in the cylinder after expiration. The mouthpiece is then replaced by a piston, used to push the sample in an adsorption tube connected to the end part of the cylinder[66].
Even if these systems are not the best solution, they are favourable in terms of their simplicity and cost. Their main limitations are the poor control of sampling conditions and the low volume collectable with a single breath.

A more sophisticated system, namely Breath Collecting Apparatus was developed by Menssana Research[57]. In this system the subject breathes through a mouthpiece in an instrument consisting of an inlet valve for ambient air inspiration and an exit valve connected to an open, stainless steel, cylindrical container held to 40°C to avoid water condensation. The sampling port, close to the mouthpiece, is connected to an adsorption tube, a flow meter and a pump. The system is controlled by a microprocessor, which, when necessary, starts the pump. The selective collection of end-tidal air in the

container is obtained by starting the pump at an appropriate time interval after expiration. The total volume collected in the adsorption tube during multiple breaths can be selected by the operator.

A breath sampler with CO_2 control has been proposed by Schubert et al. for patients who are mechanically ventilated[67]. When the percentage CO_2 volume exceeds the expected value an infrared sensor sends the data to an electronic processor, which activates a two way valve directing the flow towards an adsorbent trap[55].

As regards the commercially available sampler, it is worth noting that BioVOC does not have a system to hold the temperature at a specific value to avoid water condensation, moreover the cylinder has a small hole into which the subject breathes, which causes high back pressure, making the sampler not suitable for subjects with pulmonary dysfunctions.

The same limitations apply to the Quintron system. Although the first bag volume can be modified on the basis of the weight and height of the subject, the different pulmonary capacities are not considered. The sampler offers a lower back pressure to the breathing respect to BioVOC sampler, but no remedy is taken for water condensation.

M. Phillips, on the other hand created a good prototype sampler. The device is built with inert materials, uses a disposable mouthpiece and is heated to avoid sample condensation, however only a portion of alveolar air is sampled (when the pump is sucking) and not all the alveolar air.

From this analysis on breath samplers it is evident that there are many difficulties to overcome: back pressure during the expiration phase, sample condensation and an accurate separation of the two breath fractions.

1.7 Analytical techniques used for chemical breath characterization

The methodologies described in the literature can be divided in two main groups:

- Analytical techniques providing a sample collecting phase and subsequent analysis using different instrumental techniques;
- Analytical techniques providing an analysis in real time directly on the patients using dedicated equipments.

Gaschromatography is certainly the best technique for the separation and the analysis of volatile organic compounds (VOCs) in liquid and gaseous samples and coupled with mass spectrometry (MS) it enables us to identify analytes in complex mixtures.

After collection the pre-concentration of the analytes is required, because of the very low concentration levels in the breath sample. The pre-concentration of the compounds can be performed with thermal adsorption tubes, through which a known volume of the sample is passed through (Solid Phase Extraction, SPE), or using the solid-phase micro extraction technique (SPME)[92, 93, 94].

Information on breath composition can also be obtained from direct breath measurements performed by different mass spectrometric techniques: *Selected Ion Flow Tube* [68, 69, 70] (SIFT) or *Proton Transfer Reaction Mass Spectrometry* [71, 72, 73, 74] (PTR-MS). These techniques enable us to eliminate the collection and pre-concentration phases and give real time responses.

The SIFT technique was recently applied in the identification and quantification of gases present in air and breath samples. A primary ion current (i.e. H_3O^+) is generated from an ionic source. A quadrupole mass filter directs the primary ions into a tube, where a flow of an inert carrier gas such as helium is passed through. Most of the organic substances have a greater proton affinity than water, thus the H_3O^+ ion is a proton donor.
The gaseous sample, introduced in the tube, interacts with the primary ion stream; the collision between molecules causes the transfer of the proton from primary ion to the analyte, which receives a positive charge without any fragmentation. Before reaching the detector the produced ions are separated from each other and from the primary ion

stream, on the basis of the mass/charge ratio, using a quadrupole mass filter placed at the end of the tube.

All the produced ions are detected giving a single mass spectrum. When analyte concentrations in the sample are very low, the amounts of primary ions that react are lower than the total and the amount of ions produced by the analyte is proportional to the partial pressure in the sample[73,74].

The PTR-MS technique is similar to SIFT and is shown in Figure 1.5.

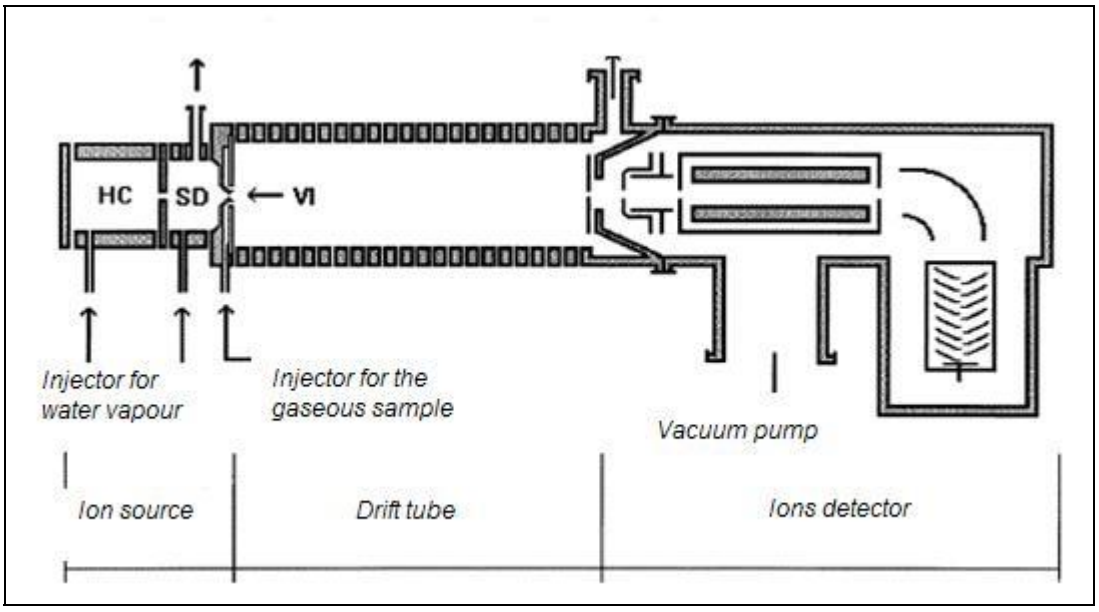

Figure 1.5 *Schema of PTR-MS*

An ion source produces H_3O^+ from water vapour molecules. The H_3O^+ ions entering the drift tube transfer the protons to volatile organic compounds, which have higher proton affinities than water.

The exothermic reaction is as follows:

$$H_3O^+ + VOC \rightarrow VOCH^+ + H_2O \qquad (4)$$

The PTR-MS technique is different from SIFT in some ways that are used to increase the sensitivity of the method. The system works with an empty cathode ion source, which produces a high density of primary ions directing them into the tube without a pre selector. Inside the tube only the sample is introduced avoiding dilution from a

transport gas. For this reason, an increase of two orders of magnitude in sensitivity can be achieved compared to the SIFT technique[74].

The two techniques can be used to take frequent and fast measurements without any pretreatment of the sample. However, the characterization of the substance only occurs on the basis of the mass/charge ratio and the chemical identification of the compounds is not easy[73, 75, 76]. Sometimes gas chromatography separation is needed to perform a quantitative, because ions produced from different analytes with the same mass/charge ratio can be present[73, 74]. Another problem is the undesirable fragmentation of the sample molecules, which complicate the situation considerably.

In recent years *Ion Mobility Spectrometry* (IMS) has been developed, which is a small and effective instrumentation for the detection of trace compounds in a gases[76, 77, 78]. IMS bases the characterization of compounds on their gas phase mobility, under a high electric field. Typically IMS is formed from an ionization chamber with β or a UV radiation source, an ion injector, a drift tube and an ion collector. The carrier gas (i.e. air or nitrogen) transfers the neutral analyte molecules in the vapour phase into the ionization chamber where a series of ion-molecule reactions occur. The reactant ions are injected into the drift tube, by periodically opening the shutter grid. The ions produced are formed in defined chemical reactions of neutral analyte molecules with reactant ions. The mobility of these produced ions may be used to identify the analyte molecules. The ions are selectively revealed on the basis of their characteristic drift time through the tube. The drift velocity (v) of the ion is related to the electric field strength (E) by the mobility (k), where[78]:

$$v = k \times E \qquad (5)$$

Therefore the mobility is inversely proportional to the drift time, which is usually measured at a fixed drift length. In order to work the IMS does not need the vacuum and the ambient air may be used as carrier gas. Often the IMS is coupled with a gas-chromatographic system because, in the case of a complex mixture, the selectivity is low[70, 71, 76, 77].

Recently spectroscopy techniques were developed to determine volatile compounds in gaseous samples. The laser spectroscopy technique for example measures the light attenuation of the laser after passing through the gaseous sample in the adsorption cell. The mid infrared region (λ=3-10 μm) is particularly interesting, because the vibrational transition of most of the organic compounds leads to adsorption lines in that spectral range. One of the main advantages of these techniques is the possibility of measuring breath components in real time, in pptv (parts per trillion in volume) concentrations. Moreover spectroscopy provides information on analyte concentrations in the sample during the different phases of respiration. This is different from other analysis techniques, which give information on the whole expiratory phase[79,70].

Alongside traditional analytical techniques, over the last few years innovative instrumental techniques based on sensor have been on the increase[4, 18, 74, 80, 81]. Sensors for ethanol and acetaldehyde analysis in breath have been created, immobilizing alcohol oxidase (AOD) and aldehyde dehydrogenase (ALDH) enzymes on electrodes[82]. Furthermore MOS (metallic oxide semiconductor) gas sensors seem to provide a good methodological approach to breath analysis, because of the wide range of detectable gases, their high sensitivity, the fast response and the simplicity of the devices involved. The poor selectivity of the sensor is a limitation that may be overcome with the use of an array of non selective sensors. The simultaneous use of more than one sensor and the application of a multivariate analysis of the data enable us to obtain useful information on the chemical composition of the sample. This is despite the fact that the sensor array response is not univocally related to the concentration of a single compound, but rather to the combination of all the compounds in the breath sample[83]. Recently these sensor arrays have been used in the medical field, to diagnose some pathologies, such as diabetes, lung cancer, asthma or other respiratory inflammations[74, 84, 85, 86, 87].

1.8 Breath pre-concentration techniques

Owing to the low concentrations of most of the compounds present in human breath (order of magnitude μg/l and ng/l), after sampling and before analysis, a pre-concentration analyte step is required. The methods used in the sampling and the pre-concentration of breath substances include a chemical method, a cryogenic method[2, 3, 5] and the most diffused adsorptive methods[3].

In the chemical methods, breath is usually bubbled through a reagent solution, which only captures a specific compound. The method is simple and direct, and the trapped sample is easily analyzed by a spectrophotometric technique. The main disadvantages are the low sensitivity and the great effort required by the subject to breathe.

In the cryogenic method, breath is passed through a trap immersed in a cryogenic fluid. After sampling the trap is heated and the trapped compounds are released and transferred to the instrument by a carrier inert gas.

The main problems encountered during the collecting phase are linked to the high content of water, a relative humidity > 95%. In the cryogenic method, if the water is not removed, the formation of ice clogs the trap[3]. In the adsorptive method on the other hand, the dipolar interactions between the water and the polar analytes reduce the trap efficiency of the adsorbent used[88].
To avoid interferences due to water, different resolutions were found which included the use of drying agents (Na_2SO_4 and $CaCl_2$) through which the sample was passed, or the use of membranes with adsorbent surfaces (MESI, *Membrane Extraction with Sorbent Interface*)[89]. These systems are limited due to the possible loss of the analytes and the contamination of the sample.

In the adsorptive method, breath comes in contact with a resin, which traps the organic compounds of interest. There are two different types of pre-concentration techniques that use adsorption in the solid phase:

- **The Solid Phase Micro-Extraction (SPME)**, which uses fused silica micro-fiber coated with an appropriate stationary phase;
- **The Solid Phase Extraction (SPE)**, in which tubes filled with a specific adsorbent phase are used.

1.8.1 The Solid Phase Micro-Extraction (SPME)

The solid phase micro-extraction technique, developed by Pawliszyn in 1989, combines the collecting and analytes extraction phases in one step. The SPME does not use any solvents[90, 91] and may be easily coupled with the most diffuse chromatographic technique, such as Gas-Chromatography (GC), High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE)[90].

The complete system used for SPME is shown in Figure 1.6

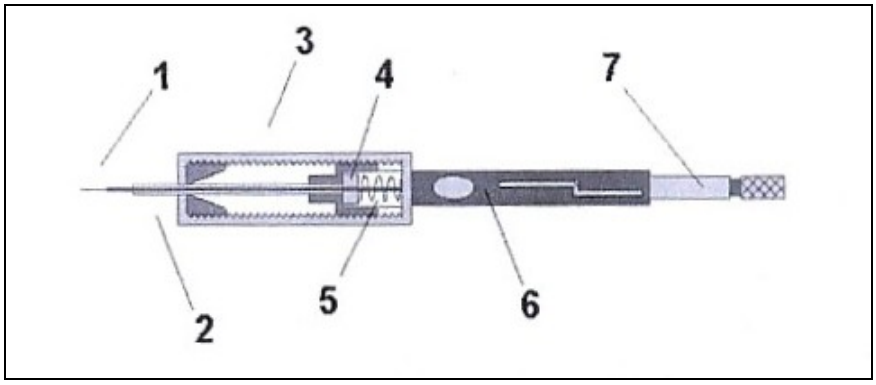

Figure 1.6 *SPME system: 1) withdrawn fused silica fiber, 2) stainless steel needle, 3) holder, 4) silicon septum, 5) spring, 6) tube support, 7) plunger.*

The pre-concentration procedure exposes the fiber to the sampler for the time required by the analytes to reach equilibrium between the sample and the fiber. At the end of the equilibration time, the fiber is inserted in to the gas-chromatography injector and the analytes are thermally desorbed. Consequently the carrier gas then transfers the analytes into the column[30, 91, 92, 93].

The fiber is coated with a thin stationary phase, made of polymers or a mixture of polymers with carbon materials. Different stationary phases, with different thicknesses and polarities are available and have affinities with different classes of compounds. Polydimethylsiloxane (PDMS), is suited to non polar compounds, carbowax-divinylbenzene (CW-DVB) and polyacrylate (PA) are suited to polar analytes and polydimethylsiloxane-divinylbenzene (PDMS-DVB) and carboxen-polydimethyl siloxane (CX-PDMS) do not have a specific application[91, 93 , 94, 95].

This technique has several advantages in terms of its applicability to all kinds of samples (solid, liquid or gaseous) without needing any particular sample preparation, its easiness to use, the speed of the analysis and the possibility to perform a chemical qualitative screening of a gaseous sample. The principle disadvantages involve the low pre-concentration factor analytes are present at trace levels. It is also limited in terms of a quantitative analysis of a complex sample matrix, due to the competition of different compounds in the distribution process[94, 95].

1.8.2 The Solid Phase Extraction (SPE)

In the solid phase extraction technique, the sample passes through an adsorption tube, filled with a suitable stationary phase and the analytes are retained on the basis of their chemical-physical properties. (Figure 1.7)

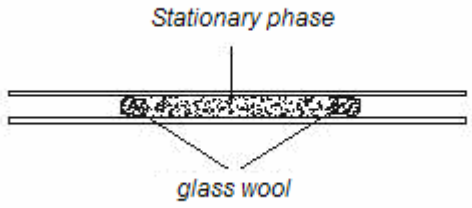

Figure 1.7 *Adsorption tube used in SPE technique: the tube can be made of stainless steel or glass*

Carbon is the main constituent of the adsorbent in terms of its high chemical inertness and its thermal stability[96].

Different kinds of carbon are available:

- Active carbon
- Carbon molecular sieve
- Graphite carbon
- Porous carbon

Each has different properties and retains different classes of compounds.

Active carbons have a complex superficial structure made up of many functional groups: phenolic, carboxylic, aldehyde, ketone , peroxy, quinone and lactone. The principle mechanism of interaction includes hydrophobic, hydrogen bond and cationic exchanges. Using active carbons the compounds were not able to be completely released due to their high affinity with the stationary phase[96].

Carbon molecular sieves have a highly porous surface with almost uniform micro-porous diameters and a wide superficial area, suited for the retention of organic compounds. The adsorption mechanisms include dispersive interaction (London or dispersion forces, Van der Waal forces) and strong dipole-dipole interactions[96]. The thermal treatment of graphite carbon eliminates volatile compounds and produces a homogeneous surface without micro-pores, where the compounds are adsorbed on the basis of their size and shape. Most of the surface has non polar sites (carbon atoms) that do not react with molecules containing functional groups, thus dispersive interactions were predominant in the adsorption mechanism[96].

Porous carbon is produced by impregnating a suitable silica gel with mixed resins of phenol-formaldehyde, phenol-hexamine, saccharose or other materials. After polymerization inside the silica porous gel, the polymer is transformed in to vitreous carbon by heating it to 1000°C. The silica portion is removed in order to form the pores. The material is then heated to 2000-2800°C in an inert atmosphere, to fit the surface, remove the micro-pores and depending on the temperature, produce a certain degree of graphitization. The porous carbon has a homogeneous hydrophobic surface and the interactions depend on the final heating and possibly on chemical treatments[96].

The choice of which adsorbent phase to use has to be made on the basis of the following properties[97]:

- **Functionality**: expresses the affinity of the phase for the different organic compounds;
- **Chemical inertness**: the reaction capacity of the phase in relation to adsorbed molecules;

- **Size and shape of the particles**: influence the hydrodynamic conditions of the sample that passes into the tube and determines the surface area value;
- **Pores dimension**: a high porosity determines a larger total surface area;
- **Surface area**: influences the recovery and the reproducibility of the adsorption process.

If the sample has a high content of water it is convenient to choose a hydrophobic material to limit water interferences. The presence of water could interfere with the detector acquisition, modify retention time and cause the column to deteriorate (referred to the chromatographic technique)[98, 99].

The use of a multi-bed tube is recommended if the sample contains compounds with different chemical-physical properties. (Figure 1.8)

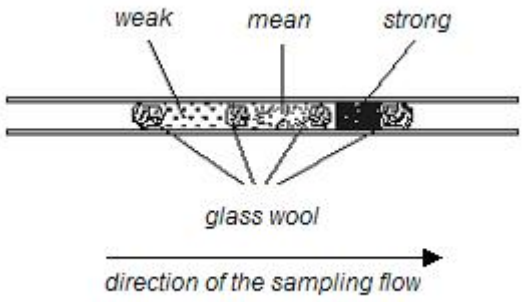

Figure 1.8 *Multi-bed tube filled with three different stationary phases with different adsorption capacities.*

The multi-bed tubes are filled with different phases in an increasing order of adsorption capacity. With these tubes the range of adsorbed compounds is wider than with the mono-phase tube provided the phases are properly chosen. Adsorption tubes are used both with gaseous and liquid samples by applying different collecting techniques.

In terms of gaseous samples, the collecting techniques are as follows:

- Diffusive sampling
- Active sampling

Diffusive sampling or passive sampling is the easiest procedure from an operative point of view. The tubes are exposed to the sample, i.e. air, for an established time; volatile compounds diffuse in the tubes and the analytes are retained by the adsorption material. This kind of sampling takes a long time. Since diffusive sampling can be performed with only mono-phase tubes, the simultaneous exposure of tubes filled with different stationary phases is required.

In the case of active sampling, a known volume of the sample passes through the adsorption tube using a pump, which maintains a constant flow. Active sampling, differently from passive sampling, enables the operator to know the amount of sample collected in the tube and for the same sampling time, a greater amount of analyte is collected.

Multi-bed tubes can be used in active sampling to retain a wide range of compounds with different boiling points and polarities, in just one collection.

The sampling flow may vary from a minimum of 10 ml/min to 200 ml/min, the optimal flow being 50 ml/min. With a flow under 10 ml/min, the analytes may diffuse along the stationary phase and with a flow higher than 200 ml/min, the recovery of the analytes may not be complete.

During the collection process, the operator must be certain that all of the analytes collected remain in the adsorbent phase. To assure that no analyte is lost during the sample collection, the Breakthrough Volume should be considered.

Breakthrough volume is defined as the calculated volume of carrier gas per gram of adsorbent resin that causes the analyte molecules to migrate from the front to the back of the adsorbent bed, at a specific temperature. This volume defines the adsorption capacity of a resin in relation to a specific analyte and depends on the affinity between the stationary phase and the considered compound, the amount of stationary phase in the tube and the temperature.

A breakthrough volume measurement can be performed flowing a gas with a known content of analyte through an adsorption tube connected directly to a detector, which

gives the signal in real time. The detector will give no response until the resin traps the analyte. When the breakthrough volume is exceeded, the detector will give a response proportional to the amount of analyte coming out. (Figure 1.9)

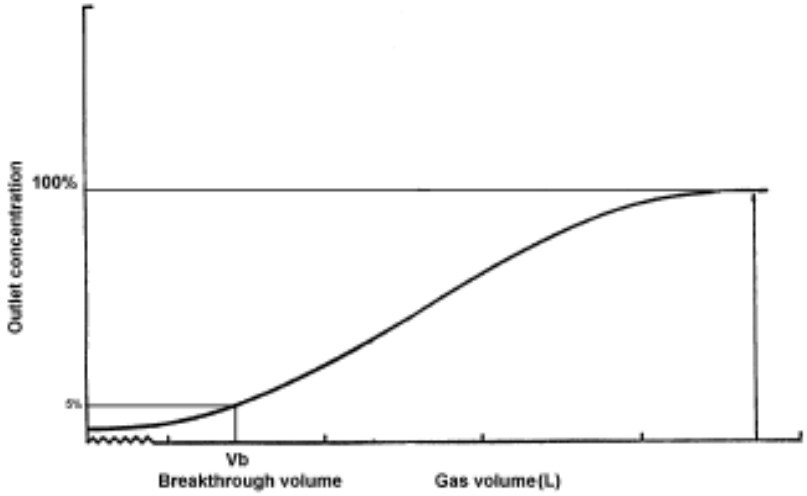

Figure 1.9 *Percentage trend of the amount of analyte coming out of the tube with respect to the volume of gas passing through (V_b is the breakthrough volume)*

For example, at a fixed temperature, if the breakthrough volume for an analyte is 1.0 litre per gram of resin, then the sample should not be purged with more than 500 ml of gas, for tubes filled with 1 g of the adsorbent phase.

The adsorbent capacity of resin is influenced by humidity, thus the breakthrough volume will also depend on the sample content of water and its evaluation should be carried out in the same humidity conditions as the sample. In fact it is appropriate to sample a maximum volume corresponding to 70% of the breakthrough volume (Safe Sampling Volume, SSV), because the amount of analyte in the sample and thus the breakthrough volume can vary significantly.

1.9 Recovery of the analytes after pre-concentration

The recovery of the sample from the stationary phase can be made by extracting a small amount of organic solvent, or alternatively by heating the adsorbent phase purging with an inert gas (thermal desorption). The absence of solvent enables us to identify the most volatile compounds, usually eluted with the solvent. In addition, the cost and the disposal of the solvent are eliminated.

The thermal desorption technique allows the volatile compounds linked to the stationary phase to be released using a rapid increase in temperature. The analytes are thus transferred to the chromatographic column, using a flow of an inert gas as a carrier. Although the sample is rapidly heated, the analyte desorption is not as rapid. Thus during the transfer to the chromatographic column, a broadening of the chromatographic signal can occur, with a consequent loss of resolution.
This problem is solved in the two stage desorption system; in fact the analytes desorbed from the tube are concentrated in a cryogenic packed trap, which has a reduced internal volume. The trap is maintained at a low temperature using a Peltier cell, in order to adsorb the analyte coming from the tube; the trap is then rapidly heated and in this case the desorption process is faster and the transfer to the column is performed with smaller carrier gas volumes. The tube and the trap are both desorbed in the opposite direction with respect to the flow during adsorption process. (Figure 1.10)

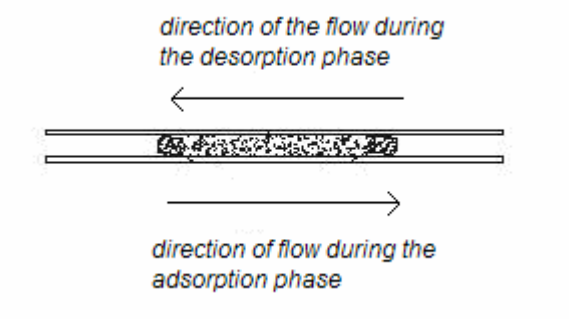

Figure 1.10 *Direction of the flow during compound adsorption and desorption of both the trap and the tube*

In order to completely release the analytes from the adsorption tube, to quantitatively adsorb the released compounds in the focusing trap and quantitatively transfer the analytes into the column using a smaller volume of carrier gas, following parameters need to be optimized:

- Packing material of the trap;
- Tube desorption, focusing and trap desorption temperatures;
- Desorption flows and times from the trap and the tube.

Since the internal trap can operate at a lower temperature than ambient temperature and the volume of gas that passes through is smaller than the volume passing through the tube, a stationary phase can be used with a lower adsorption capacity compared to the resin filling the tube.

The selection of an appropriate temperature of the tube is required, in order to completely desorb the compounds of interest and avoiding their thermal decomposition. It is also best not to desorb near the maximum temperature indicated for a specific stationary phase. This is to avoid the degradation of adsorbent material and the subsequent release of interfering compounds.

During the focusing phase, the internal trap can be cooled up to -40°C, the temperature at which most of the compounds are retained. Where there is a high water content in the sample, a temperature of around 0°C is required to avoid ice formation with the consequent obstruction of the trap. The desorption temperature also has to allow for the quantitative recovery of the adsorbed analytes. The temperature limit is the maximum provided for the stationary phase used. Trap and tube desorption times may guarantee the complete release of the compounds of interest and usually varies from 5 to 30 minutes depending on the carrier gas flow and desorption temperature.

The flow of auxiliary gas during tube desorption should be optimized. A low flow limits the extraction efficiency from the tube and the desorption time becomes too long. An extremely high flow could limit the retention capacity of the trap. Usually flows around 30-50 ml/min are suggested. During the transfer from the focusing trap to the chromatographic column, the carrier gas flow should guarantee a quantitative and fast transfer of the compounds. The lowest compatible flow is 7-8 ml/min. The carrier gas

flow through the trap is the sum of the flow into the column and the split injection flow. Capillary columns need a split flow of 5-10 ml/min with flow values in the column of 0.5-2.0 ml /min, to ensure a sufficiently rapid transfer.

One of the application fields of thermal desorption technique includes an environmental analysis for the determination of volatile and semi volatile organic pollutants in the atmosphere[26], in the working environment[30, 32], and in the emissions from building materials[28, 29]. It is also widely used for the extraction of VOCs from matrices such as solid samples, emulsions and saline solutions, which cannot be injected directly in the chromatography. Over the last few years, this technique has also been used in breath analysis, in diagnostic application or to evaluate human exposure to toxic substances[100]. A limitation of this technique obviously includes compounds that are not analyzable by gas chromatography, compounds that are easily thermally degradable and compounds with a high boiling point (C_{36} hydrocarbons, PAH, etc).

CHAPTER 2

Materials and Methods

2.1 Description of standard solution preparation

Stock A

The liquid mixture consisted of eighteen compounds and two labelled internal standards, Toluene-D8 and Isopropanol-D8 in methanol. Labelled isopropanol-D8 and toluene- D8 (purity 99.8 %) were purchased from ARMAR Chemicals and used as an internal standard, without any further purification.

Isopropanol, 2-butanone, 2-pentanone, hexanal, 2-heptanone, 4-heptanone, heptanal, benzaldehyde were purchased from AccuStandard, Inc. Chemical Reference Standard (USA).

Pentane, 2-methylpentane, hexane, 1,1,1,3,3,3-hexafluoro-2-isopropanol, dimethylsulphide, acetone, carbon sulphide and toluene puriss p.a. standard for GC grade > 99% were purchased from Fluka. Dimethyldisulphide and isoprene puriss p.a. for GC grade > 99% were supplied by Sigma- Aldrich, all compounds were used without any further purification.

A stock solution consisting of 50 μl of each 18 neat compounds was prepared (SOL 1). Stock A was obtained by adding 20 μl of the stock solution and 50 μl of an intermediate labelled solution (Stock H) to 10 ml of methanol. Compound concentrations are reported in Table 2.1.

Table 2.1 *Concentration of compounds in Stock A*

Stock A	Concentration [ng/μl]
pentane	70
isoprene	76
acetone	88
dimethylsulphide	94
carbon sulphide	141
isopropanol	87
2-methylpentane	73
hexane	73
2-butanone	89
2-pentanone	90
hexafluoroisopropanol	177
dimethyldisulphide	116
toluene	96
hexanal	91
4-heptanone	91
2-heptanone	91
heptanal	91
benzaldehyde	116
isopropanol-D8	111
toluene-D8	118

Stock B, Stock C and Stock D

Two solutions were prepared, consisting of 100 μl of twelve neat compounds (SOL 2) and 100 μl of thirteen neat compounds (SOL 3) respectively. A stock solution was prepared, consisting of 40 μl of SOL 2 and 40 μl of SOL 3 in 20 ml of methanol (Stock B) (Table 2.2).

Table 2.2 *Concentration of compounds in Stock B*

Stock B	*Concentration of compounds in Stock B* **[ng/μl]**
tetrachloroethylene	267
3-hydroxy-2-butanone	164
1-butanol	135
trichloroethylene	245
benzene	146
carbontetrachloride	265
chloroform	247
ethylacetate	150
2,3-butandione	183
1-propanol	134
acetonitrile	131
ethanol	133
pentane	96
isoprene	105
acetone	122
dimethyl sulphide	130
isopropanol	121
2-methylpentane	100
hexane	101
2-pentanone	124
hexafluoroisopropanol	246
dimethyldisulphide	161
toluene	133
4-heptanone	126
m-cresol	158

Stock B solution was diluted to obtain Stock C and D (Table 2.3):

Table 2.3 *Preparation of Stock C and D*

	Stock C	**Stock D**
Stock B (ml)	5	1
Methanol (ml)	5	9
Dilution factor	1:2	1:10

The concentrations of all compounds in the different stock solutions are reported in Table 2.4

Table 2.4 *Standard solution concentrations*

[ng/μl]	Stock B	Stock C	Stock D
pentane	96	48	10
ethanol	133	67	13
isoprene	105	52	10
acetone	122	61	12
dimethylsulphide	130	65	13
isopropanol	121	60	12
acetonitrile	131	66	13
2-methylpentane	100	50	10
hexane	101	51	10
1-propanol	134	67	13
2,3-butandione	183	92	18
ethylacetate	150	75	15
chloroform	247	124	25
carbontetrachloride	265	133	27
benzene	146	73	15
trichloroethylene	245	123	25
1-butanol	135	68	14
2-pentanone	124	62	12
hexafluoroisopropanol	246	123	25
dimethyldisulphide	161	80	16
3-hydroxy-2-butanone	164	82	16
toluene	133	67	13
tetrachloroethylene	267	133	27
4-heptanone	126	63	13
m-cresol	158	79	16

50 μl of Stock H consisting of Toluene-D8 and Isopropanol-D8, were added to the three standard stock solutions. The concentrations of the internal standard compounds in each solution are reported in Table 2.5

Table 2.5 *Concentrations of the internal standards in stock solutions B, C, and D*

[ng/μl]	Stock B, C, D
Isopropanol-D8	111
Toluene-D8	118

Stock E

A gaseous Stock E sample was obtained by introducing 20 μl of liquid SOL 1 into a 2 L glass flask, held at 40 °C. The preparation of gaseous standards was carried out inside a oven held at 40°C, to avoid any condensation of the compounds. The corresponding concentrations are reported in Table 2.6.

Table 2.6 *Concentration of compounds in Stock E*

Stock E	Concentration [μg/l]
pentane	348
isoprene	378
acetone	439
dimethylsulphide	470
carbon sulphide	703
isopropanol	436
2-methylpentane	363
hexane	366
2-butanone	447
2-pentanone	449
hexafluoroisopropanol	887
dimethyldisulphide	581
toluene	481
hexanal	453
4-heptanone	454
2-heptanone	456
heptanal	454
benzaldehyde	581

Stock F

5 μl of both isopropanol-D8 and toluene-D8 were evaporated in a 2 L glass flask, held at 40°C, to prepare a gaseous sample for use as an internal standard. The corresponding concentrations are reported in Table 2.7.

Table 2.7 *Concentration of the internal standards in Stock F*

Stock F flask concentration [ppmv]	
Isopropanol-D8	839
Toluene-D8	605

Stock G

20 μl of stock solution (Stock G) consisting of 100 μl of the neat compounds reported in Table 2.8 were vaporized into a 2 L glass flask, held at 40°C.

5 ml of this gaseous mixture were then introduced into a Nalophan bag filled with 15 L of pure air.

Table 2.8 *Concentration of compounds in the flask and in Stock G.*

Compounds	Concentration of Stock G [ppmv]	Concentration in bag [ppbv]
Acetone	519	173
Isopropanol	628	209
Hexane	292	97
Methylethylketone	423	141
Dimethyldisulfide	429	143
Toluene	359	120

Stock H

An intermediate labelled solution was prepared consisting of 25 μl of isopropanol-D8, 25 μl of toluene-D8 and 950 μl of methanol.

2.2 Chromatographic, mass spectrometric and thermal desorption methods

Samples were transferred into adsorption tubes using a membrane pump (NMP 50, SKC ITALY) at a constant flow rate measured by a soap bubble flow meter, (mod. Humonic Inc).

Thermal desorption (TD) and GC-MS analysis were performed by an automated two-stage thermal desorption unit (STD 1000, DANI Instrument, Italy) equipped with an internal focusing trap packed with 70 mg of Tenax GR (DANI Instrument, Italy), a GC device (Trace GC Ultra, Thermo Electron Corporation, USA) equipped with a DB-624 capillary column (6% cyanopropyl phenyl siloxane and 94% dimethylpolysiloxane), (60 m x 0.25 mm x 1.4 μm film thickness, from Agilent J&W) and a quadrupole mass spectrometer (Trace DSQ, Thermo Electron Corporation, USA)

A description of the conditions used for thermal desorption, chromatographic and spectrometric methods is given in Table 2.9, 2.10, 2.11

Table 2.9 *Chromatographic and mass spectrometric method*

MTD 1	
Chromatographic method	
Inlet temperature	200 °C
Oven temperature ramps	40°C for 10 min; 10 °C/min until 200°C for 5 min
Column head Pressure	215 KPa
Inlet mode	split
Split Flow	10 ml/min
Transfer line temperature	260 °C
Mass Spectrometric method	
Ion source temperature	250 °C
Scan mode	Full Scan 10-200 amu
Scan rate	835.1 amu/sec
Solvent delay	5 min

Table 2.10 *Chromatographic, mass spectrometric and thermal desorption method*

MTD 2		
Chromatographic method		
Inlet temperature	200 °C	
Oven temperature ramps	35°C for 10 min; 4°C/min until 200°C; 20°C/min until 250°C for 10 min	
Flow	2.4 ml/min constant flow	
Inlet mode	split	
Split Flow	14 ml/min	
Transfer line temperature	260 °C	
Mass Spectrometric method		
Ion source temperature	250 °C	
Scan mode	Full Scan 10-200 amu	
Scan rate	345.2 amu/sec	
Solvent delay	0 min	
Thermal Desorption method		
	Tenax GR	**Carbopack Y, Carbopack B, Carboxen 1003**
Valve and transfer line temperature	200 °C	200 °C
Tube desorption Temperature	250 °C	330 °C
Tube desorption Time	5 min	5 min
Tube desorption Pressure	72 kPa	72 kPa
Trap focusing Temperature	5 °C	-10 °C
Trap desorption Temperature	250 °C	250 °C
Trap desorption Time	5 min	5 min

Table 2.11 *Chromatographic, mass spectrometric and thermal desorption method*

MTD 3	
Chromatographic method	
Inlet Temperature	200 °C
Oven temperature ramps	35°C for 10 min; 8 °C/min until 200°C for 0.50 min; 25 °C/min until 260°C for 5 min
Column head Pressure	225 kPa
Inlet mode	splitless
Split flow	10 ml/min
Transfer line temperature	260 °C
Mass Spectrometric method	
Ion source temperature	250 °C
Scan mode	Full Scan 15-200 amu and SIM
Scan rate	1801.10 amu/sec
Solvent delay	5 min
Thermal Desorption method	
	Tenax GR
Valve and transfer line temperature	170 °C
Tube desorption Temperature	300 °C
Tube desorption Time	5 min
Tube desorption Pressure	72 kPa
Trap focusing Temperature	-10 °C
Trap desorption Temperature	250 °C
Trap desorption Time	5 min

In Table 2.12 are shown the ions of the compounds that were identified by MS operating in selected ion mode (SIM).

Table 2.12 *Characteristic ions in SIM mass spectra used in MTD3*

Compound	SIM Mass	Compound	SIM Mass
pentane	43	1-butanol	56
ethanol	45	2-pentanone	43
isoprene	67	hexafluoroisopropanol	99
acetone	58	dimethyldisulphide	94
dimethylsulphide	62	3-hydroxy-2-butanone	45
isopropanol	45	toluene	91
acetonitrile	41	tetrachloroethylene	166
2-methylpentane	43	4-heptanone	71
hexane	57	m-cresol	108
1-propanol	31		
2,3-butandione	43	Isopropanol-D8	49
ethylacetate	43	Toluene-D8	98
chloroform	83		
carbontetrachloride	119		
benzene	78		
trichloroethylene	95		

2.3 Adsorbent phases

Table 2.13 *Tenax GR properties*

TENAX GR (250 mg, supply by SUPELCO)	
Chemical Structure	2,6-diphenylene-oxide polymer resin plus 30% graphite
Temperature Limit	350 °C
Affinity for Water	low
Specific Surface Area	24.1 sq. m/g
Pore Volume	2.4 cc/g
Average Pore Size	200 nm
Density	0.55 g/cc
Mesh size	60/80 mesh

Table 2.14 *Multi-bed adsorption tube phase properties*

	Carbopack Y	**Carbopack B**	**Carboxen 1003**
Chemical Structure	graphitized carbon black		carbon molecular sieve
Temperature Limit	400 °C		
Affinity for Water	Relatively low		
Specific Surface Area	24 sq. m/g	100 sq. m/g	1000 sq. m/g
Density	0.42 g/cc	0.35 g/cc	0.46 g/cc
Mesh size	60/80 mesh		40/60 mesh

Anhydrous sodium sulphate (Na_2SO_4) was purchased from SKC, magnesium perchlorate ($Mg(ClO_4)_2$) from SerCon and calcium sulphate ($CaSO_4$) from QuinTron.

2.4 Bag materials

Nalophan bags were made from a 20 μm thick roll of Nalophan tube, (Tillmanns S.p.a.,Milan). A 50 cm long chunk was cut from the roll, then one end was wrapped and closed with nylon cable ties; the other end was wrapped and closed around a Teflon tube connected to a valve.

Tedlar bags (film thickness 10-50 μm) were purchased from SKC (USA) while Cali-5-bond bags were purchased from Alltech (Italy).

Cali-5-bond bags are made from a sandwich foil consisting of five different layers, a 75 μm polyethylene sheet, in contact with the sample, a 40 μm polyamide layer, a 12 μm aluminium foil, a 3–4 μm polyvinyl dichloride (PVDC) and a 12 μm polyester (PTEP) as the external layer.

To test CO_2 bag stability a 5% CO_2 standard gaseous mix, (Sol Group Spa, Milan) was used. CO_2 bag contents were measured using an infrared sensor (Capnostat Mainstream CO_2 Sensor, Respironics, USA).

Ultra pure water used during the experiments was obtained using a PureLab Classic Pro, USF Elga instrument.

CHAPTER 3

Experimental Part

3.1 Effect of instrument tuning

Before any sample analysis, it is necessary to demonstrate that the mass spectrometry is operating satisfactorily. The instrument was tuned using one or two calibration compounds. Tuning involves adjusting source settings, analyzer settings, and gas flows to produce optimal peak intensities.

Perfluorotributylamine (PFTBA) is used to establish tuning performance prior to the analysis of volatile compounds. The calibration gas has a molecular weight of 671, and fragment ions over a wide m/z range. The relative abundances of ions must fall within a predefined range. PFTBA spectra and ion abundance criteria are shown in Figure 3.1 and Table 3.1, respectively.[101]

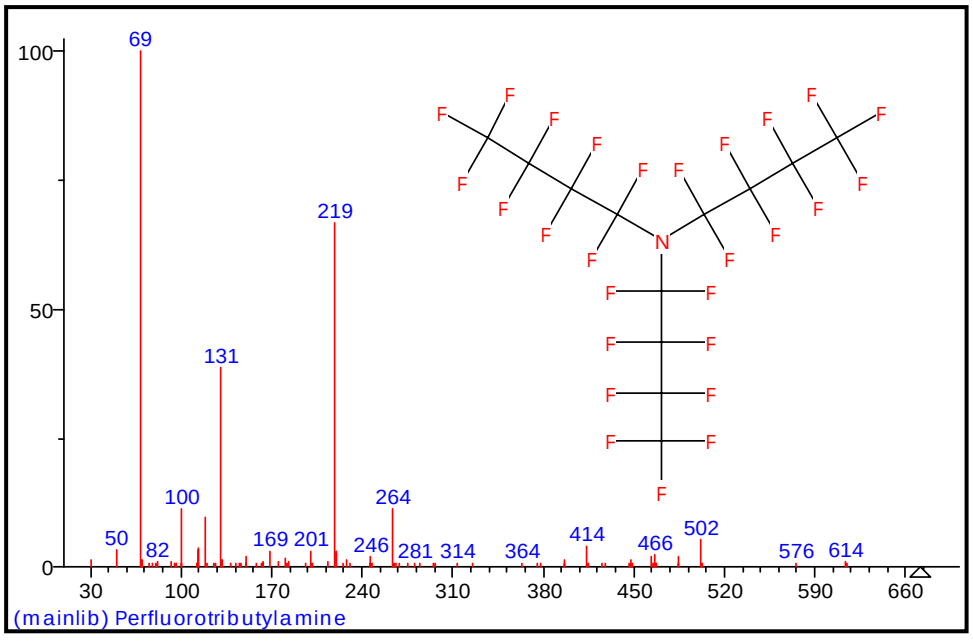

Figure 3.1 *PFTBA spectra and molecular structure*

Table 3.1 *PFTBA fragment ions and their relative abundances*

Perfluorotributylamine			
m/z	*Rel. Abundance*	*m/z*	*Rel. Abundance*
69	**100**	314	1.8
72	1.1	**414**	**3.4**
93	1.3	464	4.5
100	17.7	**502**	**2.6**
114	7.9		
119	13.0		
131	**47.9**		
132	1.7		
145	1.0		
150	2.8		
164	1.4		
169	4.0		
176	1.5		
181	1.8		
214	1.2		
219	**50.7**		
220	2.3		
226	1.0		
264	13.4		

This calibration procedure ensures that all samples are analysed with respect to a known mass spectrometer reference point.[102]

Firstly tune parameters were therefore set. (Figures 3.2 and 3.3)

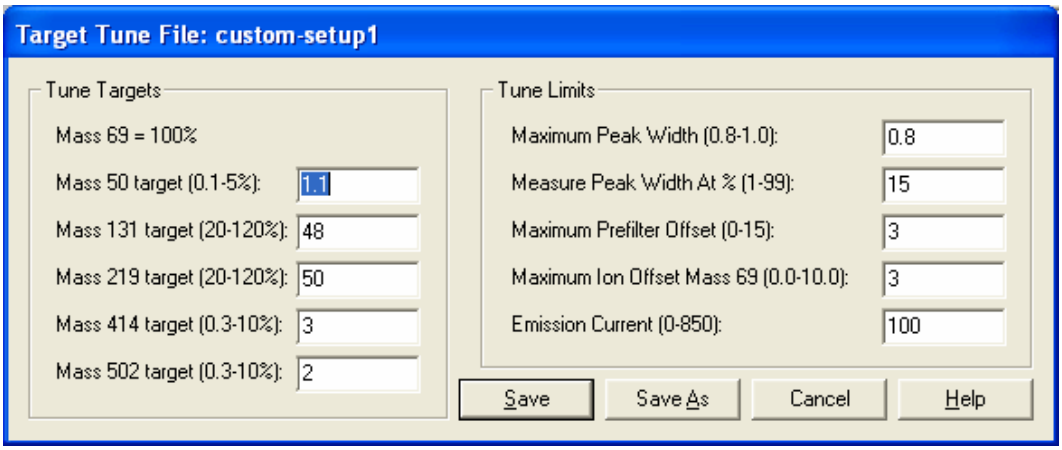

Figure 3.2 *Instrument tune parameters*

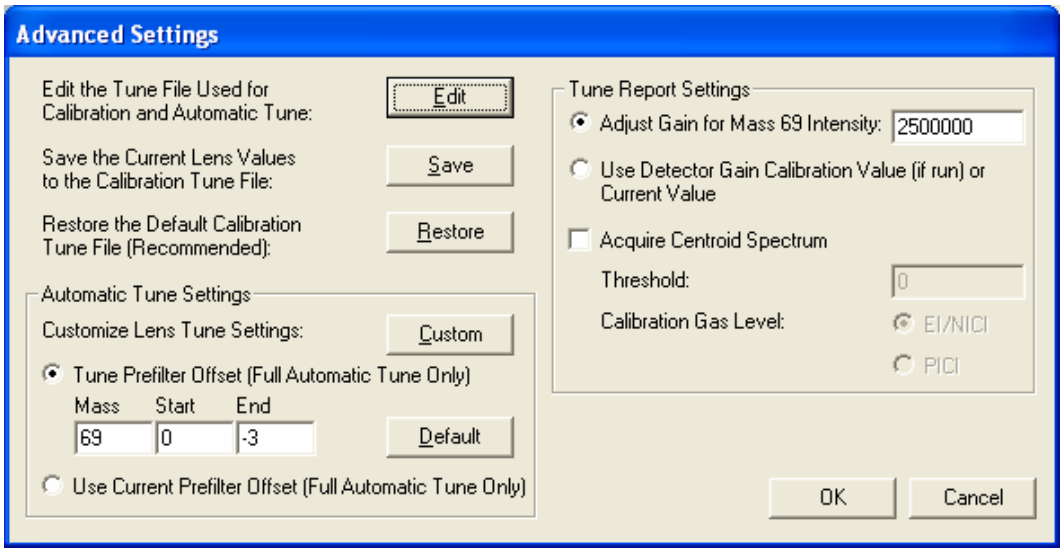

Figure 3.3 *Instrument tune advanced setting*

In order to evaluate instrument tuning maintenance and reproducibility over time, repeated direct injections of 1 µl of a standard liquid solution Stock A were performed in the GC/MS during tuning variation. The liquid mixture was composed of eighteen compounds and two labelled internal standards compounds, Toluene-D8 and Isopropanol-D8. The concentrations of the compounds are reported in Table 2.1

After the instrument was tuned, six injections of Stock A per day were performed for two days. The instrument was then tuned again and another six daily injections of Stock A were performed for three days. The chromatographic and mass spectrometric methods are described in Table 2.9.

The graphs below show peak area trends for Toluene-D8 and Isopropanol-D8 (Figures 3.4 and 3.5) and their peak area ratio trends (Figure 3.6), during tuning variation.

The measurements taken with the first tuning are highlighted with circles. The shape is relative to the tuning (circles: first tuning; squares: second tuning) and the fill is relative to the injections performed in different days.

It is possible to notice that the second tuning (squares) gives higher response respect to the first one (circles).

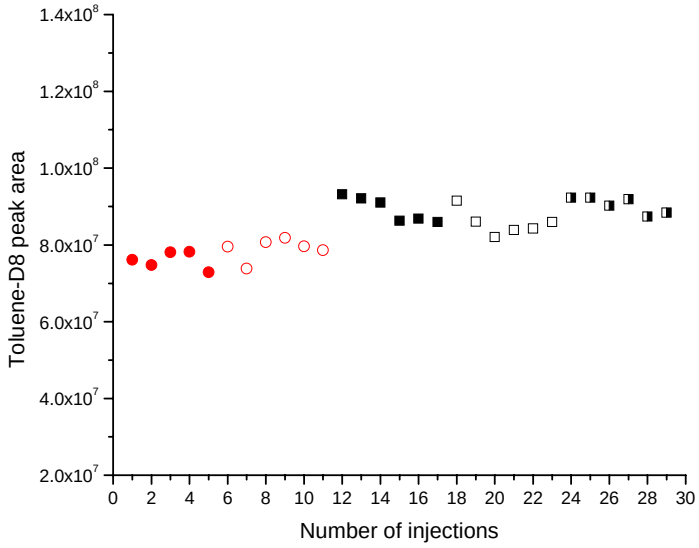

Figure 3.4 *Toluene-D8 peak area trends, during instrument tuning variation*

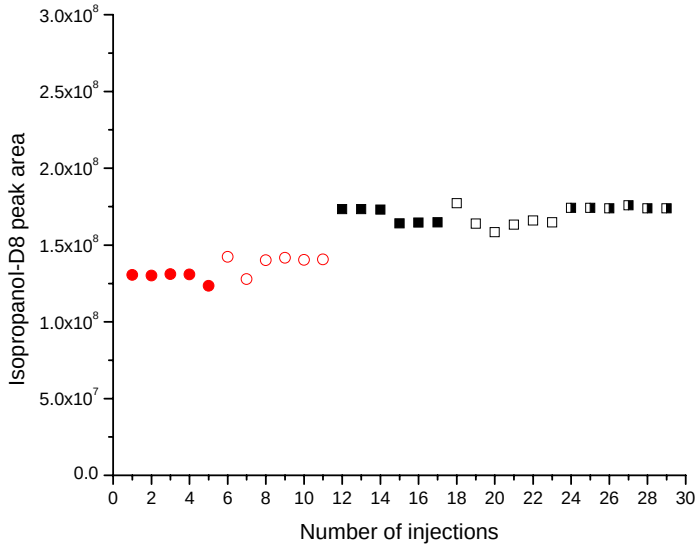

Figure 3.5 *Isopropanol-D8 peak area trends, during instrument tuning variation*

In Figure 3.6 is reported the Isopropanol-D8 and Toluene-D8 peak area ratio trends, which confirm that the instrument response is stable in time.

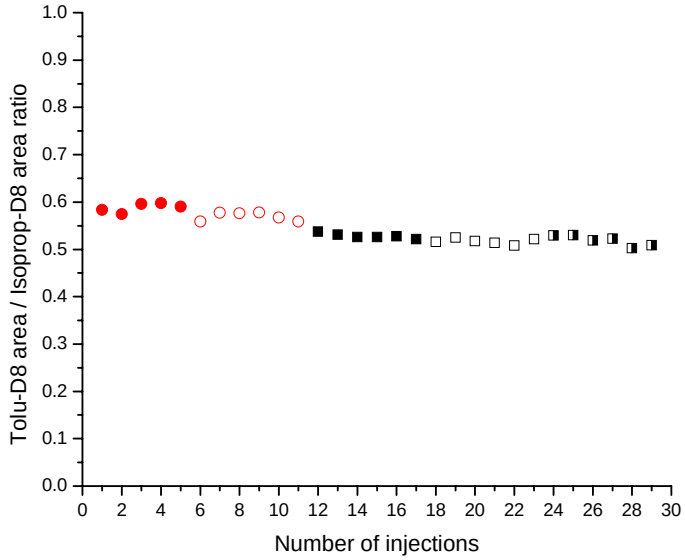

Figure 3.6 *Isopropanol-D8 and Toluene-D8 peak area ratio trends, during instrument tuning variation*

Average and relative standard deviation percentage (RSD%) of the two internal labelled standard injections are shown in Table 3.2.

Table 3.2 *Average and RSD% of Toluene-D8 and Isopropanol-D8, during tuning variation*

First Tuning			
	Isopropanol-D8	**Toluene-D8**	**Toluene-D8 / Isopropanol-D8 Ratio**
Tuning Average	1.34E+08	7.76E+07	0.58
Tuning RSD%	5%	4%	2%
1° Day Average	1.29E+08	7.60E+07	0.59
1° Day RSD%	3%	3%	2%
2° Day Average	1.39E+08	7.90E+07	0.57
2° Day RSD%	4%	4%	2%
Second Tuning			
	Isopropanol-D8	**Toluene-D8**	**Toluene-D8 / Isopropanol-D8 Ratio**
Tuning Average	1.70E+08	8.84E+07	0.52
Tuning RSD%	3%	4%	2%
1° Day Average	1.69E+08	8.92E+07	0.53
1° Day RSD%	3%	4%	1%
2° Day Average	1.66E+08	8.56E+07	0.52
2° Day RSD%	4%	4%	1%
3° Day Average	1.74E+08	9.04E+07	0.52
3° Day RSD%	0.4%	2%	2%
Total Average	1.56E+08	8.43E+07	0.54
Total RSD%	12%	7%	6%

Figures 3.7, 3.8, 3.9, 3.10 and 3.11 show peak area trends for some of Stock A compounds, during tuning variation. For each compound the first figure represents peak area trends, the second represents peak area trends normalized to Toluene-D8 (a) and the third the peak areas trends normalized to Isopropanol-D8 (b).

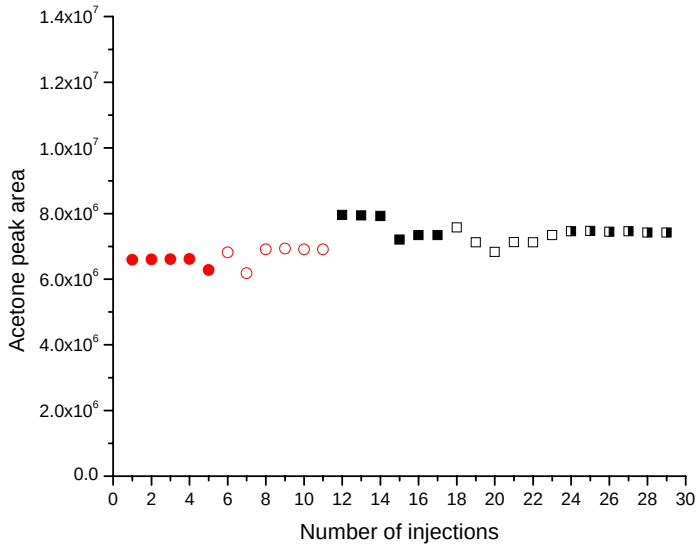

Figure 3.7 *Acetone peak area trends, during instrument tuning variation*

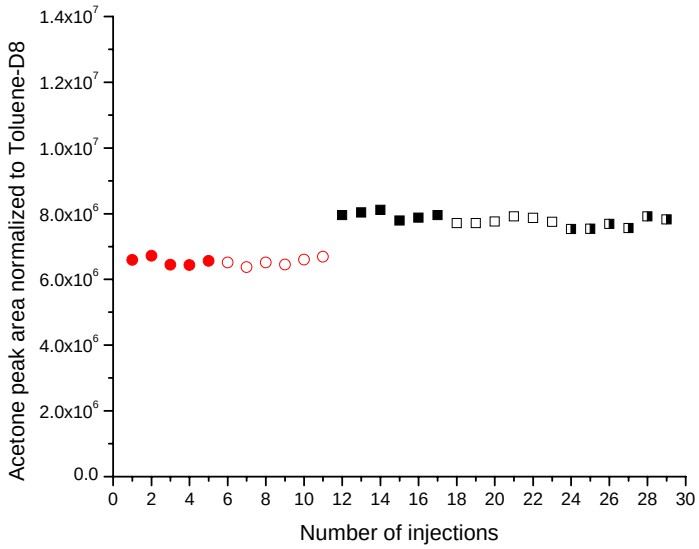

Figure 3.7a *Acetone peak area trends normalized to Toluene-D8, during tuning variation*

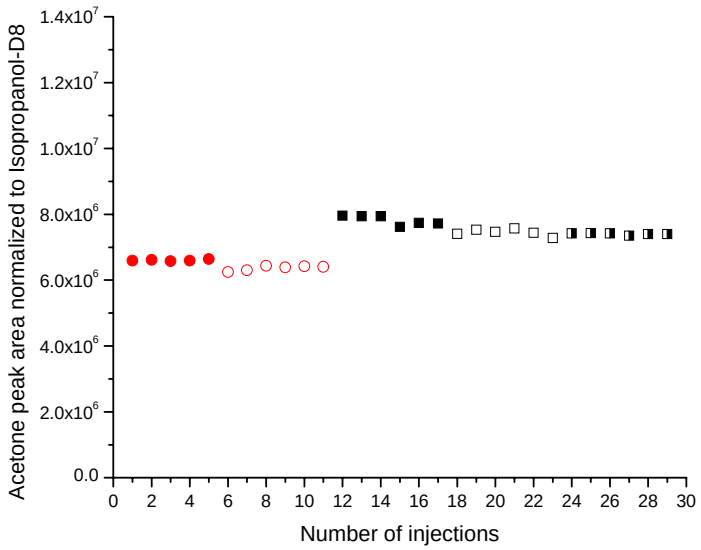

Figure 3.7b *Acetone peak area trends normalized to Isopropanol-D8, during tuning variation*

Average and relative standard deviation percentage (RSD%) of Acetone injections are shown in Table 3.3.

Table 3.3 *Average and RSD% of Acetone, during tuning variation*

ACETONE			
First Tuning			
	Peak Area	**Peak Area Normalized toToluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	6.67E+06	6.54E+06	6.48E+06
Tuning RSD%	4%	2%	2%
1° Day Average	6.54E+06	6.55E+06	6.61E+06
1° Day RSD%	2%	2%	0%
2° Day Average	6.78E+06	6.53E+06	6.37E+06
2° Day RSD%	4%	2%	1%
Second Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	7.42E+06	7.81E+06	7.56E+06
Tuning RSD%	4%	2%	3%
1° Day Average	7.62E+06	7.96E+06	7.82E+06
1° Day RSD%	5%	1%	2%
2° Day Average	7.19E+06	7.79E+06	7.45E+06
2° Day RSD%	3%	1%	1%
3° Day Average	7.45E+06	7.68E+06	7.40E+06
3° Day RSD%	0.3%	2.1%	0.4%
Total Average	7.13E+06	7.33E+06	7.15E+06
Total RDS%	7%	9%	8%

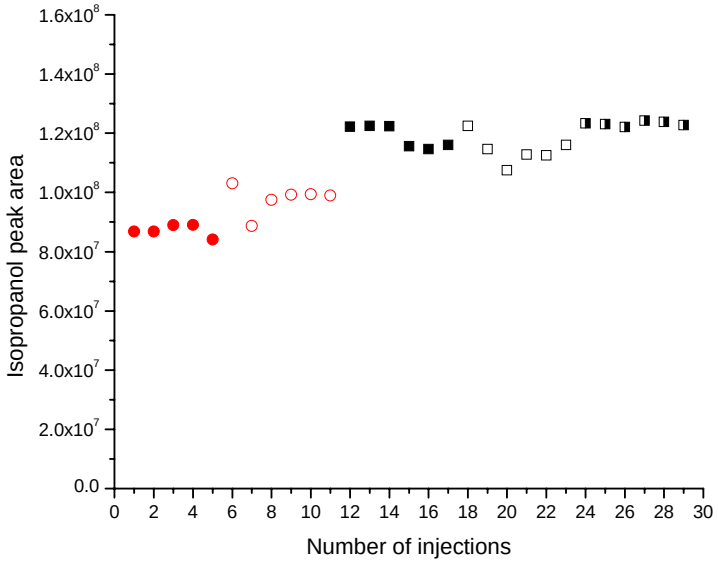

Figure 3.8 *Isopropanol peak area trends, during instrument tuning variation*

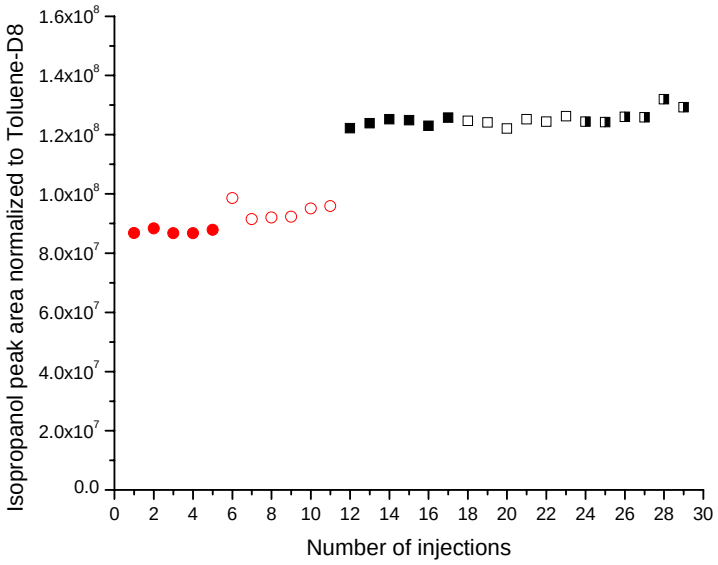

Figure 3.8a *Isopropanol peak area trends normalized to Toluene-D8, during instrument tuning variation*

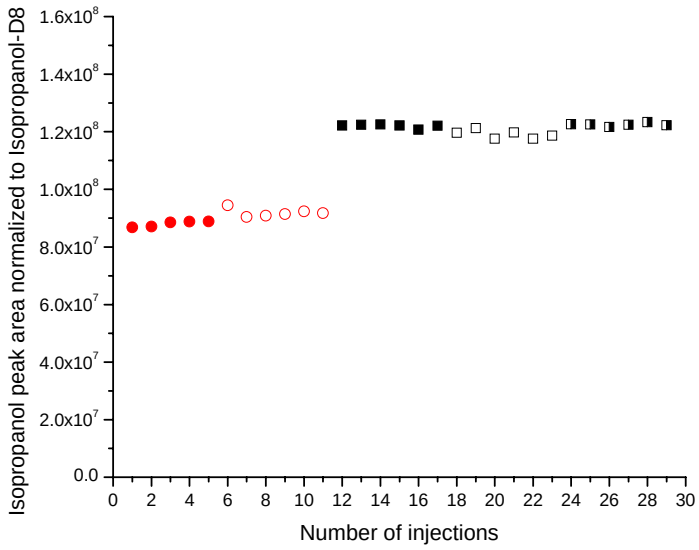

Figure 3.8b *Isopropanol peak area trends normalized to Isopropanol-D8, during instrument tuning variation*

Average and relative standard deviation percentage (RSD%) of Isopropanol injections are shown in Table 3.4 and 3.4a.

Table 3.4 *Average and RSD% of Isopropanol, during tuning variation*

ISOPROPANOL			
First Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	9.30E+07	9.11E+07	9.01E+07
Tuning RSD%	7%	5%	3%
1° Day Average	8.71E+07	8.73E+07	8.80E+07
1° Day RSD%	2%	1%	1%
2° Day Average	9.78E+07	9.42E+07	9.19E+07
2° Day RSD%	5%	3%	2%
Second Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	1.19E+08	1.25E+08	1.21E+08
Tuning RSD%	4%	2%	1%
1° Day Average	1.19E+08	1.24E+08	1.22E+08
1° Day RSD%	3%	1%	1%
2° Day Average	1.14E+08	1.24E+08	1.19E+08
2° Day RSD%	4%	1%	1%
3° Day Average	1.23E+08	1.27E+08	1.22E+08
3° Day RSD%	0.6%	2.4%	0.4%
Total Average	1.09E+08	1.12E+08	1.09E+08
Total RDS%	13%	15%	14%

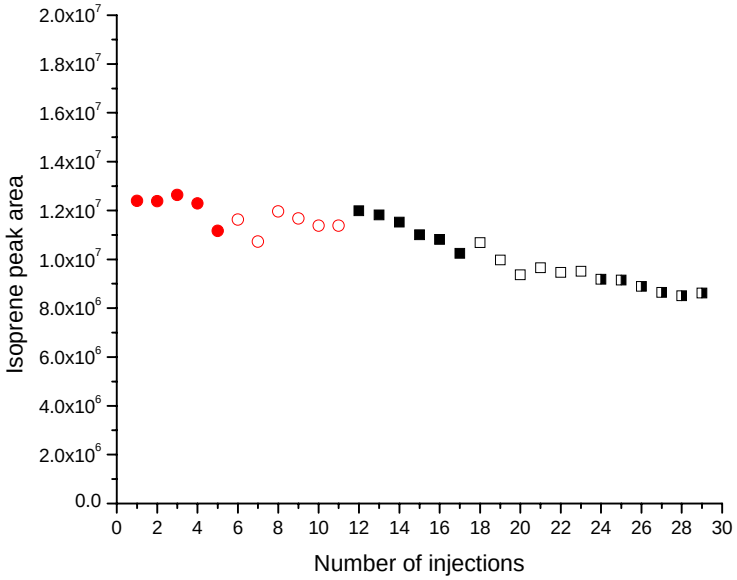

Figure 3.9 *Isoprene peak area trends, during instrument tuning variation*

Isoprene had a trend during tuning variation and the instrument response seemed to decrease in time. This is probably referable to the loss of isoprene from the methanol solution, due to its high volatility and poor affinity for the solvent.

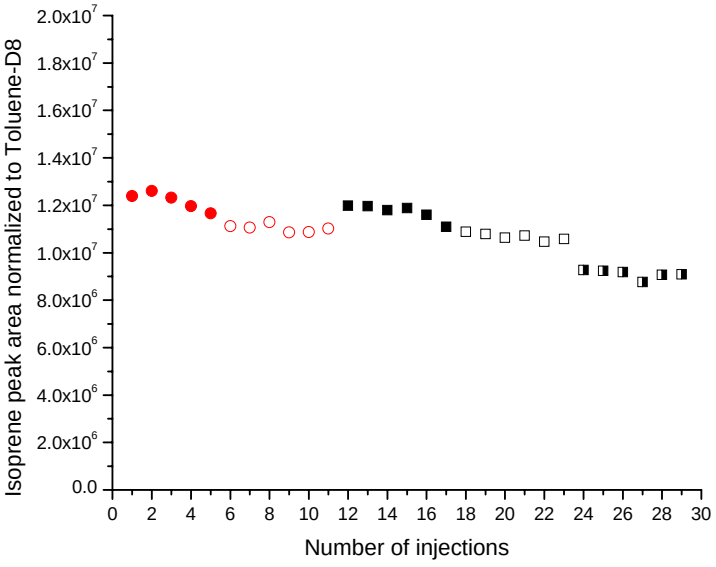

Figure 3.9a *Isoprene peak area trends normalized to Toluene-D8, during instrument tuning variation*

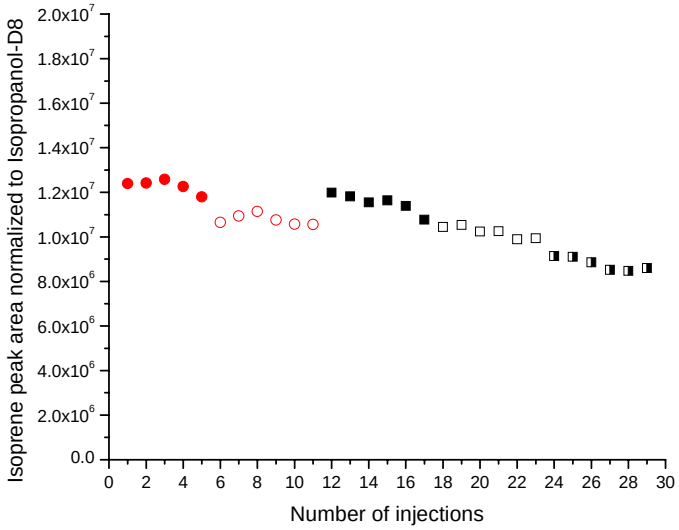

Figure 3.9b *Isoprene peak area trends normalized to Isopropanol-D8, during instrument tuning variation*

Average and relative standard deviation percentage (RSD%) of Isoprene injections are shown in Table 3.5.

Table 3.5 *Average and RSD% of Isoprene, during tuning variation*

ISOPRENE			
First Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	1.18E+07	1.16E+07	1.15E+07
Tuning RSD%	5%	6%	7%
1° Day Average	1.22E+07	1.22E+07	1.23E+07
1° Day RSD%	5%	3%	2%
2° Day Average	1.15E+07	1.10E+07	1.08E+07
2° Day RSD%	4%	1%	2%
Second Tuning			
	Peak Area	**Peak Area Normalized toToluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	9.95E+06	1.05E+07	1.02E+07
Tuning RSD%	11%	11%	12%
1° Day Average	1.12E+07	1.17E+07	1.15E+07
1° Day RSD%	6%	3%	4%
2° Day Average	9.77E+06	1.07E+07	1.02E+07
2° Day RSD%	5%	1%	3%
3° Day Average	8.83E+06	9.10E+06	8.78E+06
3° Day RSD%	3.3%	2.0%	3.4%
Total Average	1.06E+07	1.09E+07	1.07E+07
Total RDS%	12%	10%	12%

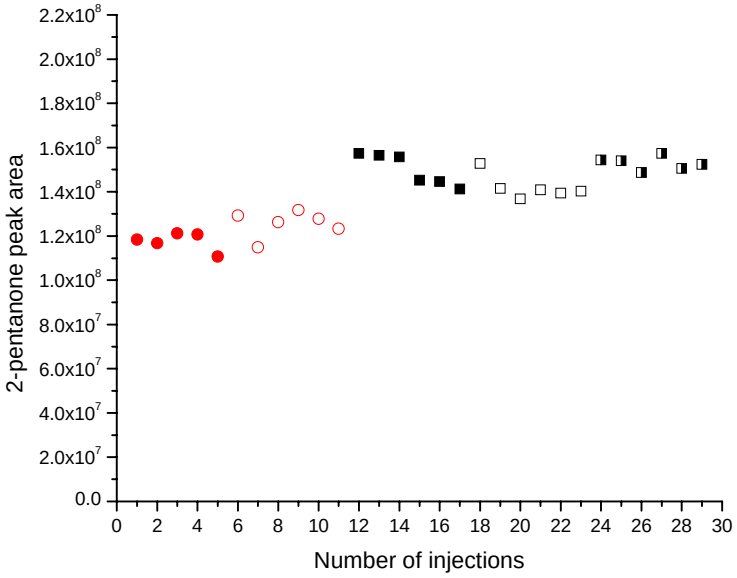

Figure 3.10 *2-pentanone peak area trends, during instrument tuning variation*

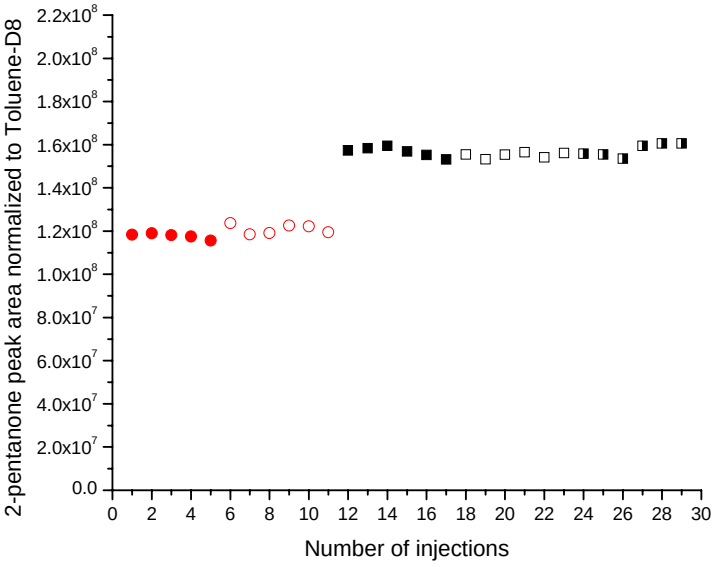

Figure 3.10a *2-pentanone peak area trends normalized to Toluene-D8, during instrument tuning variation*

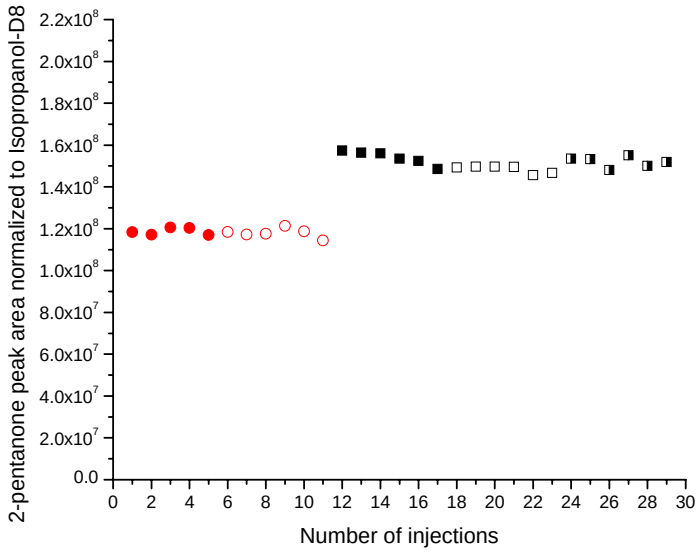

Figure 3.10b *2-pentanone peak area trends normalized to Isopropanol-D8, during instrument tuning variation*

Average and relative standard deviation percentage (RSD%) of 2-pentanone injections are shown in Table 3.6.

Table 3.6 *Average and RSD% of 2-pentanone, during tuning variation*

2-PENTANONE			
First Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	1.22E+08	1.19E+08	1.18E+08
Tuning RSD%	5%	2%	2%
1° Day Average	1.18E+08	1.18E+08	1.19E+08
1° Day RSD%	4%	1%	1%
2° Day Average	1.26E+08	1.21E+08	1.18E+08
2° Day RSD%	5%	2%	2%
Second Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	1.48E+08	1.56E+08	1.51E+08
Tuning RSD%	5%	2%	2%
1° Day Average	1.50E+08	1.57E+08	1.54E+08
1° Day RSD%	5%	1%	2%
2° Day Average	1.42E+08	1.55E+08	1.48E+08
2° Day RSD%	4%	1%	1%
3° Day Average	1.53E+08	1.58E+08	1.52E+08
3° Day RSD%	2.0%	1.9%	1.7%
Total Average	1.38E+08	1.42E+08	1.39E+08
Total RDS%	11%	13%	12%

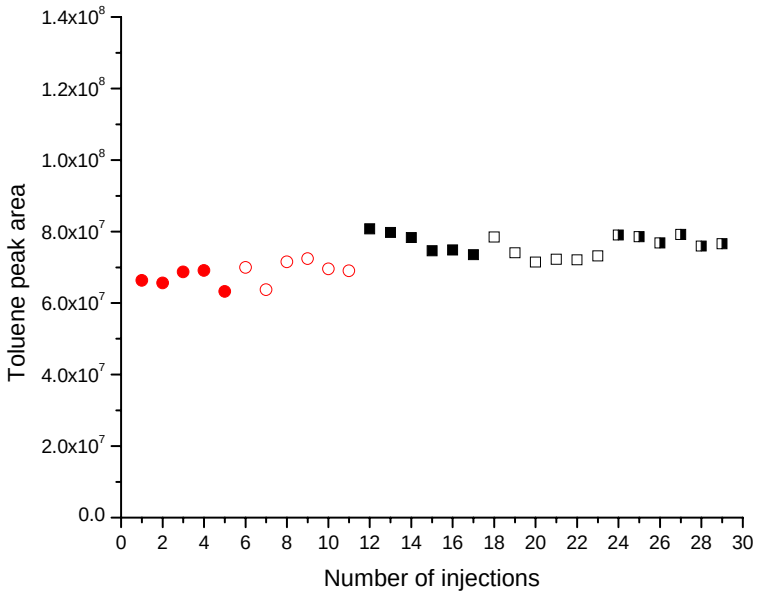

Figure 3.11 *Toluene peak area trends, during instrument tuning variation*

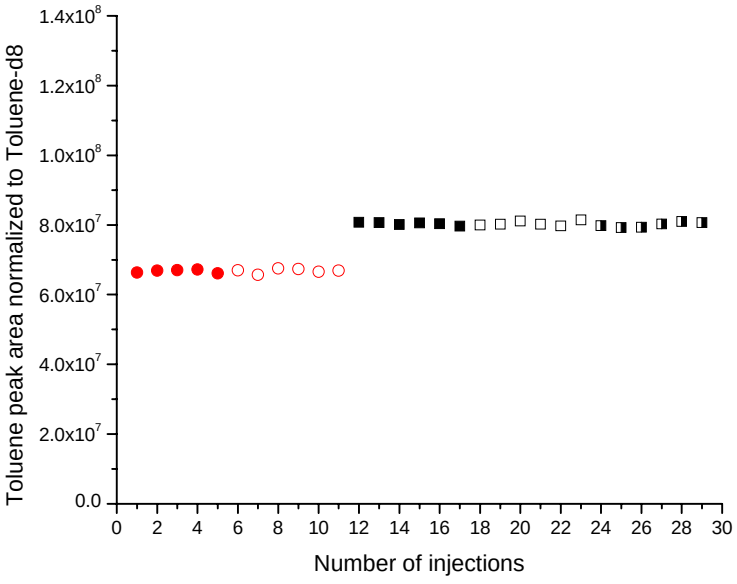

Figure 3.11a *Toluene peak area trends normalized to Toluene-D8, during instrument tuning variation*

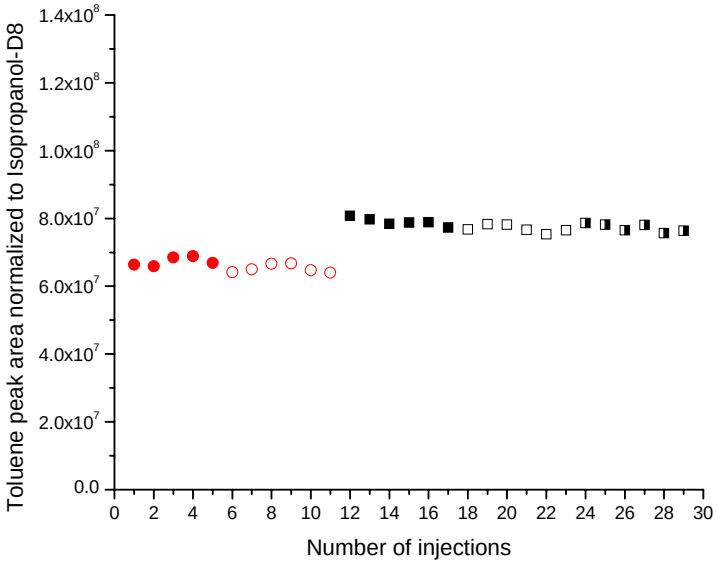

Figure 3.11b *Toluene peak area trends normalized to Isopropanol-D8, during instrument tuning variation*

Average and relative standard deviation percentage (RSD%) of Toluene injections are shown in Table 3.7.

Table 3.7 *Average and RSD% of Toluene, during tuning variation*

TOLUENE			
First Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	6.81E+07	6.68E+07	6.61E+07
Tuning RSD%	4%	1%	2%
1° Day Average	6.66E+07	6.67E+07	6.73E+07
1° Day RSD%	4%	1%	2%
2° Day Average	6.94E+07	6.68E+07	6.52E+07
2° Day RSD%	4%	1%	2%
Second Tuning			
	Peak Area	**Peak Area Normalized to Toluene-D8**	**Peak Area Normalized to Isopropanol-D8**
Tuning Average	7.61E+07	8.03E+07	7.77E+07
Tuning RSD%	4%	1%	2%
1° Day Average	7.70E+07	8.04E+07	7.90E+07
1° Day RSD%	4%	1%	1%
2° Day Average	7.36E+07	8.04E+07	7.70E+07
2° Day RSD%	4%	1%	1%
3° Day Average	7.77E+07	8.01E+07	7.72E+07
3° Day RSD%	1.8%	0.9%	1.6%
Total Average	7.31E+07	7.52E+07	7.33E+07
Total RDS%	7%	9%	8%

During the same tuning a good reproducibility of the injections was obtained, with a RSD% < 5%, but every time the instrumental tuning was changed, it was necessary to make an instrument calibration with standard solutions. Moreover daily average injections gave good results. For some compounds a RSD% of around 5% was obtained and if the data were corrected for the internal standard, the RSD% decreased to 2%.
In any case it was necessary to verify instrument responses every day with a standard solution, even during the same tuning.

The normalization of data was differentiated on the basis of m/z fragments chosen for quantification. Compounds quantified on m/z in the range of 30 to 60 were normalized with respect to Isopropanol-D8 (m/z = 49), compounds quantified on m/z in the range of 61 to 120 were normalized with respect to Toluene-D8 (m/z = 98). For example acetone was quantified on m/z = 58 and for this reason it was normalized with respect to Isopropanol-D8. Sometimes the normalization made on the basis of the quantified ion mass was not confirmed from data obtained in the previous measurements. In fact, Toluene quantified on m/z = 91 should be normalized with respect to Toluene-D8, but the data obtained during the second tuning (Table 3.7) have shown that the variability is lower, normalizing with respect to Isopropanol-D8 then Toluene-D8. So in these cases we had to find the better compromise.

3.2 Selection of the adsorbent phase and evaluation of adsorption tube performances

There are varieties of adsorbents used in the field of thermal desorption. Often choosing the right adsorbent can be difficult. The aim is to choose one that can retain a specific or group of analytes for a specified sample volume. However, just as importantly, the adsorbent must also be able to release the analytes during the desorption process.
Most commonly used adsorbent phases are Tenax TA®, Tenax GR™, Carbopack™, Carbotraps™, Carboxens™, Carbosieve™.

There is no one adsorbent available that can both retain and release all the analytes.[103] The selection of an adsorbent resin for an application is based on a large number of variables.

- Breakthrough Volume Data for Analytes of Interest
- Bed Volume of Adsorption Tube
- Affinity of Resin for Water
- Sample Collection Flow Rate
- Backpressure in the Adsorption Tube
- Desorption Temperature Required to Purge off Analytes
- Gas Volume Required to Purge off Analytes
- Need for an Initial Purge to Remove Water or other Solvents

Adsorbent resins such as Tenax® TA, Tenax® GR and a wide variety of activated charcoals are widely used to trap and analyse volatile and semi-volatile organics from gas, liquid or solid matrix samples. Adsorbent resins are specified in several EPA methods for air and water testing.[104]

Breakthrough Volume charts are helpful for determining the usefulness of a particular resin for a particular analyte or a range of analytes in a group of organics such as hydrocarbons or alcohols. However, when comparing different adsorbent resins, the amount of data can be somewhat overwhelming. Bar chart histograms comparing the different adsorbent resins for a range of analytes of a class of organics were therefore developed by Manura J. (SIS Inc.).[105] A lot of information is condensed into these charts. (Figure 3.12 and 3.13)

The numbers at the top of the chart indicate the number of carbons in the analyte carbon chain. The numbers at the bottom of the chart indicate the boiling point of the analyte. The chart is divided into 5 vertical bar columns indicating the broad classification of analytes as gases, volatile, low boiler, medium boiler or semi-volatile.

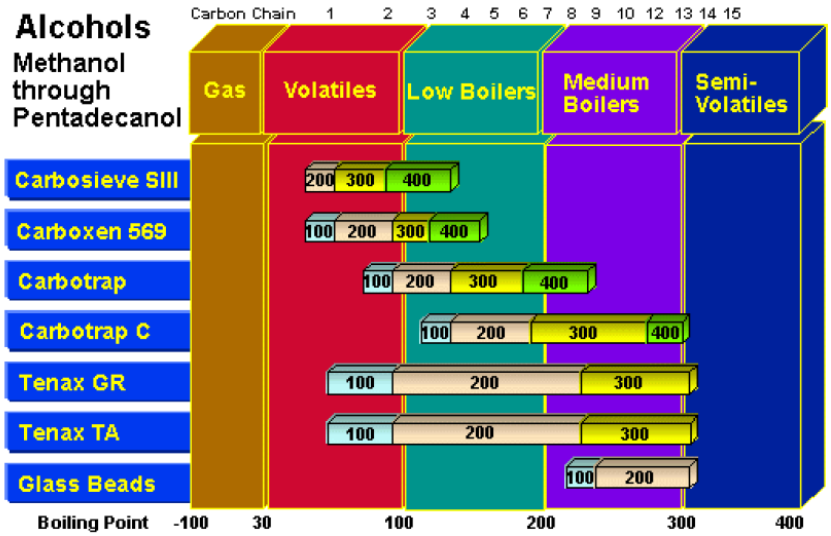

Figure 3.12 *Bar chart histogram comparing the different adsorbent resins for alcohols*[105]

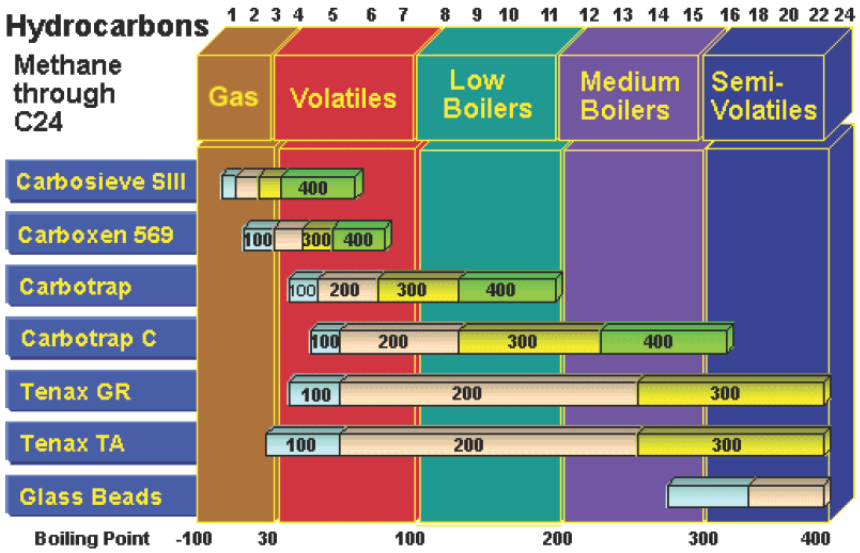

Figure 3.13 *Bar chart histogram comparing the different adsorbent resins for hydrocarbons*[105]

The four horizontal bars of the chart indicate the range of analytes that can be effectively analyzed with each of the seven adsorbent resins. In addition, each of the horizontal bars is divided into smaller sections, which indicate the temperature required to desorb the analytes off the adsorbent resin.

These charts can be easily used to compare adsorbent resins. For example, the charts show that Carbosieve SIII can be used to analyze hydrocarbons between ethane (C_2)

and hexane (C_6). Hydrocarbons greater than hexane can not be desorbed off this resin below 400 °C.

Tenax TA can be used to analyze hydrocarbons between pentane (C_5) and tetracosane (C_{24}). Hydrocarbons smaller than pentane would not be effectively trapped on Tenax TA at room temperature and compounds larger than C_{24} would not be effectively desorbed off this resin at

300 °C.

A mixed bed resin of Carbosieve SIII and Tenax TA could be used to analyze hydrocarbons from ethane (C_2) through tetracosane (C_{24}).

From these charts, the analyst can determine the optimum resin for a particular application or to develop a mixed bed resin to expand the range of analytes that can be trapped and analyzed via the purge and trap thermal desorption methods.[105]

On the basis of this information two different adsorption tubes were tested. One was packed with Tenax GR and the other was a multi-bed desorption tube, packed with Carbopack Y, Carbopack B, and Carboxen 1003.

In the literature many examples of the use of Tenax GR for sampling ambient air and human breath are reported.[106] This porous adsorbent phase can retain large amounts of compounds with different chemical properties. In fact it retains alcohols, glycols, aldheydes, ketones, hydrocarbons, aromatic compounds and halogens.

Tenax™ GR is a composite material of Tenax TA and 30% graphite. The resulting material gives a higher breakthrough volume for most volatile organics, yet still has a low affinity for water. In addition Tenax™ GR maintains its high temperature stability up to 350 °C. (Table 2.13)

These properties make Tenax™ GR an ideal adsorbent for the trapping of volatiles from air, water and solid samples. Due to its low affinity for water, Tenax™ GR is especially useful for the purging of volatiles from high moisture content samples including the analysis of volatile organic compounds in water. Since its density is twice that of Tenax™ TA, this enables a larger amount of the Tenax™ GR resin to be placed inside the desorption tube, thereby increasing the ability to retain volatiles on a small resin bed.[101]

A number of adsorbent materials have proved useful for trapping organics and for desorption particularly using thermal techniques. However, none of these materials

individually can fully accommodate a complete range of organic volatilities or polarities, and thus they must be used in combination with each other. It is apparent from published results, that no single sorbent material is practical for collecting all solutes. In some instances this is because the sorbents for example, do not retain low-molecular mass materials; in other cases it is because the analytes are too strongly retained to be easily desorbed off the heavier sorbents (activated charcoal for example).
Given the lack of a universal sorbent, the best approach was to construct tubes containing several different materials to attain the desired collection and desorption characteristics, the so co-called multi-bed sorbent, where the principle of sequential trapping takes place. The adsorption layers are arranged in such a way that compounds with the lowest molecular mass go through the initial layer(s), protect the next layer(s) and are trapped on the last layer[95].
The multi-bed adsorption tubes were used in the NIOSH Method 2549 for volatile organic compounds screening in the air.[107]
These phases have a limited adsorption range, but they can retain small volatile compounds, such as C_2-C_3 hydrocarbons and alcohols, and differently from Tenax™ GR, they have a rather affinity for water and maintain their high temperature stability up to 400 °C. (Table 2.14)

In order to evaluate the performance of the adsorption tubes, systematic measurements were taken with a standard gaseous mixture.

3.2.1 Comparison between several drying agents

Breath samples contain large amounts of water (RH > 90%) that can affect the trapping of compounds in the tubes, so it was necessary to test the adsorption efficiency of different desiccants. Anhydrous sodium sulphate (Na_2SO_4), magnesium perchlorate ($Mg(ClO_4)_2$) and drierite, (97% anhydrous $CaSO_4$ and 3% $CoCl_2$) were tested.
In order to evaluate desiccant adsorption efficiency, 20 g of each desiccant were introduced into Nalophan bags, and the bags were then filled with breath. The desiccants had been in contact with the sample for a couple of hours. Five hundred ml of the breath sample were transferred into the multi-bed tube, because Tenax GR is

unaffected by water. The chromatographic, mass spectrometric and thermal desorption methods are described in Table 2.10.

Figure 3.14 shows water chromatographic peaks in breath samples obtained from the bags with Na_2SO_4, $Mg(ClO_4)_2$ and Drierite.

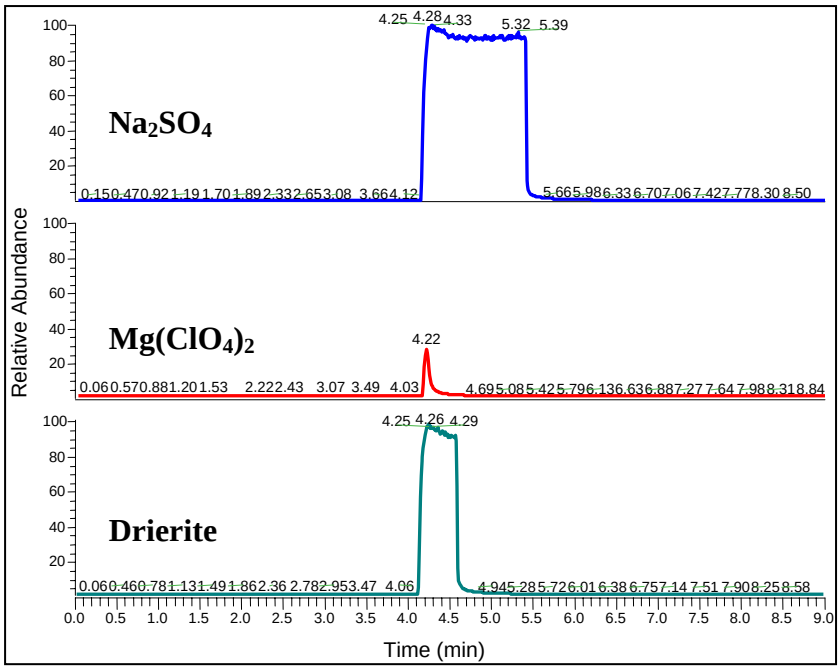

Figure 3.14 *Water chromatographic peaks (m/z=18) in breath samples obtained from the bags with Na_2SO_4 (blue; first), $Mg(ClO_4)_2$ (second) and drierite (third).*

The chromatograms underline excellent water adsorption efficiency for magnesium perchlorate compared to the other two salts, but show significant losses, about 50% of the oxygenate compounds, particularly acetone. (Figure 3.15)

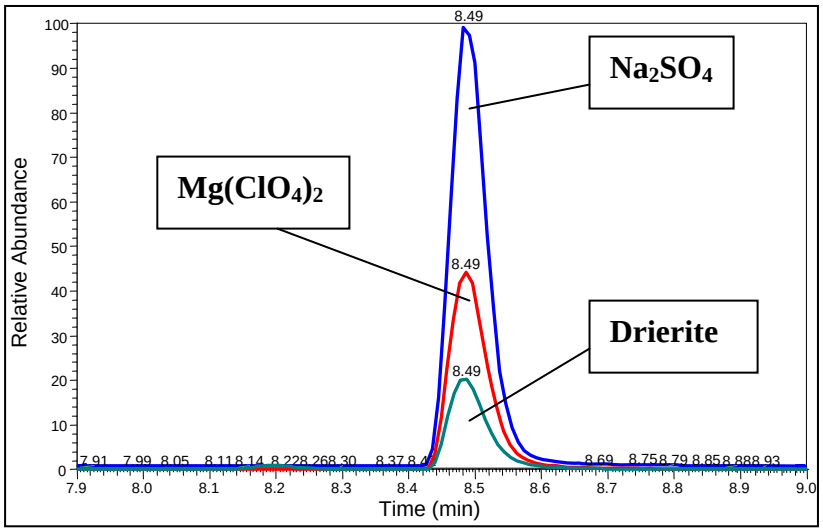

Figure 3.15 *Acetone chromatographic peaks (m/z=58) in breath samples obtained from the bags with Na_2SO_4, $Mg(ClO_4)_2$ and drierite.*

To conclude, sodium sulphate seemed to be the most appropriate desiccant for our purposes, even if there was a minor loss of water.

3.2.2 Determination of breakthrough volumes

In order to simplify the experimental procedure, a solution containing a small number of compounds was used for the breakthrough volume measurements, which was representative of the class of compounds present in the breath samples.

In the solution there were alcohols (isopropanol), ketones (acetone and methylethylketone), alkanes (hexane), aromatics (toluene) and sulphur compounds (dimethyldisulphide).

Five ml of Stock G were introduced into a Nalophan bag filled with 15 L of pure air. The concentration of compounds in the bag is given in Table 2.8.

Tenax GR and multi-bed adsorption tube breakthrough volumes were evaluated transferring increasing volumes (0.25 L, 0.5 L and 1 L) of the gaseous mixture from the bags into the tubes (flow 100 ml/min). The sampling bag is heated at 40 °C to prevent water condensation. The sample is drawn from the bag, through a desiccant cartridge, into the adsorption tube by a pocket pump. The flow is set up on the pocket pump and controlled by a flow meter. (Figure 3.16)

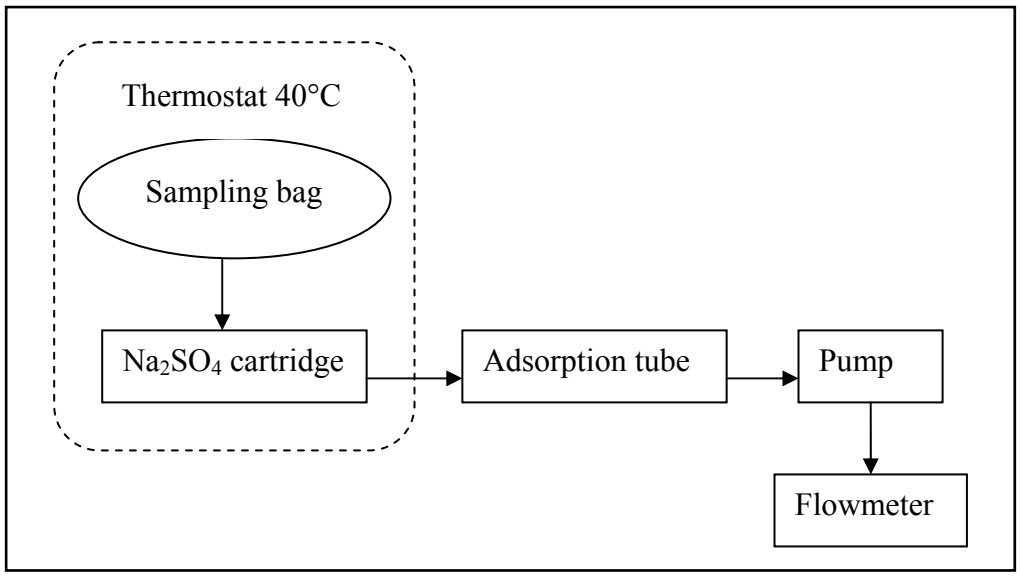

Figure 3.16 *Schematic diagram of sample transfer in the adsorption tube*

The operative conditions are reported in Table 2.10.

Tenax GR and multi-bed breakthrough volumes for the compounds in the mixture are reported in the figures below.

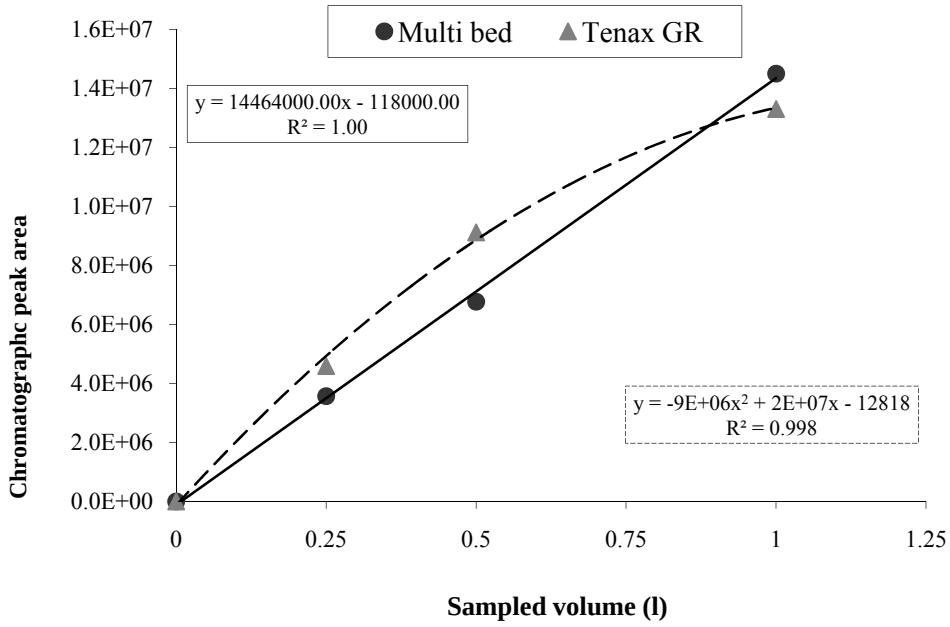

Figure 3.17 *Acetone breakthrough volume*

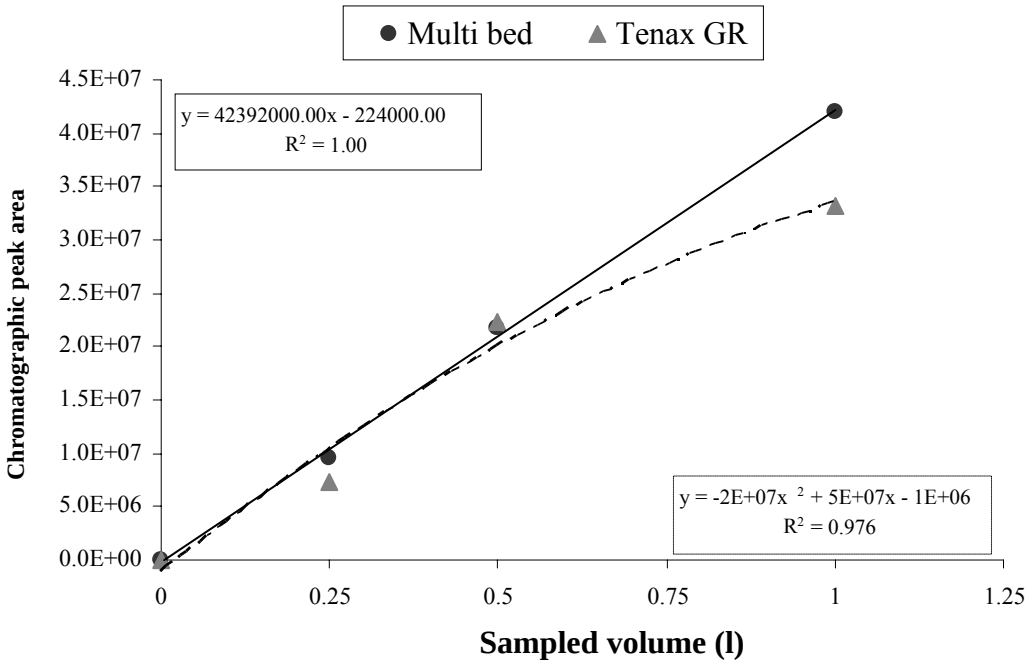

Figure 3.18 *Isopropanol breakthrough volume*

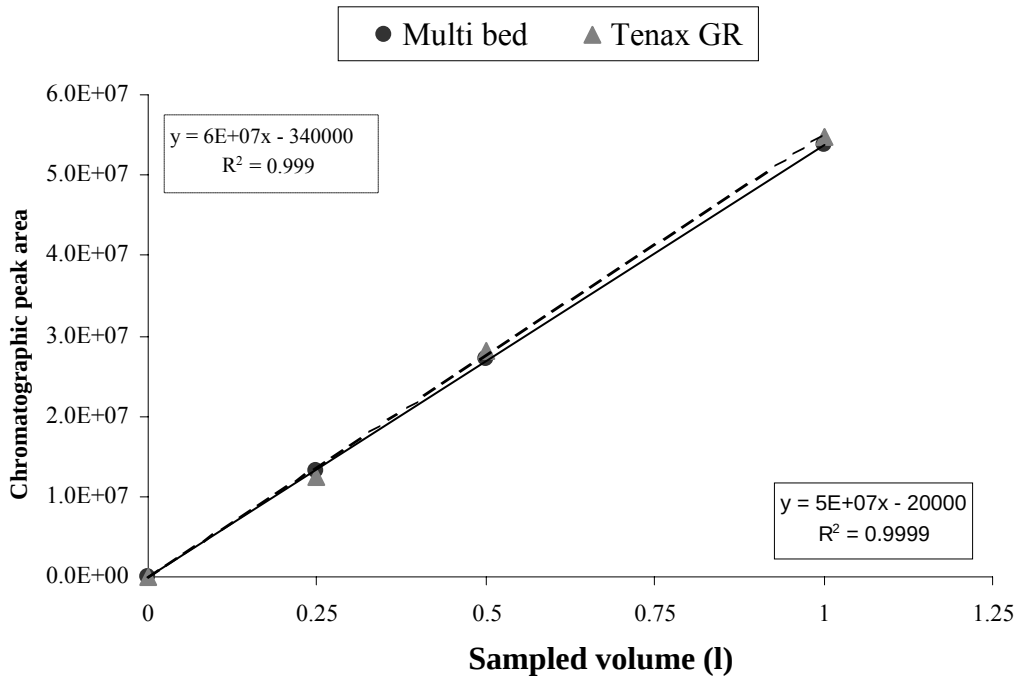

Figure 3.19 *Methylethylketone breakthrough volume*

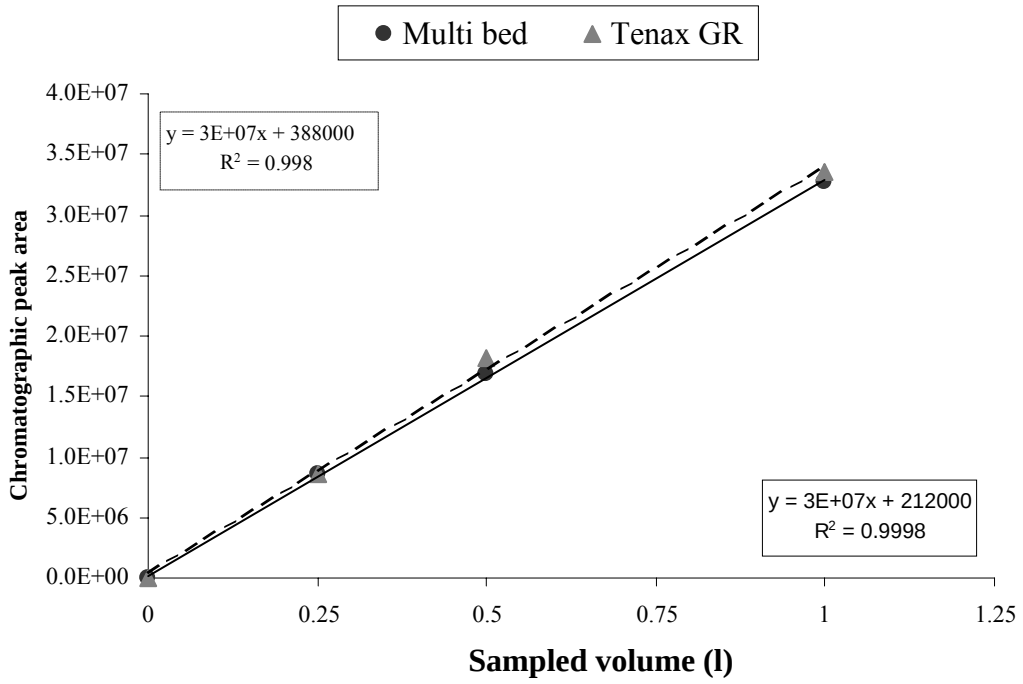

Figure 3.20 *Hexane breakthrough volume*

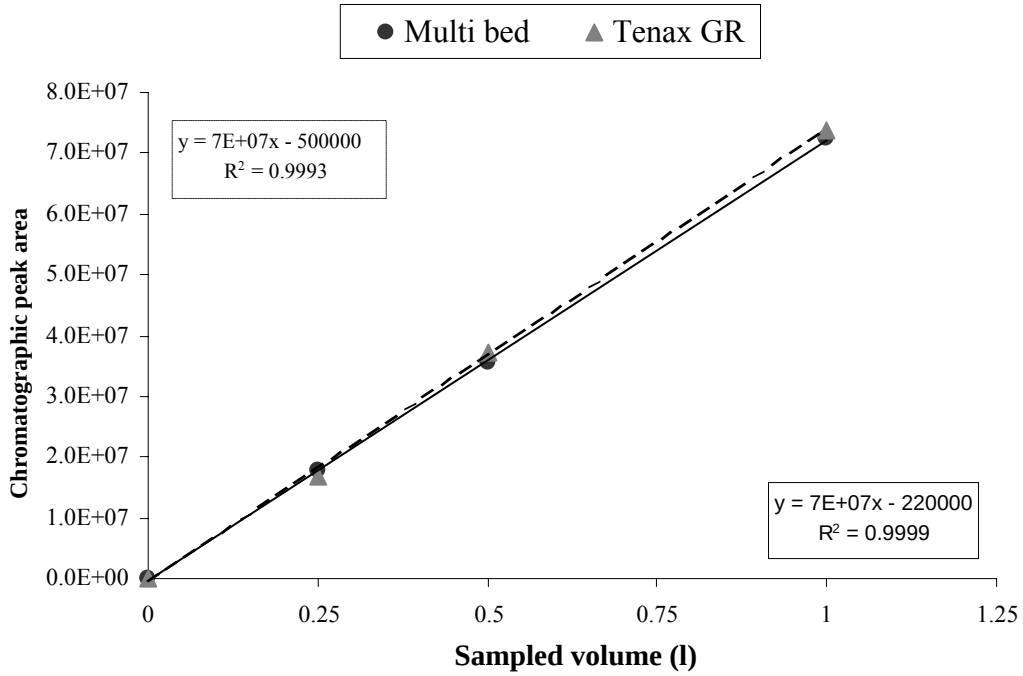

Figure 3.21 *Dimethyldisulphide breakthrough volume*

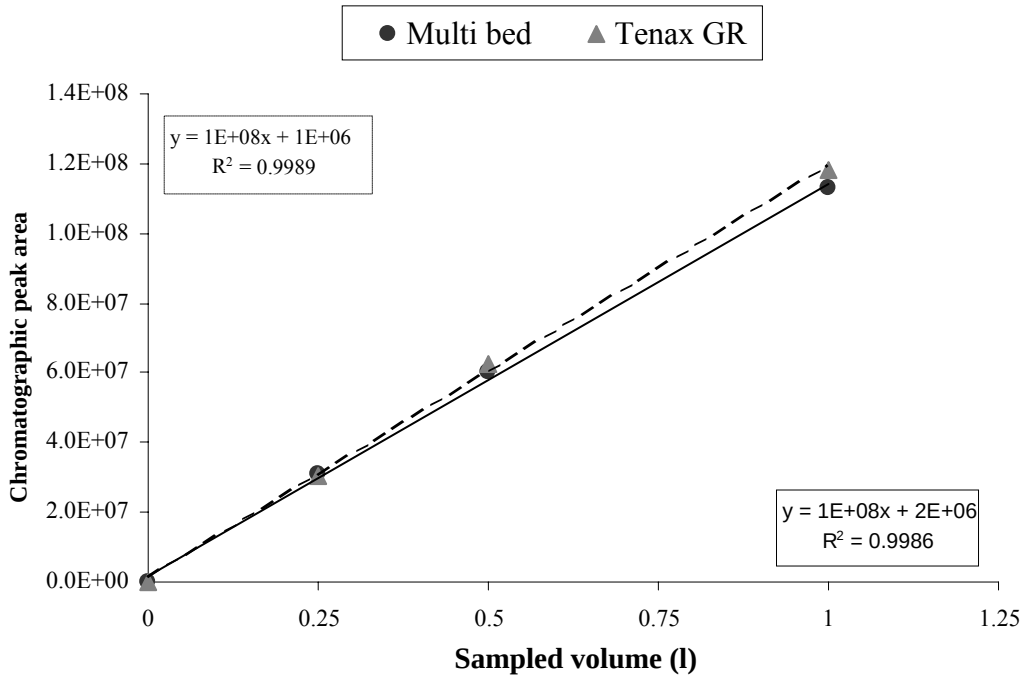

Figure 3.22 *Toluene breakthrough volume*

The data showed that all the compounds had a good linearity (showed by the R^2 values) in the case of the multi-bed adsorption tubes, whereas in the Tenax GR tubes deviations from linearity for volumes over 500 ml were noticed for acetone and isopropanol.

Multi-bed adsorption tubes seemed to be better in recovery but presented critical issues in terms of water.

Carbon molecular sieves are strong hydrophobic sorbents with micro porous surfaces, used as the last and most active layer in multilayer traps, to intercept very volatile and polar compounds. Initially, the carbon sieves had the reputation of not sorbing water at all, since their hydrophobic surface consisted of carbon atoms. However, both laboratory practice and specific studies have since showed that their water sorption capacity is surprisingly large and approximates to the micro pores volume.[108]

Once the relative humidity surpasses a threshold value, a particular mechanism becomes operative, micro pores volume filling. This mechanism was studied extensively by Stoeckli for the active carbon–water system.[109] It involves water vapour condensation inside the micro pores rather than the usual physical adsorption on the surface. The faster the water saturation, the less free micro pores are available for organics, and the faster the decrease in the adsorption capacity for such compounds.[110]

For these reasons Tenax GR tubes were chosen to take measurements.

3.2.3 Blank evaluation of adsorption tubes

In order to verify Tenax GR adsorption tubes and trap blanks, systematic measurements were taken on conditioned tubes.

The aim was to control the conditioning procedure and the storage of the adsorption tube after conditioning. Thus the chromatograms below were compared:

1. A tube analyzed immediately after conditioning
2. A tube analyzed after 6 hours, maintained in the thermal desorption with a black cap
3. A tube analyzed after 9 hours, maintained in the thermal desorption with a black cap
4. A tube analyzed after 6 days, maintained in a Nalophan bag with a black cap
5. A tube analyzed after 6 days, maintained in a Nalophan bag with a screw cap
6. A tube analyzed after 6 days, maintained in the thermal desorption with a black cap
7. A tube loaded with a breath sample

Figure 3.23 *Black cap*

Figure 3.24 *Screw cap*

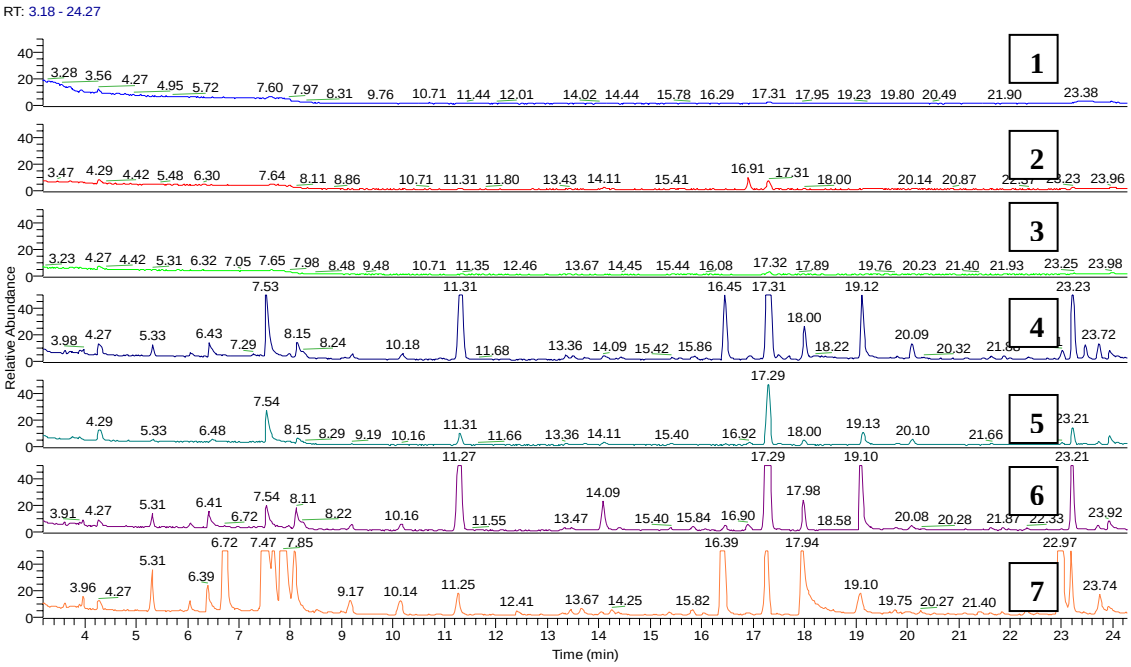

Figure 3.25 *Comparison of the seven chromatograms; fixed scale at 1.00 * 10^6; Mass range selected 35-200.*

In the following Table are reported a list of the compounds present in the chromatograms.

Table 3.8 *Compounds present in chromatograms*

COMPOUNDS	RT	Chromatogram
acetaldeyde	4.28	4,5,6,7
2-methylbutane	5.33	4,5,6,7
pentane	6.06	4,6,7
ethanol	6.42	4,6,7
acetone	7.53	4,5,6,7
isoflurane	8.27	4,6,7
hexane	11.30	4,5,6,7
methylethylketone	14.09	6
2-methyldioxane	16.42	4,7
benzene	16.92	2,4,6
isooctane	17.30	2,4,5,6,7
acetic acid	17.98	2,4,5,6,7
hepatne	18.01	4,6
butanol	19.12	4,5,6,7
toluene	23.22	4,5,6,7

The chromatograms in Figure 3.25 show that the conditioned tube maintained in the thermal desorption with a black cap (chromatogram No.2 and 3), was not contaminated in a time span of 9 hours. If a conditioned tube was left in the thermal desorption or in a Nalophan bag with a black cap for 6 days (chromatogram No.4 and 6), a comparable contamination from room air was detected, but this was negligible when compared with the breath samples (chromatogram No.7).

The best way to store conditioned tubes is to put them in a Nalophan bag, closed with a screw cap (chromatogram No. 5).

Some compounds present in the chromatograms came from ambient air contamination, for example isooctane was used as solvent for analytical measurements performed in the same room.

3.3 Determination of response factors for compounds quantification

Instrumental responses, in particular mass spectrometry detectors could change over time and it is not always a good practice to compare compound peak areas. So when a large number of samples are collected over a long period, it is necessary to quantify the amount of compounds present in the sample to compare them.

Since breath is a gaseous matrix, a gaseous standard mixture was prepared in order to calculate for each compound a response factor (K) and determine its concentration.

Adsorption tubes were loaded with Stock E and a labelled internal gaseous standard mixture, composed of Toluene-D8 and Isopropanol-D8 (Stock F). The response factor for each compound was calculated with respect to labelled compounds according to the following relation:

$$K = \frac{A_i \cdot C_{D8}}{A_{D8} \cdot C_i} \qquad (6)$$

A_i and C_i are chromatographic peak areas (a.u.) and theoretical amounts (ng loaded in the desorption tube) of *i- compound*, A_{D8} and C_{D8} are chromatographic peak areas (a.u.) and theoretical amounts (ng loaded in the desorption tube) of internal labelled standard added (toluene-D8).

A_i and A_{D8} were obtained by injecting 250 µl of Stock E and 50 µl of Stock F mixtures through a septum into the aspiration flow, during sampling of 250 ml of pure air in the adsorption tube. In order to verify response factor accuracy, the concentration of compounds in a known gaseous sample was determined.

A Nalophan bag was filled with 10 L of pure air. Twenty ml of Stock E mixture were introduced into the air flow through a septum, whilst the bag was being filled.

From the bag 250 ml of the sample were loaded into the adsorption tube, and analyzed, adding 50 µl of the Stock F through a septum.

Compound concentrations were calculated as follows:

$$C_i^{sper} = \frac{A_i \cdot C_{D8}}{A_{D8} \cdot K} \cdot \frac{1}{V} \qquad (7)$$

A_i is the chromatographic peak area of generic compound (a.u.), A_{D8} e C_{D8} are chromatographic peak areas (a.u.) and theoretical amounts (ng loaded in the desorption tube) of internal standard added (toluene-D8), K is the response factor and V is the sampled volume (l).

Table 3.9 *Comparison between expected and experimental concentrations of Stock E mixture in Nalophan bag.*

Compound	Expected Concentration [ng/l]	Experimental Concentration [ng/l]	Recovery %
Pentane	626	618	99%
Isoprene	700	768	93%
Acetone	790	760	97%
Dimethylsulfide	847	931	93%
Carbon sulfide	1260	1317	97%
Isopropanol	990	906	94%
2-methylpentane	653	629	97%
Esane	659	637	98%
2-butanone	800	799	99%
2-pentanone	800	800	100%
Hexafluoroisopropanol	1618	1821	92%
Dimethyldisulfide	1046	1147	93%
Toluene	870	963	93%
Hexanale	800	712	92%
4-Heptanone	800	863	95%
2-Heptanone	800	797	99%
Heptanale	800	837	97%
Benzaldehyde	1050	999	96%

This calibration method produced good results; in fact experimental concentrations were in good agreement with theoretical values within ± 7%.

Some problems were encountered over time in terms of the reproducibility in the preparation of gaseous mixtures, so we decided to test liquid solutions, for the instrumental calibration and compound quantification.

3.4 Instrumental calibration with liquid mixture

In order to avoid compound degradation or reactions, two new liquid stock solutions composed of twelve compounds (SOL 2) and thirteen compounds (SOL 3) were prepared. A stock solution composed of 40 µl of SOL 2 and 40 µl of SOL 3 in 20 ml of methanol (Stock B) was made (Table 2.2). Stock B was diluted in two other solutions, according to the schedule in Table 2.3.

Fifty µl of an internal labelled standard solution in methanol, composed of Toluene-D8 and Isopropanol-D8 (Stock H) were added to the three standard solutions (Table 2.5).

To obtain calibration curves Tenax GR tubes were loaded with 1µl of each standard solution. Each point of the calibration was analysed in triplicate. The mass spectrometric, chromatographic and thermal desorption methods are reported in Table 2.11.

The acquisitions were carried out in full-scan and selected ion monitoring during the same analysis. This enabled us to identify interferences or other compounds not present in the standard solutions. (Tables 2.12)

The data obtained were processed with GC software (Xcalibur). The results in terms of the correlation coefficient are reported in Table 3.10 and 3.10 (a).

Table 3.10 *Correlation coefficient of each compound present in the solution*

Compounds	R squared
pentane	0,997
etanolo	0.995
isoprene	0.998
acetone	0.998
dimethylsulphide	0.995
isopropanol	0.999
acetonitrile	0.998
2-methylpentane	0.999
2,3-butandione	0.999
ethylacetate	0.999
chloroform	0.998
carbonthetrachloride	0.999
benzene	0.996
trichloroethylene	0.999
1-butanol	0.998
2-pentanone	0.996
hexafluoroisopropanol	0.999

Table 3.10 (a) *Correlation coefficient of each compound present in the solution*

Compounds	R squared
dimethyldisulphide	0.999
3-hydroxy-2-butanone	0.998
toluene	0.999
tertachloroethylene	0.999
4-heptanone	0.998
m-cresol	0.855

Correlation coefficient values for most of the compounds were greater than 0.99, which confirmed the linearity of the calibration curves in the concentration range considered.

Only the correlation coefficient of m-cresol was < 0.90. In fact the RSD% between the three replicates for each point is high. (Figure 3.26)

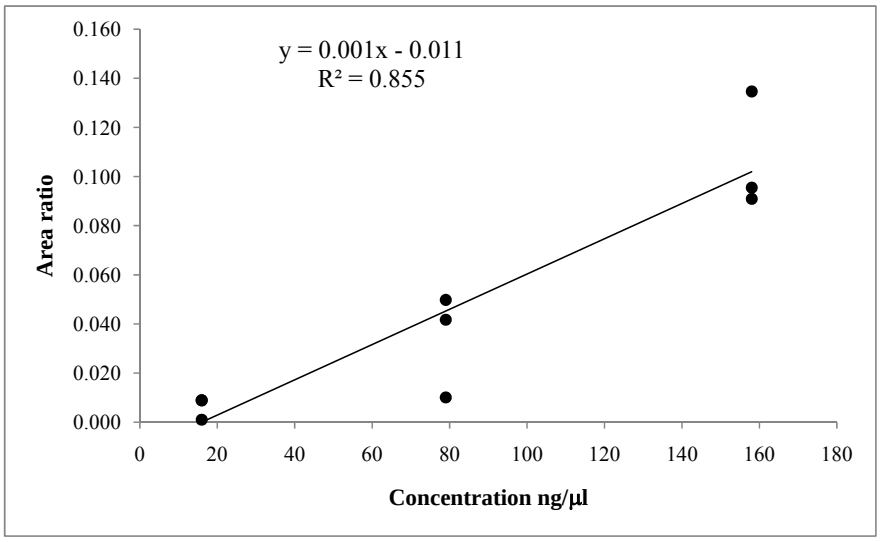

Figure 3.26 *Calibration curve for m-cresol*

The internal trap desorption efficiency for m-cresol was verified, in order to understand the previous results. Three tubes were loaded with 1µl of Stock B. The tubes were analyzed maintaining the same tube desorption temperature (300°C), and increasing the trap desorption temperature (250°C; 275°C; 300°C). The tubes were desorbed twice. (Table 3.11)

Table 3.11 *m-cresol peak area response, increasing trap desorption temperature*

	Trap Desorption Temperature		
	250°C	**275°C**	**300°C**
First analysis (peak area)	3481198	638861	1499564
Second analysis (peak area)	5093473	1355890	5387632
First / Second analysis RATIO	0.68	0.47	0.28

Increasing the trap desorption temperature, the recovery of m-cresol did not increase. On the contrary, despite the three tubes were loaded with the same amounts, the area counts were significantly different from each other. For each analysis the m-cresol did not seem to be released from Tenax GR in the same amounts and most of it was released during the second analysis.

The third tube was then desorbed another three times, with the tube and trap desorption temperature at 300°C. Data show that the m-cresol contribute is negligible only to the fifth analysis (13% compared to the second analysis) and is retained by TenaxGR in the tube. (Table 3.12)

Table 3.12 *m-cresol peak area after fifth analysis*

m-cresol	*Trap and Tube desorption temperature 300°C*
First analysis	1499564
Second analysis	5387632
Third analysis	2514613
Fourth analysis	1252170
Fifth analysis	693087

For this reason m-cresol was not quantifiable.

In order to evaluate if the non-released m-cresol could interfere with the quantification of other compounds, the following analyses were performed:

- Tube 1 was emptied (to verify trap and thermal desorption line cleaning)
- Tube C was conditioned (to verify conditioning efficiency)
- Tube loaded with 1µl of Stock C solution (the tube was analyzed twice, but the second one was not acquired)

- Tube 2 was emptied (to verify trap and TD line cleaning after analysis of a tube loaded with a standard solution)

Different contributes were quantified using the previous calibrations. The percentage ratios between the area counts obtained from the two empty tubes and the conditioned tube, divided by the area counts obtained from the tube loaded with the standard solution are reported in the Table 3.13.

Table 3.13 *The contribute percentage of each compound with respect to Stock C solution, in the following: Empty tube 1; Conditioned tube; Empty tube 2*

	First empty tube	**Conditioned tube**	**Second empty tube**
Pentane	<0.05%	<0.05%	<0.05%
Ethanol	0.1%	0.1%	0.1%
Isoprene	<0.05%	<0.05%	<0.05%
Acetone	7.4%	8.6%	4.1%
Dimethyldisulphide	<0.05%	<0.05%	<0.05%
Isopropanol-D8	0.1%	<0.05%	<0.05%
Isopropanol	0.3%	0.2%	0.1%
Acetonitrile	0.1%	0.2%	0.1%
2-methylpentane	0.1%	<0.05%	<0.05%
Hexane	0.2%	0.2%	<0.05%
1-propanol	0.1%	0.1%	<0.05%
2,3-butandione	0.1%	0.1%	<0.05%
Ethylacetate	0.1%	<0.05%	<0.05%
Chloroform	0.1%	<0.05%	<0.05%
Carbon tetrachloride	0.1%	<0.05%	<0.05%
Benzene	1.8%	13.0%	0.3%
Trichloroethylene	0.1%	<0.05%	<0.05%
1-butanol	0.2%	0.5%	1.4%
2-pentanone	0.2%	<0.05%	<0.05%
Hexsafluoroisopropanol	<0.05%	<0.05%	<0.05%
Dimethyldisulphide	0.1%	<0.05%	<0.05%
3-hydroxy-2-butanone	0.1%	<0.05%	<0.05%
Toluene-D8	0.2%	<0.05%	<0.05%
Toluene	0.4%	0.1%	0.1%
Tetrachloroethylene	0.3%	<0.05%	<0.05%
4-heptanone	0.2%	<0.05%	0.1%

The data show that after two desorption of the tube loaded with Stock C solution, the instrument line and the trap are cleaned (Table 3.13 third column) and m-cresol does not interfere, thus it is possible to proceed with the other tube analyses.

Furthermore in a time span of about one month another three measurements of the standard solutions were taken on different days. The calibration curves obtained were in agreement with the previous one, within 10%.

3.5 Comparison between different sampling bags

Breath can be obtained non-invasively using a variety of fairly simple collection techniques.[111]

Three types of sample collectors are often used to collect and transport air samples for later analysis: glass bulbs, stainless steel canisters and bags.

However, each collector has several drawbacks. Glass bulbs are fragile, and their volume is limited. Canisters are heavy and require a special device for cleaning and air sampling.

They are also considered as expensive equipment for occasional analyses. Bags are easy to manipulate and transport but are quite fragile (explosion or puncturing).[112]

They are used in different studies: for example, bags have been used to collect exhaled air[113] from volunteers and also used to sample indoor air[114].

Analytical standards were prepared by volumetric injection into the bags and storage stability in three different bags was studied.

Three different bag materials were compared in order to evaluate sample stability and the release of interfering compounds from bag walls. (Paragraph 2.4)

1. **Nalophan**: polyethylenterephtalate (PET)
2. **Tedlar** : polyvinylfluoride (PVF)
3. **Cali-5-bond**: five different layers of materials are bonded together to form a single, flexible material 140 μm thick.

First of all the bags' background was evaluated by filling the bags with purified air; after 30 minutes, 500 ml of the sample from each bag were transferred into Tenax GR adsorption tubes. Chromatographic, mass spectrometric and thermal desorption methods are described in Table 2.10. Figure 3.27 show the chromatograms of Nalophan, Tedlar, Cali-5-bond bag background respectively.

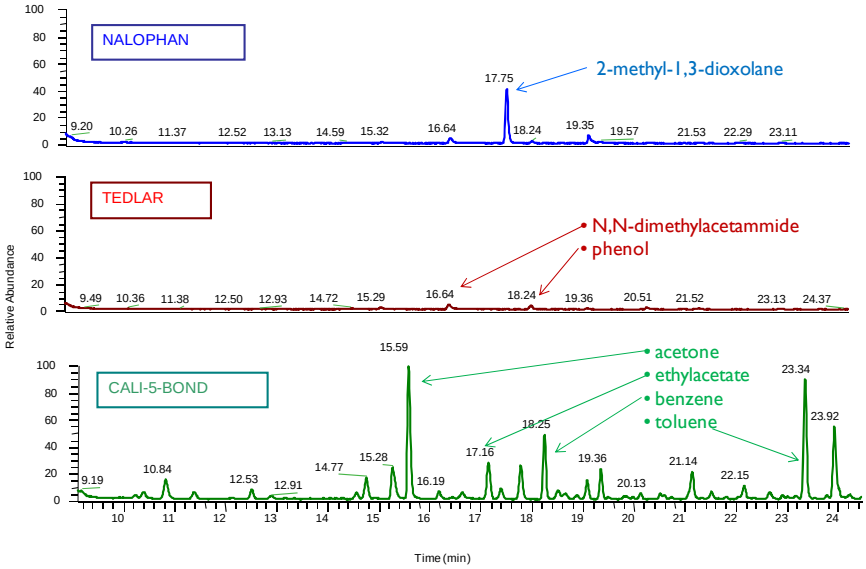

Figure 3.27 *Nalophan, Tedlar, Cali-5-bond bag background*

Nalophan and Tedlar bags released less then Cali-5-bond bag. In Table 3.14 the mainly identified compounds for each bag are reported.

Table 3.14 *Compounds present in bag background*

Compound	RT	Bag
2-methyl-1,3-dioxolane	17.75	Nalophan
N,N-dimethylacetammide	32.99	Tedlar
phenol	36.80	Tedlar
acetone	8.52	Cali-5-bond
ethylacetate	15.59	Cali-5-bond
benzene	18.25	Cali-5-bond
2-ethyl-3-methyl-1-pentene	23.34	Cali-5-bond
toluene	24.82	Cali-5-bond
1-metoxi-2-propylacetate	30.75	Cali-5-bond
2,2,4,6,6-penthamethylpentane	34.88	Cali-5-bond

To evaluate sample stability in the bag, a gaseous standard mix of eighteen compounds (Stock E) was used. The compounds, with different polarities and volatilities represented the molecules in breath and in ambient air.

Twenty ml of Stock E were injected in the flow of pure air while simultaneously filling Nalophan, Cali-5-Bond, Tedlar bags with 10 L, 10 L and 30 L respectively (Table 3.15).

Table 3.15 *Concentration of gaseous standard mix in the bags.*

Compound	**Concentration in the bags**	
	Nalophan and Cali-5-Bond [ng/l]	**Tedlar [ng/l]**
Pentane	696	232
Isoprene	757	252
Acetone	879	293
Dimethylsulphide	940	313
Carbon sulphide	1407	469
Isopropanol	872	291
2-methylpentane	726	242
Hexane	732	244
2-butanone	894	298
2-pentanone	899	300
Hexafluoroisopropanol	1773	591
Dimethyldisulphide	1162	387
Toluene	961	320
Hexanal	906	302
4-Heptanone	908	303
2-Heptanone	911	304
Heptanal	908	303
Benzaldehyde	1161	387

The gaseous internal standard mix was prepared injecting 5 µl of Isopropanol-D8 and Toluene-D8 in 2 L glass flask.

Two hundred and fifty ml of the sample were transferred into the adsorption tubes from each bag, using a membrane gas pump, see Figure 3.16. Tubes loaded with the sample were repeated in triplicate after 3, 6, 24, 48 and 72 hours after the bags had been prepared.

During the transfer of the sample in the tube, 50 µl of the internal gaseous labelled standard were added to the aspiration flow.

All samples were desorbed from the adsorption tubes and concentrated in an automated two-stage thermal desorption, followed by gas chromatographic separation and identified in a mass spectrometer. Chromatographic, mass spectrometric and thermal desorption methods are described in Table 2.10.

In the following figures for each time span, the average area count for each compound corrected by the internal labelled standard and normalized with respect to the area count at time zero are reported.

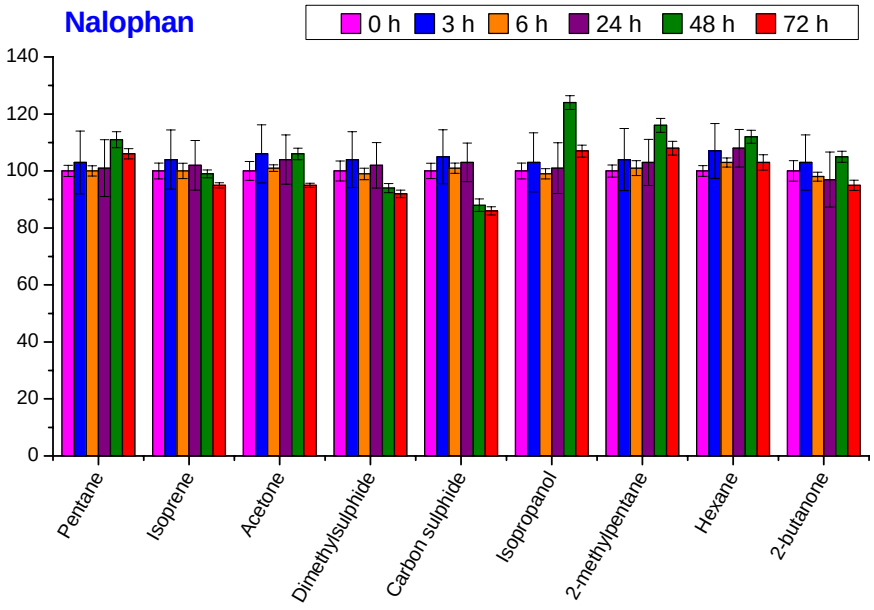

Figure 3.28a *Nalophan bag*

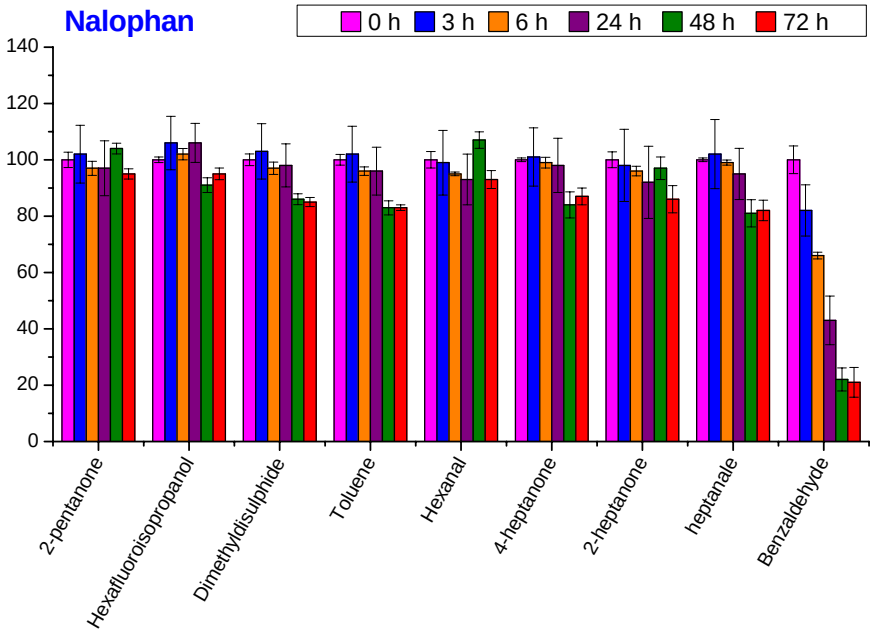

Figure 3.28b *Nalophan bag*

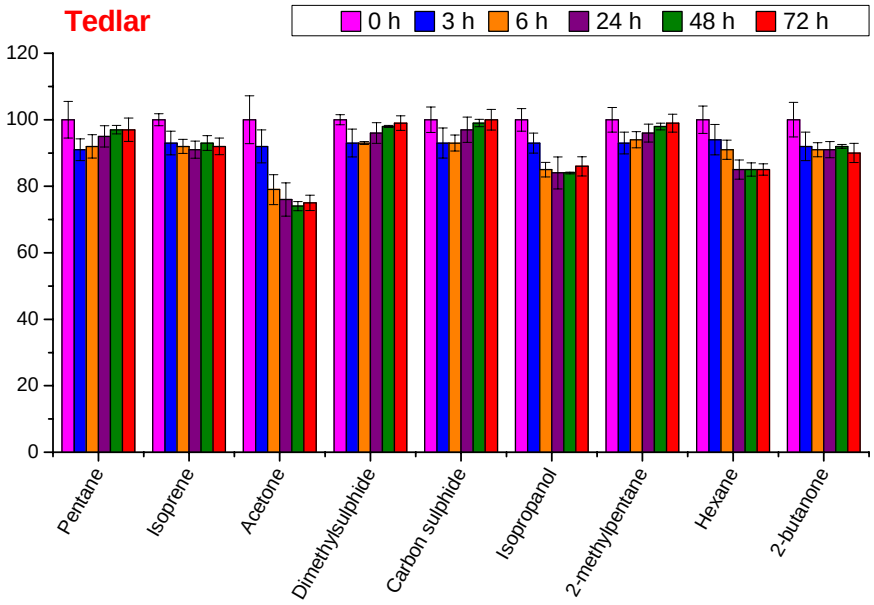

Figure 3.29a *Tedlar bag*

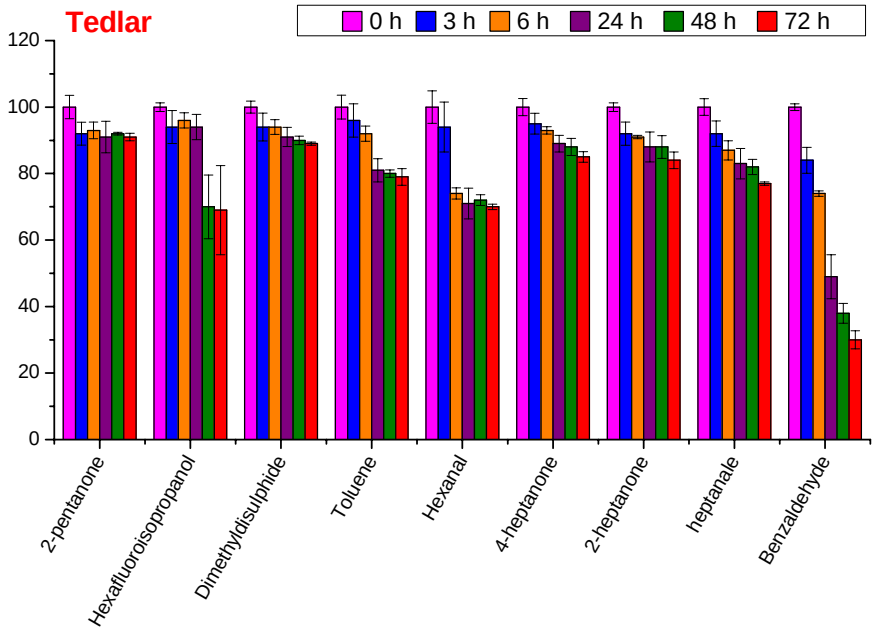

Figure 3.29b *Tedlar bag*

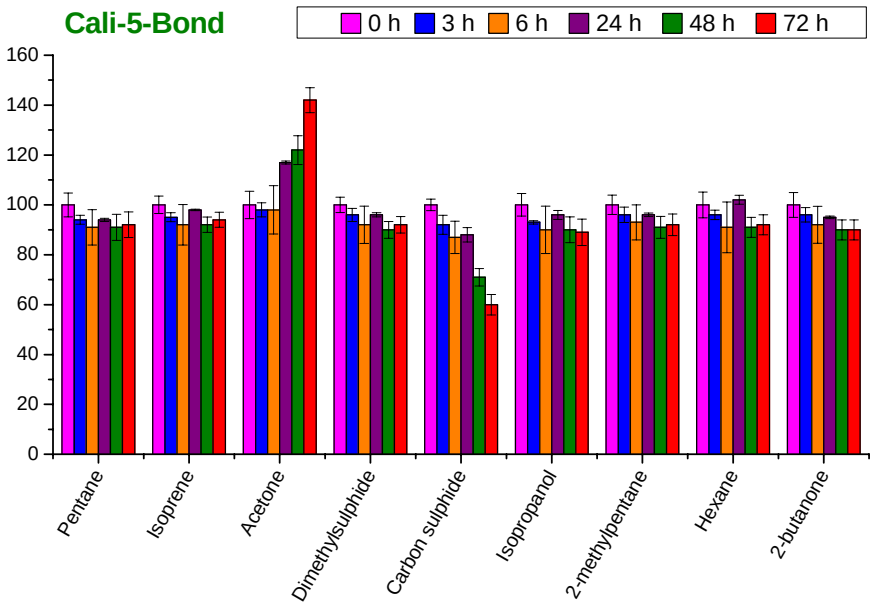

Figure 3.30a *Cali-5-bond bag*

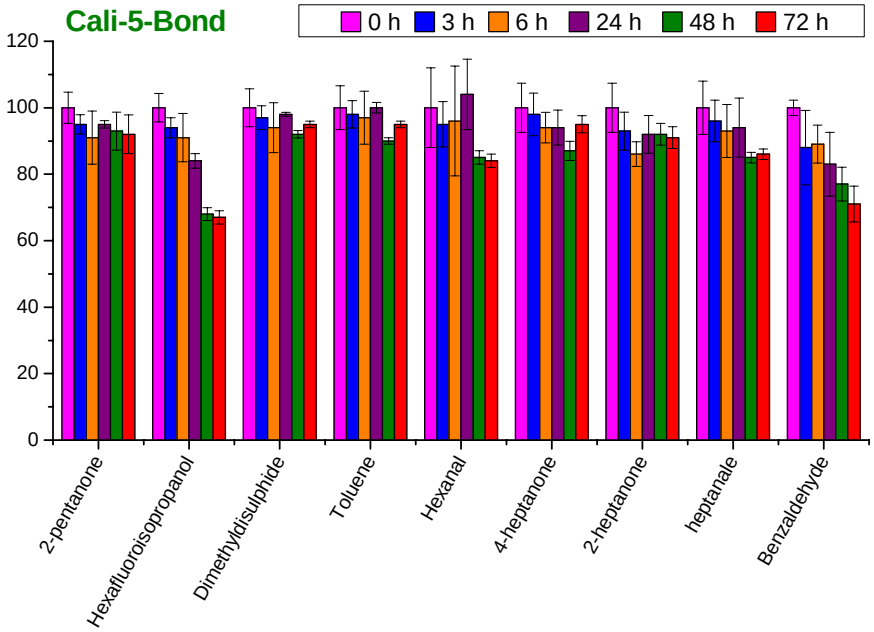

Figure 3.30b *Cali-5-bond bag*

On the basis of these results the following considerations were made:

a) Nalophan

- Within 24 hours there were no significant variations, confirmed by the ANOVA test
- Within 72 hours dimethylsulphide, toluene and heptanal showed a 20% loss

- The benzaldehyde bag content decreased over time: 20% after 3 hours, 35% after 6 hours, 60% after 24 hours, 70% after 48 hours, 80% after 72 hours

b) Tedlar

- Within 6 hours acetone, isopropanol and hexanal showed a 20% loss that remained constant over the subsequent hours
- Within 24 hours toluene had a variation of 20%, that remained constant over the subsequent hours
- Within 48 hours hexafluoroisopropanol presented a 30% loss
- Benzaldehyde showed the same behaviour as the Nalophan bag

c) Cali-5-Bond

- Within 24 hours there were no significant variations except for acetone
- Within 48 hours hexanal, heptanal, benzaldehyde, carbon sulphide and hexafluoroisopropanol presented a significant variation (25%)
- After 24 and 48 hours acetone showed signal increases of about 20% and then 40%, probably due to a release from the bag's wall.

In addition CO_2 stability in the three bags was tested. The bags were filled with a 5% CO_2 standard gaseous mix. CO_2 bag content was measured immediately and after 1, 3, 5, 24, 29, 31, 32, 48 hours, with an infrared sensor (Paragraph 2.4). Each measurement was taken by pumping the sample from the bag through the sensor. The bag volumes were 6 L for the Nalophan bag, 3 L for the Tedlar bag and 30L for the Cali-5-bond bag.

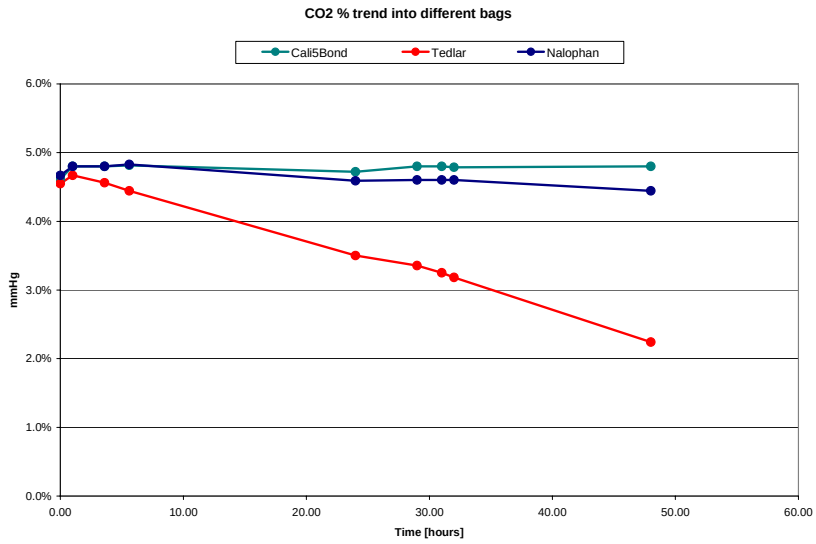

Figure 3.31 *CO_2 bags trend*

The data show that CO_2 was stable in the Cali-5-bond bag. For the Nalophan bag, there was a decrease of 7% within 40 hours and there was a loss of importance of 50% only for the Tedlar bag.

The drop in content of some compounds in the Nalophan and Tedlar bags was probably due to diffusive processes through the bag walls; the variations for the Cali-5-Bond on the other hand were linked to the adsorption phenomena.

Table 3.16 *Summarizing table of bags of different materials*

	Background	**Sample stability**	**Handy**	**Cost**
Nalophan	GOOD	GOOD	GOOD	CHEAP
Tedlar	GOOD	GOOD	GOOD	EXPENSIVE
Cali-5-bond	BAD	EXCELLENT	GOOD	VERY EXPENSIVE

The Nalophan bag was chosen for our purposes due to its low background contamination and its extremely low cost that enabled it to be disposed of thus avoiding any need for cleaning.

3.6 Extension of analytical procedure to samples with high water contents

Since breath samples have high water content, measurements of sample stability in Nalophan bags were repeated with the same mixture saturated with water.

To prepare humid samples pure air was bubbled through milli Q water before filling the bags.

Dry tubes, filled with 9 g of sodium sulphate, were used to transfer sample from the bags into the adsorption tubes.

The volume of pure air introduced in the bags was calculated measuring the flow and time of filling. Two hundred and fifty ml of the sample were transferred into the adsorption tube (flow 50 ml/min).

Tubes loaded with the sample were repeated after 3, 6, 24, 48 e 72 hours, after bag preparation. During the transfer of the sample in the tube, 50 µl of internal gaseous labelled standard were added to the aspiration flow.

In the following figures for each time span, the average area counts for each compound corrected by the internal labelled standard and normalized respect to the area count at time zero are reported.

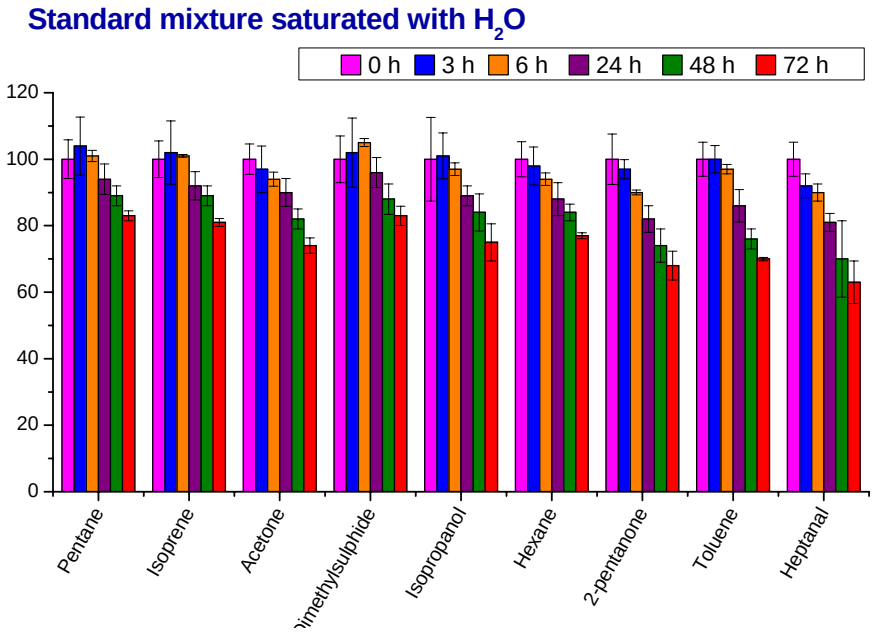

Figure 3.32 *Stability of the gaseous mixture saturated with water in the Nalophan bag*

Within 24 hours for the compound of interest present in breath the variation was around 15%, so compound storage stability was also confirmed for humidity samples.

3.7 Bags preparation reproducibility

In order to evaluate bag preparation and sampling tube reproducibility, repeated measurements on three Nalophan bags were taken.

Twenty ml of Stock E were transferred into three Nalophan bags during bag filling with 10 L of pure air (flow 500 ml/min).

Three tubes were loaded with 250 ml of the sample for each bag. During sample transfer, 50 µl of the internal labelled standard gaseous mixture were added (isopropanol-D8, toluene-D8).

In the Tables below are reported the average area counts results obtained for some of the compounds present in the mixture.

Table 3.17 *Total compound area counts average and RSD %*

	Total compounds area counts	
Compounds	**Average**	**RSD %**
pentane	3.70E+07	4%
isoprene	3.83E+07	4%
acetone	1.46E+07	3%
dimethylsulphide	4.01E+07	5%
carbon sulfide	1.32E+08	4%
isopropanol	4.72E+07	5%
2-methylpentane	4.17E+07	4%
hexane	3.62E+07	3%
2-butanone	5.38E+07	5%
2-pentanone	6.47E+07	5%
hexafluoroisopropanol	2.87E+07	3%
dimethyldisulphide	7.47E+07	5%
toluene	1.22E+08	6%
hexanal	9.82E+06	7%
4-heptanone	5.95E+07	5%
2-heptanone	5.19E+07	7%
heptanal	1.73E+07	5%

Table 3.18 *Total average and RSD % of compound area counts corrected by the internal labelled standard*

	Total compounds area counts	
Compounds	**Average**	**RSD %**
pentane	3.82E+07	5%
isoprene	3.96E+07	5%
acetone	1.51E+07	5%
dimethylsulphide	4.14E+07	6%
carbon sulfide	1.37E+08	5%
isopropanol	4.87E+07	5%
2-methylpentane	4.30E+07	5%
hexane	3.74E+07	5%
2-butanone	5.56E+07	5%
2-pentanone	6.68E+07	5%
hexafluoroisopropanol	2.96E+07	5%
dimethyldisulphide	7.71E+07	6%
toluene	1.26E+08	6%
hexanal	1.01E+07	6%
4-heptanone	6.14E+07	5%
2-heptanone	5.36E+07	6%
heptanal	1.79E+07	6%

The procedure gave good results in terms of reproducibility, with a RSD% around 6%. Not always normalizing the data it is possible to obtain a better reproducibility; in any case it is important to control the internal standard in order to avoid errors coming from the instrument response.

CHAPTER 4

Clinical Applications

4.1 Breath variability

Diagnosis of disease is based on physical examination and a number of different tests, some of which have standard reference values for normal subjects. These reference values are based on thousand of subject. In breath analysis there is also a need to create a database of reference values and evaluate inter and intra personal variability in healthy people. In fact breath composition is affected by many factors, such as food ingestion, physical activity, pulmonary ventilation and blood rate.
In the literature differences in breath samples between people on a diet and people eating regularly have been found. In fact the compounds in food enter metabolic pathways, which are then absorbed by the organism.
Sulphur compounds were found in breath samples from people after garlic or onion ingestion. In people who drink, much more ethanol was found than in people who do not drink. Brushing teeth could also introduce interferences; in fact toothpaste contains compounds such as menthol, traceable in breath. Isoprene concentration was found to change with the heartbeat.

We thus decided to study daily and weekly individual breath composition variability in two volunteers, in order to evaluate how personal habits influence breath composition. In particular the trend in time of the two principal compounds in breath, acetone and isoprene was observed.

Ethics and respect of cultural differences, privacy, interests, rights and feelings of people has been a primary concern, even beyond compliance to current legislation, regulations and ethical codes of conduct. Nobody was asked to give a breath sample

that had not been informed regarding the aim and modalities of the present study and really willing to contribute. In the initial phase of the study, when breath samples were only needed to test the analytical procedure without a real interest for the presence of markers, breath samples were obtained from the author or from colleagues who spontaneously offered to provide breath samples.

The large majority of patients were enrolled in the Institute of Clinical Physiology. These people are consented when entering the Institute about their willingness to provide biological samples for research purposes, and only those who had provided a positive answer to the questions were contacted. The studies on markers were approved by the Ethical Committee of Pisa University -Hospital, Reference Authority of Italian National Commission of Bioethics and Tuscany Regional Ethical Commission with the deliberation n° 2328; written informed consensus was collected from both patients and nominally healthy subjects involved in these studies.

Breath samples from the same person were collected in a time span of one week. The volunteer had to fill one bag in the morning three hours after breakfast and one in the afternoon after lunch. In both cases room air was collected.

The breath sampling system consisted of a disposable mouthpiece, a one way valve, a Nalophan bag and a Teflon tube. (Figure 4.1) The mouthpiece and the one way valve were sterilized by the sterilized used in the Institute of Clinical Physiology. The Teflon tube enabled us to connect the bag to the adsorption tube in order to transfer the breath sample.

The 10 L Nalophan bags were home made. A piece of Nalophan was cut from a 1 Km Nalophan cylindrical roll, one tip was wrapped to a Teflon tube, the other tip was closed with nylon cable ties.

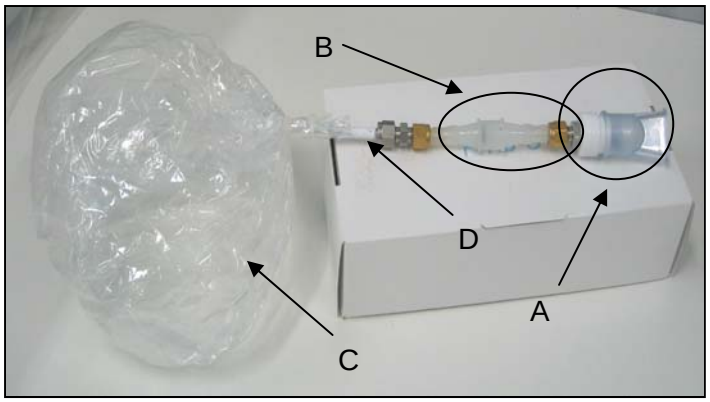

Figure 4.1 *Breath sampling system: A) disposable mouthpiece; B) a one way valve; C)Nalophan bag; D) Teflon tube*

Breath samples were analyzed according to the methods described in Table 2.11.

The following graphics show acetone and isoprene breath trends for the two volunteers.

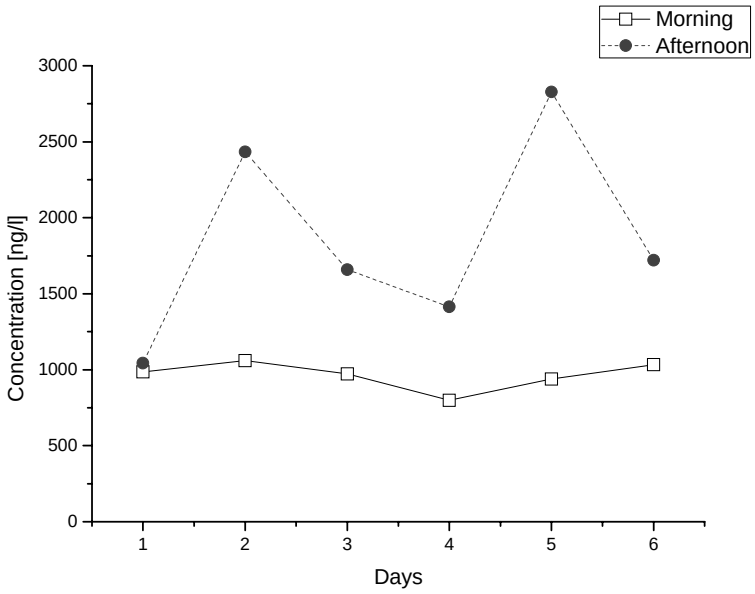

Figure 4.2 *Subject 1 acetone trend*

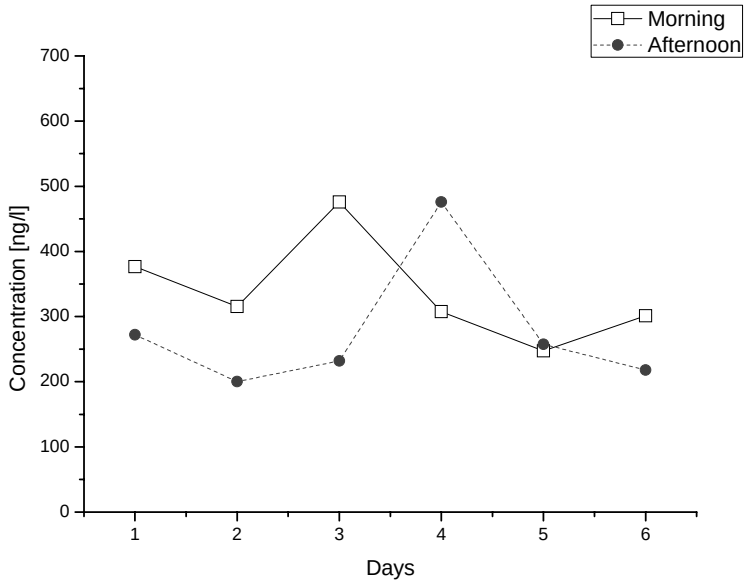

Figure 4.3 *Subject 1 isoprene trend*

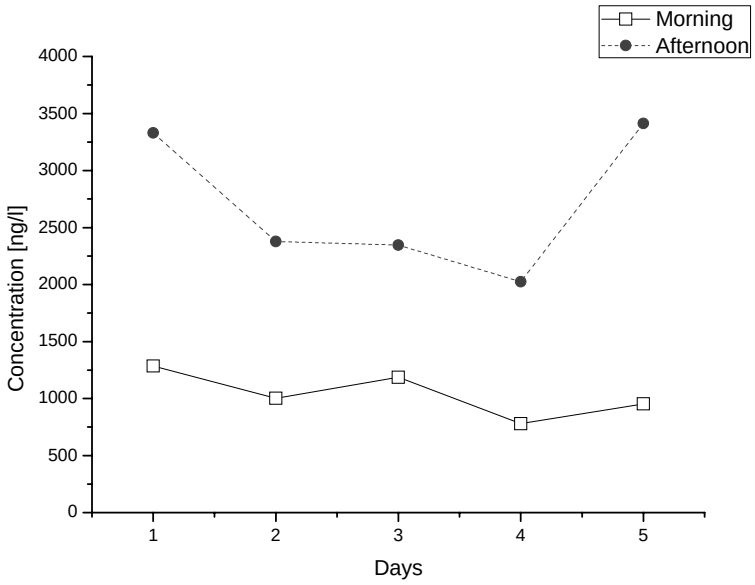

Figure 4.4 *Subject 2 acetone trend*

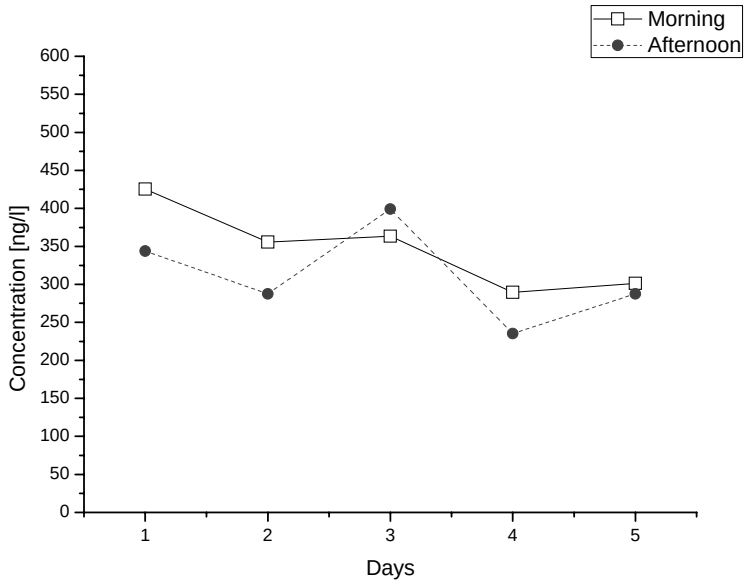

Figure 4.5 *Subject 2 isoprene trend*

Comparing concentrations and variability of acetone and isoprene in the morning and afternoon, in the same subject, it transpired that isoprene did not seem to be affected by the ingestion of a meal during the day. Acetone, on the other hand, showed a major variability and its concentration in the afternoon was doubled, probably due to the kind of meal that the subject had eaten (a greater or lower quantity of carbohydrates, fats or proteins).

In Tables 4.1 and 4.2 the acetone and isoprene mean concentrations and percentage relative standard deviations in the morning and afternoon for each subject are reported.

Table 4.1 *Acetone and Isoprene average concentrations and RSD% for Subject 1*

	Subject 1			
Day	**Isoprene [ng/l]**		**Acetone [ng/l]**	
	Morning	***Afternoon***	***Morning***	***Afternoon***
1	377	272	986	1043
2	316	200	1059	2434
3	476	232	972	1658
4	308	476	799	1415
5	247	258	939	2828
6	301	218	1033	1721
Average	337	276	965	1850
RSD%	*24%*	*37%*	*10%*	*36%*

Table 4.2 *Acetone and Isoprene average concentrations and RSD% for Subject 2*

	Subject 2			
Day	**Isoprene [ng/l]**		**Acetone [ng/l]**	
	Morning	***Afternoon***	***Morning***	***Afternoon***
1	425	344	1285	3330
2	356	288	1001	2378
3	363	399	1186	2345
4	289	235	780	2024
5	301	287	952	3414
Average	347	311	1041	2698
RSD%	*16%*	*20%*	*19%*	*23%*

The data confirm that a meal can influence breath composition. For this reason if possible, it is better to collect a breath sample in the morning before eating.
The data show also a wide inter-variability between different people. It is thus important to find a parameter that enables us to normalize the data and reduce that variability.

In the literature this role is in part given to the carbon dioxide present in breath. In fact carbon dioxide is the main product of the respiration process and derives from

organisms breaking down sugars, fats and amino acids, thus it is closely connected to the metabolism processes. It is the amount, or more exactly the pressure of carbon dioxide in the air in the lungs, and therefore also in the arterial blood flowing from the lungs, that acts so importantly on the respiratory centre. In normal conditions the volume of air that is breathed is kept in exact proportion to the amount of carbon dioxide in the blood.[115]

For this reason mixed breath samples were collected from four volunteers on different days, measuring bag CO_2 content with an IR sensor. (Figure 4.6)

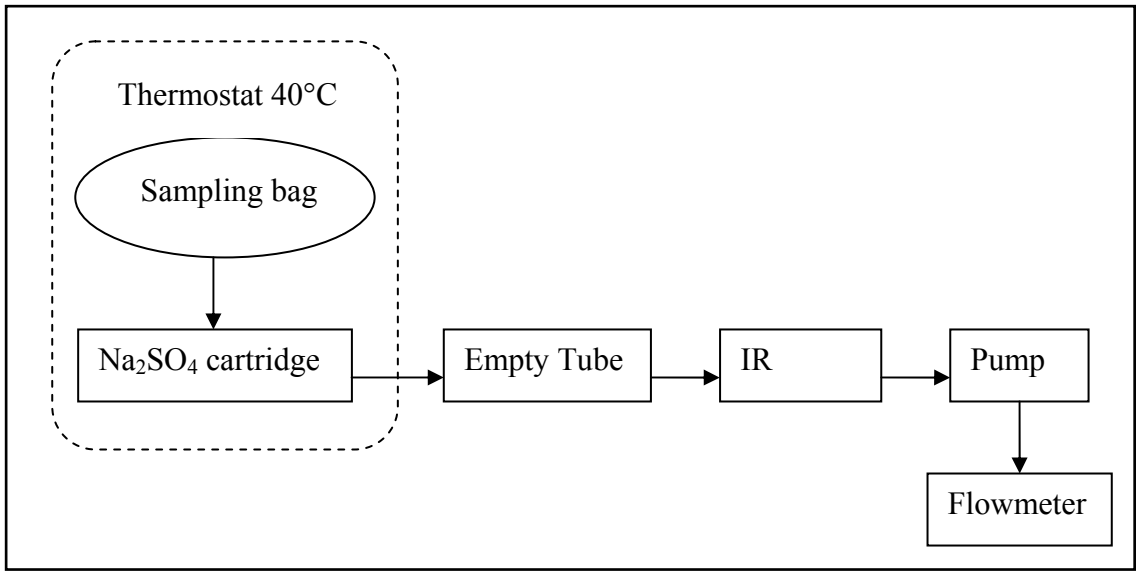

Figure 4.6 *Scheme of the system used to measure CO_2 bag content.*

In Table 4.3 the mean concentration corrected and not corrected by the CO_2 bag content of different compounds over a time span of three days is reported, with the RSD%.
The corrected concentration value was obtained dividing the measured concentration value for CO_2 bag content and multiplying for a CO_2 reference value

Table 4.3 *Mean compound concentration corrected and not corrected by CO_2 bag content and RSD%, in a time span of three days*

	Normal values		**Values corrected by CO_2**	
Compound	**Average [ng/l]**	**RSD%**	**Average [ng/l]**	**RSD%**
Isoprene	415	39%	365	37%
Acetone	1053	62%	967	70%
DMS	15	90%	14	90%
Isopropanol	23	35%	20	40%
n-propanol	23	80%	19	70%

The correction of the compound concentration by CO_2 bag content determines a small variation of the mean concentration and interpersonal variability, but these few measurements didn't show a significant improvement of the results after CO_2 correction. So the correction was not applied to the data presented in next paragraphs.

4.2 Human breath analysis medical applications

The diagnostic methods used in medicine require that a sample of body fluid be taken, which is quite invasive and uncomfortable for the donor. Breath analysis should be added to medical diagnostic techniques, especially since breath sampling is non-invasive and totally painless. The major challenge is to be able to identify and quantify the compounds present in breath with sufficient accuracy to be useful in diagnosis, despite the relatively simple matrix[5].

The final aim is to find a correlation between a compound in breath and a specific pathology, in order to develop sensors able to give fast and accurate responses, in a completely non-invasive way.

Thus preliminary studies on breath analysis application for specific pathologies were started.

4.2.1 Diabetes mellitus

In the literature many papers have reported the correlation between blood acetone and diabetes mellitus.[116,117]

Diabetes mellitus is characterized by high glucose levels in blood (hyperglycaemia) due to an abnormal quantity or function of insulin. Insulin is the hormone, produced by the pancreas, which permits glucose to enter into cells to be used as source of energy. When this mechanism is altered, glucose accumulates in the blood circulation. Once the

glucose haematic and renal threshold has been exceeded, the effects of glycosuria (glucose in urine) and polyuria (loss of water and electrolytes) appear.

The organism therefore tries to produce glucose through other metabolic pathways such as lipolysis (fat acids oxidation), which causes an increase in ketone bodies in blood (acetone, β–hydroxybutyric acid, acetoacetic acid).

Acetone diffuses from the blood to the lung through the alveolar membrane and is usually one of the compounds present in large amounts in breath, due to its volatility. This compound should be a breath diabetes marker.

In order to estimate the performance of the procedure, 32 healthy subject samples and 20 diabetes patient samples were collected in the morning and analyzed.

Table 4.4

Control Subject	Age range	Location	Diabetes Patient	Age range	Location
14 male	25-63	Institute workers	16 male	50-80	11 patients
18 female			4 female		9 ambulatory patients

In Figure 4.7 diabetes breath acetone concentrations are reported in ascending order, compared with the average acetone concentrations of healthy people (control group).

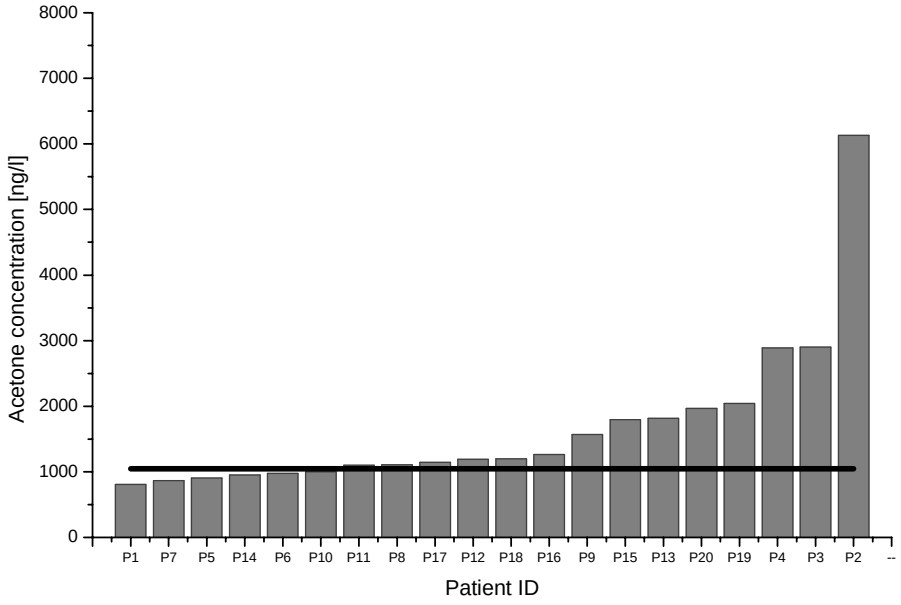

Figure 4.7 *Diabetes acetone concentrations (bars) compared with healthy people acetone average concentrations (straight line)*

Mean breath acetone concentrations in the control group was 1048 ng/l, with a standard deviation of 407 ng/l. The diabetes patients acetone concentration on the other hand was 1682 ng/l (1.6 times higher), with a standard deviation of 1216 ng/l.
Results showed a good difference between controls and average patient acetone concentrations, but also show a large variability in acetone breath concentrations. Only in a few cases was breath acetone in patients significantly different from breath control samples.
These data make the discrimination between the two groups critical and highlight an apparent contradiction with what is reported in the literature, concerning the possibility of using these parameters to discriminate diabetes patients from healthy people.

Also isopropyl alcohol was investigated because, as reported in literature this compound is related to acetone content in blood.[118] It is involved in an acetone/isopropanol enzymatic equilibrium, linked to lipolysis and regulated by oxidized NAD (NAD^+) and reduced NAD (NADH) ratio.

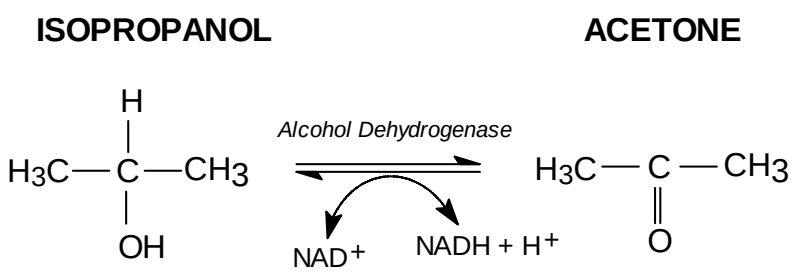

Figure 4.8 *Enzymatic acetone- isopropanol equilibrium*

Isopropanol concentration in breath of patients and healthy people was determined. In Figure 4.9 patients are reported in the same order as the acetone Figure 4.7.

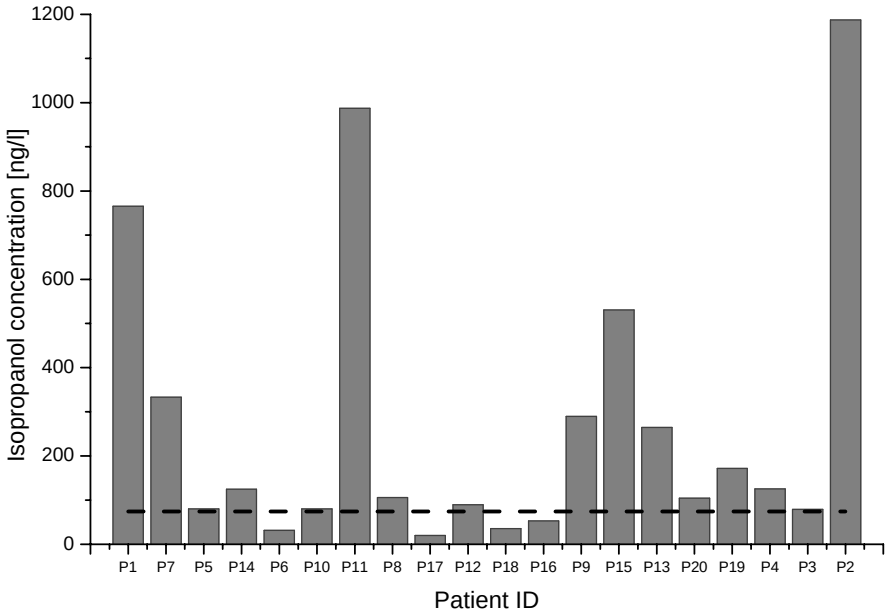

Figure 4.9 *Isopropanol breath concentrations in diabetes patients (bars) compared to isopropanol average concentrations in healthy people (dot line)*

The average concentration of isopropanol in diabetes patients was 273 ng/l, 2.7 times higher than the control group (74 ng/l). The data in this case also had a high variability. In most of the samples there was no correlation between the two compounds. Only in one case did a high amount of acetone correspond to an elevated amount of isopropanol (P2). Furthermore only few patients had a higher level of isopropanol compared to the control group.
It is possible to conclude that isopropanol is also not capable of discriminating between diabetes and healthy people.

On the bases of these unsatisfactory results, a critical evaluation of all steps of the procedure was made, particularly on the possibility of contamination.

During breath sampling of another group of diabetic patients, room air samples were also collected using a glass cylindrical container. A bag was attached to the top of the container and inserted. On the cover there were two holes, one was for the inlet of room air and the other was to empty the container, in order to fill the bag. Room air samples were transferred from the bag into the adsorption tube and analyzed as breath samples.

From the results acetone was present in the room air but at a lower concentration level than the breath concentration levels. Isopropanol on the other hand resulted in a higher level compared to the breath samples. (Figure 4.10)

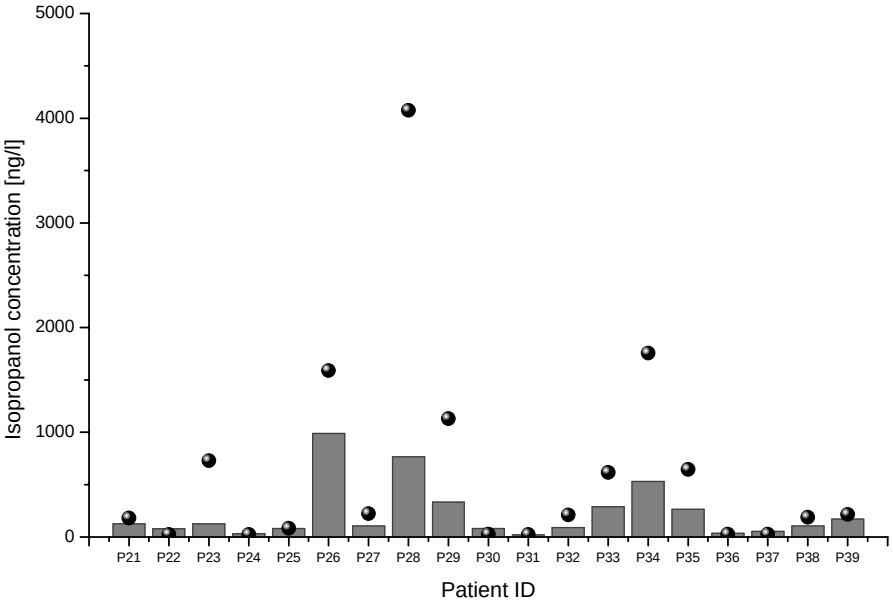

Figure 4.10 *Isopropanol breath concentrations in diabetes patients (bars) compared to isopropanol room air concentrations (circles)*

A primary cause of the contamination was found to be the disinfectant used in the hospital. In fact one of the principal components of disinfectant is isopropanol.

In order to confirm the existence of the enzymatic equilibrium between acetone and isopropanol various measurements were carried out. One volunteer inhaled in different days a small amount of acetone-D6 and then isopropanol-D8.

The aim of these preliminary measurements was to verify the production of a compound as a consequence of the exposition of another compound, related by a metabolic pathway.

Breath samples were collected at different times after the exposition in order to evaluate the removal kinetics of the compounds.

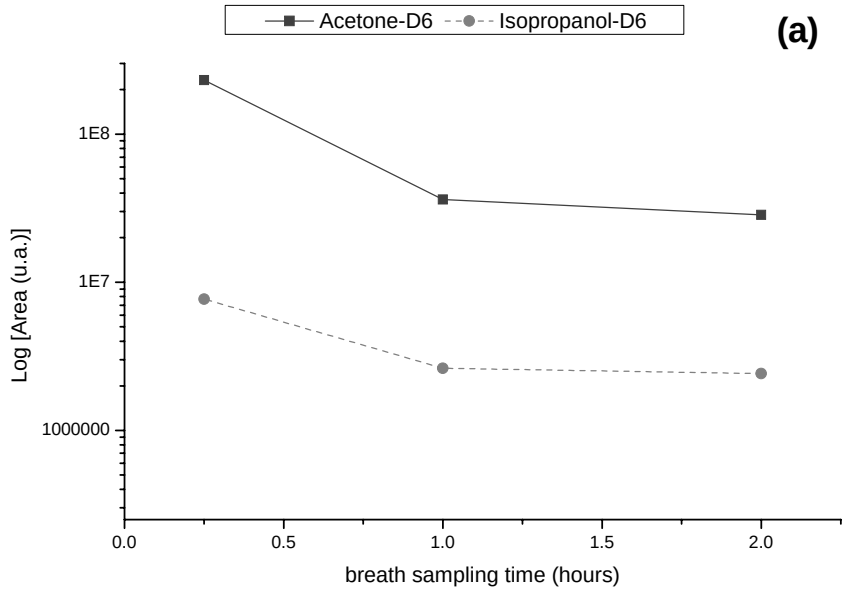

Figure 4.11 *Isopropanol-D6 trend after inhalation of acetone-D6 (logarithmic scale)*

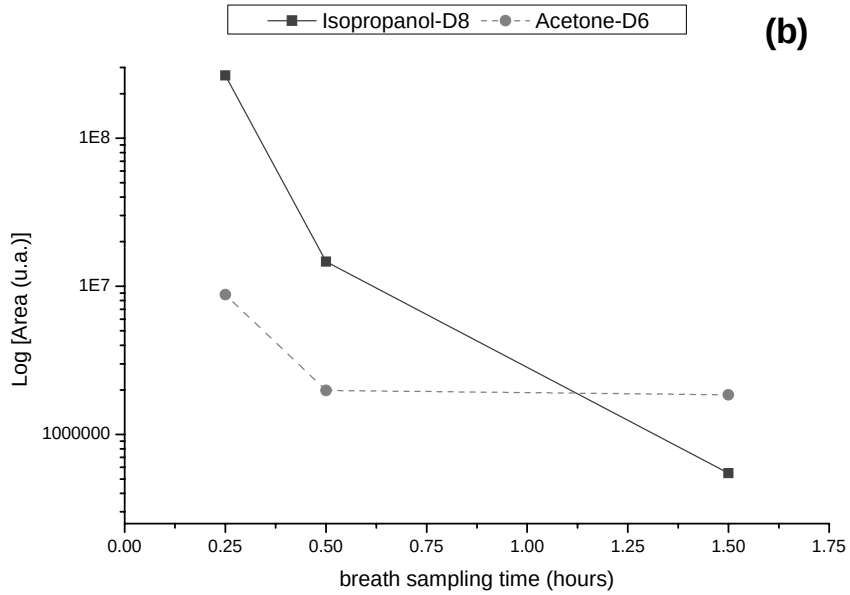

Figure 4.12 *Acetone-D6 trend after inhalation of isopropanol-D8 (logarithmic scale)*

The data show the presence of labelled isopropanol after labelled acetone inhaling and vice versa.

In a time span of two hours it was possible to observe a decay in breath labelled compound content.

In the presence of such a contamination and due to the metabolic relation between acetone and isopropanol, it is pretty easy to explain the high variability in acetone and

isopropanol breath contents, particularly in subjects exposed for a long time. This highlights the importance of understanding the relationship between the presence and the cause of a compound in breath. The concentration of a compound from a specific metabolic pathway can be altered by exposition to the same compound or to other exogenous molecules related to several metabolic pathways.

We decided to follow the acetone breath trend of people submitted to oral glucose tests (OGT), in order to evaluate if there could be a correlation between blood glucose and breath acetone trends in time. The OGT is a diabetes diagnostic test and monitors glycaemia in the subsequent hours after dispensing a known amount of glucose. After dispensing glucose, glycaemia rises. Within two hours, in subjects without diabetes, glycaemia reduces to 1400 mg/l; if this doesn't happen the OGT is considered positive and diabetes is diagnosed. Sixteen samples were collected from subjects submitted to the OGT in day hospitals. Before breath collection, room air was analysed, to be sure there was not any contamination. Breath samples were collected before glucose dispensing (Time 0) when the subject had gone without eating and after glucose dispensing, evenly to blood taking, in order to determine glycaemia. Acetone breath concentration trends and glycaemia values versus time, for different patients are reported in the following figures.

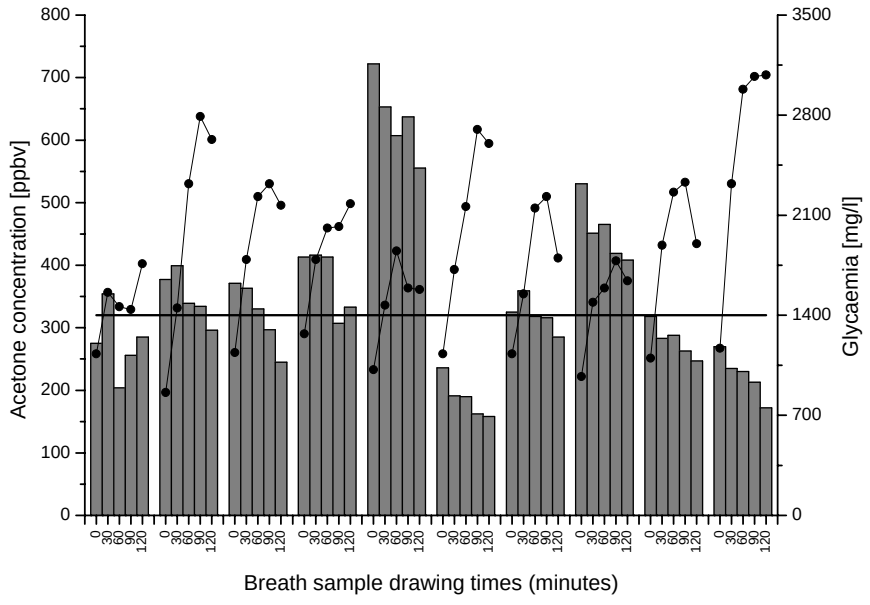

Figure 4.13 *Breath acetone trend and glycaemia blood trend in time during OGT, in glycaemia pathological curves*

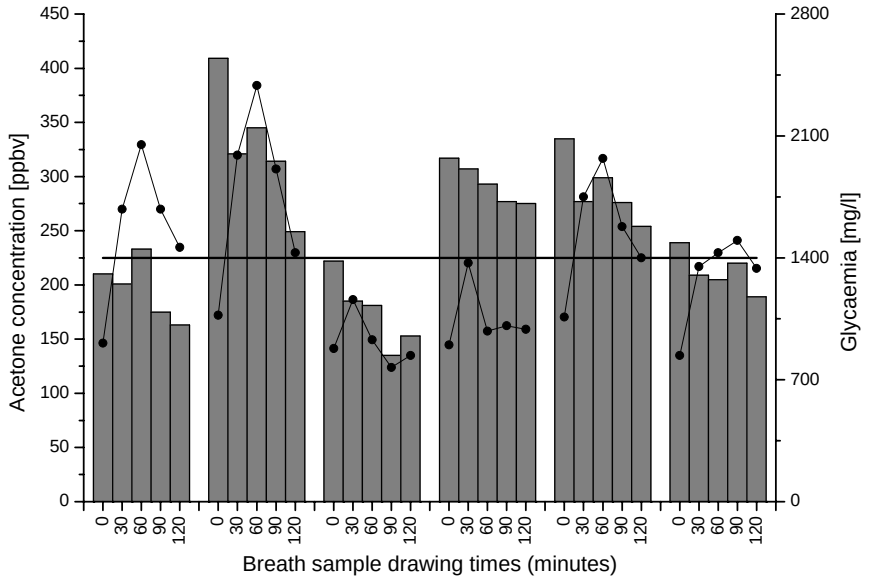

Figure 4.14 *Breath acetone trend and glycaemia blood trend in time during OGT, in glycaemia non pathological curves*

It was not found a correlation between acetone and glycaemia trend during OGT, but the results have shown the capacity of the procedure to appreciate small variation in breath composition, in consequence of the glucose dispensing.

4.2.2 Hepatic cirrhosis

Cirrhosis is a condition that causes irreversible scarring of the liver. As scar tissue replaces normal tissue, blood flow through your liver is affected. This makes it increasingly difficult for the liver to carry out essential functions, such as detoxifying harmful substances, purifying the blood and manufacturing vital nutrients.[119] When damage is so severe that liver function is seriously impaired, a liver transplant may be the only option.

Preliminary measurements of the breath of cirrhotic patients were taken. The aim was to find the compounds in breath that would enable us to diagnose a possible rejection crisis or to monitor recovery of hepatic functionality.

Breath samples from 7 cirrhotic patients (6 men and 1 woman, age range 19-63) were collected before and after a liver transplant.

Data show the presence of the anaesthetic (sevoflurane) used during the operation and its metabolite (hexafluoroisopropanol) in all breath patient samples[120]. The graphics (Figure 4.14 and 4.15) show the two compounds post-transplant peak area trends.

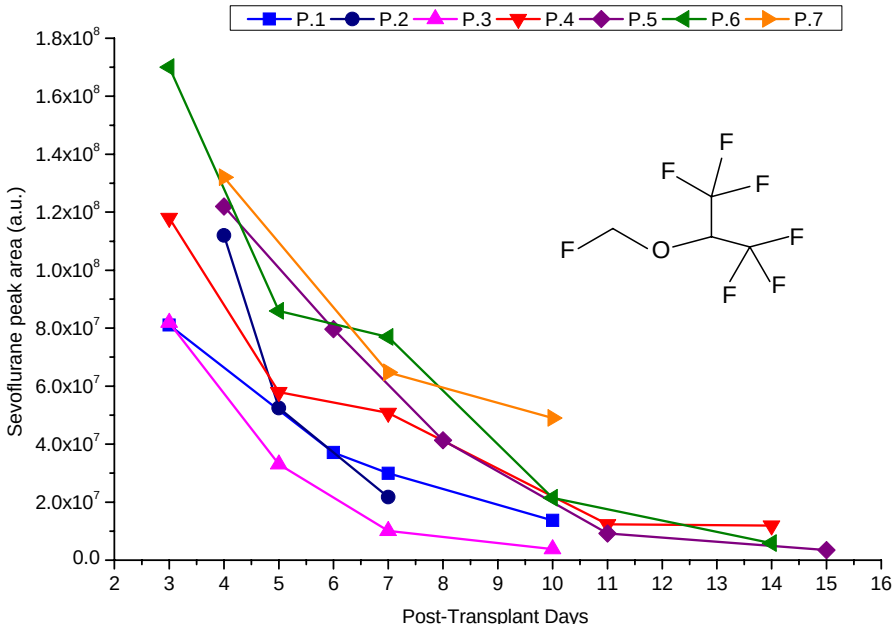

Figure 4.14 *Post liver transplant breath sevoflurane trend*

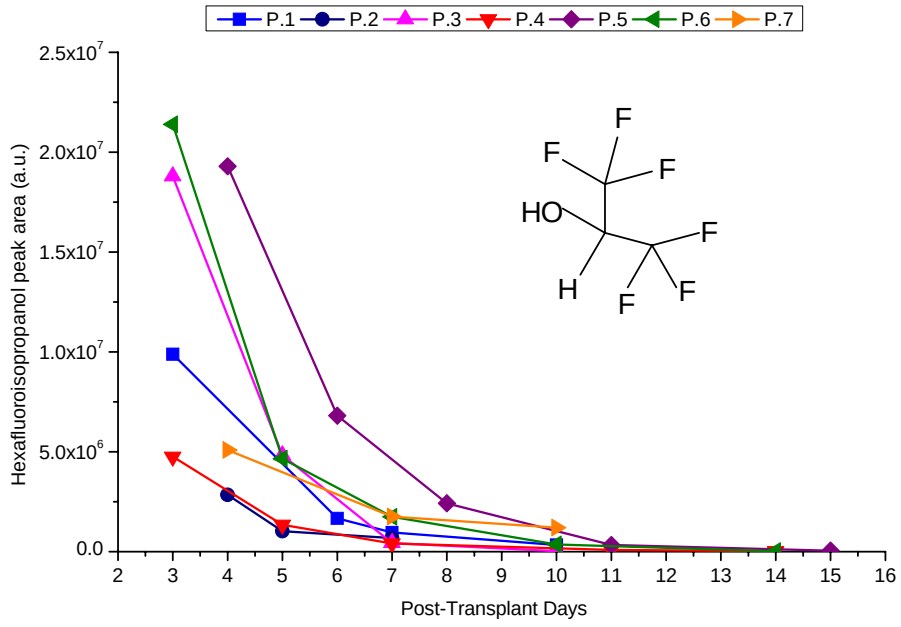

Figure 4.15 *Post liver transplant breath sevoflurane metabolite trend*

These preliminary results highlighted the capacity of this procedure to follow a throughput kinetic (clearance) of an anaesthetic and its metabolite.
Since hepatic metabolism is the principle pathway of drug elimination and more serious hepatic diseases can alter that metabolism, it may be possible to obtain information on the recovery of hepatic functionality, following post-transplant anaesthetic metabolite breath trends.
Obviously the procedure needs to be standardized, the patients' statistics has to be greater and it is necessary to compare the anaesthetic metabolite throughput kinetic of patients with healthy people.
This is interesting if we consider that surgeons have only one way to verify liver rejection crisis: by a biopsy, which is an invasive technique.

4.2.3 Dialysis

The kidney is an organ with many functions: it eliminates hydrophilic metabolites, regulates hormonal functions, maintains the fluid balance and acid-base equilibrium, manages calcium and phosphorus metabolism. Renal insufficiency is the inability of the kidney to purify the blood effectively.
Dialysis is a treatment used to manage renal insufficiency; it removes excess liquids and uremic toxins, assures the fluid balance and acid-base equilibrium, but partially replaces the renal function.
Haemodialysis is a process that needs vascular access and a machine called artificial kidney, which includes the following: peristaltic pump, dialysis filter, dialysis liquid. During haemodialysis the patient's blood is drawn using a peristaltic pump and passed through a filter dipped into the dialysis liquid. There is an exchange of liquids, uremic toxins and electrolytes between the blood and dialysis liquid. At this point the purified blood is returned to the patient. The blood purification occurs thanks to three phenomena which take place in the dialysis filter, where the blood and dialysis solution meet: diffusion, convection and osmosis. A semi permeable membrane (dialysis filter) interfaces the blood to a dialysis solution, which has a specific composition to bring back uremic toxins.

Dialysis filters have pores with different diameters, depending on the membrane type; they permit the transfer of water, small and medium size molecules, but stop large molecules, such as albumin and plasmatic proteins.
Usually a patient needs three treatments per week, each one lasts 3-5 hours. The parameter used to calculate the end of dialysis is the patient's weight loss.
The aim of collecting breath from patients before and after dialysis is to evaluate whether breath composition changes after dialysis, in order to identify an additional parameter to monitor the process. Toxins can be big molecules, such as proteins, but also small, such as volatile compounds. Since blood is in equilibrium with alveolar air, changes in breath content should be possible.

Breath samples from healthy people were also collected to compare compound concentration values of dialyzed patients.
Only breath compounds that had a concentration considerably higher than room air concentration were considered. Compounds present in concentrations higher or comparable with those of the breath sample were excluded.
In the Table 4.5 are shown breath compounds concentration range of healthy people and the relative variation coefficient within the group.

Table 4.5 *Breath concentrations of different compounds in healthy people*

Control group	**Concentration Range [ng/l]**	**Average [ng/l]**	**CV %**
Isoprene	187-471	295	30%
Acetone	378-2093	800	74%
Dimethylsulphyde	2-28	11	81%
Isopropanol	11-31	16	40%
n-propanol	5-34	20	70%
2,3-butandione	1-5	2	53%
3-hydroxy-2-butanone	0.1-2	0.5	81%

Table 4.6 *Breath concentrations of different compounds in patients before and after dialysis*

	P1		P2		P3	
Compounds concentration [ng/l]	**before dalysis**	**after dialysis**	**before dalysis**	**after dialysis**	**before dalysis**	**after dialysis**
Isoprene	110	459	192	309	300	304
Acetone	391	2183	1208	1656	2419	1136
Dimethylsulphide	2,1	6,2	3	4,8	10	3,4
Isopropanolo	43	615	70	430	1363	390
n-propanolo	1,5	24,4	1,5	17,2	19	1,9
2,3-butandione	2,7	6,4	10	4,3	20	2,7
3-hydroxy-2-butanone	4,1	4,9	13	2,1	33	0,7

Table 4.6 (a) *Breath concentrations of different compounds in patients before and after dialysis*

	P4		P5	
Compounds concentration [ng/l]	**before dalysis**	**after dialysis**	**before dalysis**	**after dialysis**
Isoprene	250	270	444	764
Acetone	1298	664	3682	2220
Dimethylsulphide	8	1,0	26	33,2
Isopropanolo	313	277	1962	655
n-propanolo	3,7	0,0	48	49,7
2,3-butandione	19	2,6	62	24,0
3-hydroxy-2-butanone	13	0,6	59	46,7

In contrast with our expectation, it was not generally possible to observe a decrease in compound breath concentrations after the dialysis. Breaths of different patients seemed to have a more similar composition after treatment, and for some compounds a minor dispersion of data was observed. This could be due to the fact that after four hours of dialysis, their blood had been cleaned, more or less in the same way, and they breathed the same air. In fact the room air was not changed during the dialysis, so for four hours the patient breathed in what they had breathed out.

There were only two compounds that decreased after the dialysis, 2,3-butandione and 3-hydroxy-2-butanone. (Figure 4.16 and 4.17) These two compounds should become an additional parameter to follow the dialysis trend, but to confirm that, a major number of

measures have to be done and it also important to understand which metabolic pathway they come from.

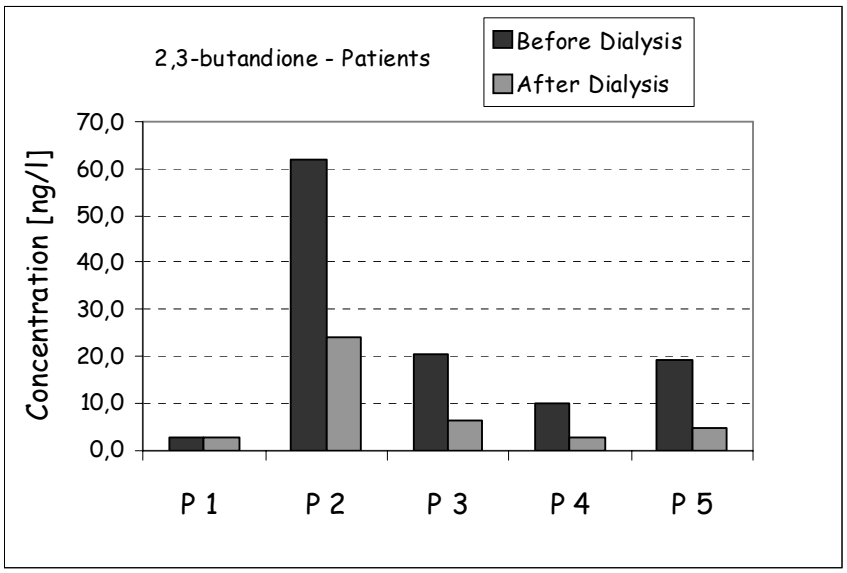

Figure 4.16 *2,3-butandione concentration in patient breath before and after dialysis*

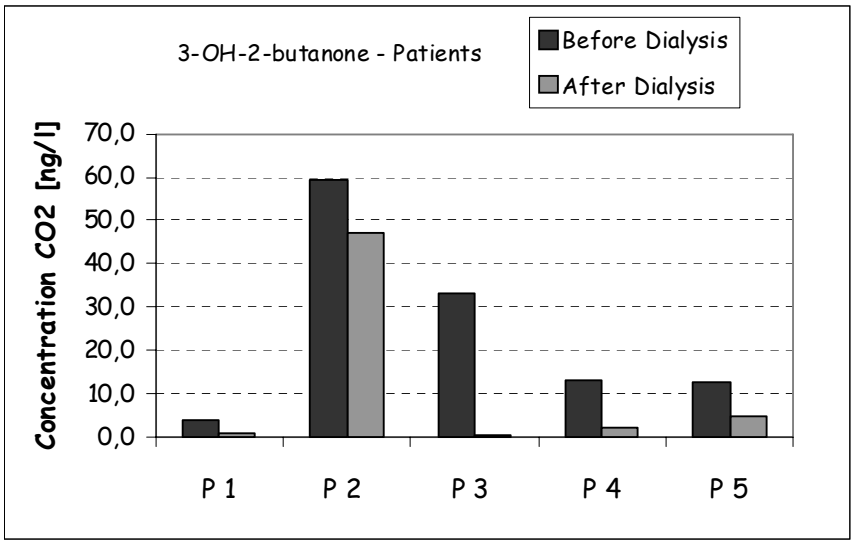

Figure 4.17 *3-hydroxy-2-butanone concentrations in patients breath before and after dialysis*

In order to compare data, a statistical analysis was made. The t-Student test was applied to concentration values of each endogenous compound to estimate if there was a significant difference between the control group and patients before and after dialysis treatment. (Tables 4.7 and 4.8)

Table 4.7 *t-Student test between healthy people and patients before dialysis*

	Control group Vs Patients before dialysis			
Compound	***t***	***Degrees of freedom***	***P (significance level)***	***Differences between observed averages p<0.05***
Isoprene	0.3541	9	0.7314	NO
Acetone	1.2590	9	0.2397	NO
Dimethylsulfide	0.1292	9	0.9001	NO
Isopropanol	2.6128	9	0.0281	YES
n-propanol	0.2673	9	0.7952	NO
2,3-butandione	2.0519	9	0.0704	YES
3-hydroxy-2-butanone	2.9306	9	0.0167	YES

Table 4.8 *t-Student test between healthy people and patients after dialysis*

	Control group Vs Patients after dialysis			
Compound	***t***	***Degrees of freedom***	***P (significance level)***	***Differences between observed averages p<0.05***
Isoprene	0.5156	9	0.6186	NO
Acetone	1.9184	9	0.0873	NO
Dimethylsulfide	0.0464	9	0.9640	NO
Isopropanol	8.7704	9	0.0000	YES
n-propanol	0.0890	9	0.9310	NO
2,3-butandione	1.5264	9	0.1613	NO
3-hydroxy-2-butanone	1.4333	9	0.1856	NO

For most of the compounds significant differences were not found between healthy people and patients. Isopropanol seemed to be higher in dialyzed patients but only because this compound was present as a contaminant in the air of the dialysis room. Acetone, as demonstrated in paragraph 4.2.1, is closely related to isopropanol so cannot be considered.

The t-test for 2,3-butandione and 3-hydroxy-2-butanone supported our hypothesis confirming significant differences between breath concentrations in patients and healthy people before the dialysis and no important differences after.

CHAPTER 5

Future Developments

5.1 Breath sampling system

In the light of the results obtained so far, it is clear that a breath collection protocol is needed. In fact the literature and preliminary measurements highlight that breath intra and inter-variability is high, even in healthy subjects.

In terms of respiration physiology, the volume of expired air can be divided in two fractions: dead volume and alveolar air. The former is the air that is inhaled into the body when breathing, but is not involved in gas exchange with blood; the latter is the air in the pulmonary alveoli and alveolar sacs, where there are oxygen-carbon dioxide exchanges with pulmonary capillary blood.[121]

Endogenous and exogenous compounds are distributed within these two fractions.

Relevant information can be obtained not only from the compounds present in the alveolar air in blood, but also from the compounds produced in tissues and mucosa of superior and inferior airways, as in the dead volume.

This considerations lead to the conclusion that the separate sampling of different breath fractions could provide a valuable contribution to breath research. When interest is focused on blood/air gas exchanges at an alveolar level, a further advantage of separate sampling is that it avoids diluting the end-tidal with dead space air. Such a dilution varies among different individuals and also in the same individual depending on sampling conditions. Thus its variability can prevent a correct quantification of compound concentrations in end-tidal air and alter the multivariate data patterns obtained in measurement campaigns[42]. A better reproducibility of data is obtained when only the end-tidal fraction of breath is analysed[122]. Compounds with an alveolar origin give higher concentrations in end-tidal samples than in mixed expiratory samples. This is a positive effect since low concentration levels of breath markers are always a challenge from an analysis point of view[6].

A prototype for breath sampling (Breath Sampler) was designed and produced in collaboration with Loccioni, in Jesi (Ancona, Italy) a company which has worked on the same problems.

This system is based on the same principle as Schubert's,[67] but with different modalities. In fact in Schubert's system, an infra red sensor sends data to a control system connected to a two way valve. This valve changes the flow direction when the percentage CO_2 volume exceeds the set value, directing the sample to an adsorbent trap. In the prototype, breath is sampled in a bag and the pre-concentration of analytes in an absorption tube is then performed off line. The system integrates a flow meter and a carbon dioxide measurement unit based on wavelength modulation spectroscopy. Flow direction is controlled by a system of valves that can be triggered by the attainment of either volume or carbon dioxide concentration values. A breath by breath calculation of the volume of anatomical dead space is achieved using an algorithm implementing Fowler's method, while end-tidal CO_2 concentration values can be inferred from the CO_2 concentration data. The system enables large volumes to be sampled on multiple breaths. (Figure 5.1)

Figure 5.1 *Breath Sampler prototype*

5.1.1 Description of the breath sampler prototype

The prototype was designed to collect different breath fractions separately (dead volume and end-tidal) according to a series of demanding constraints. Minimum pressure losses (a few ml of water) are required so that subjects do not have to make any effort when blowing and the subject's safety has to be ensured from a bacteriological and electrical point of view. For the construction, suitable materials were chosen to limit the contamination of breath samples, both in terms of the release of chemical substances from the wetted parts of various components and in terms of memory effects related to cross contamination between different samples (absorption/desorption of breath compounds onto wetted internal surfaces). Fast sensors control the valves, which enable breath fractions to be separated into different sampling bags.

The interaction of a subject with a machine tends to alter normal respiration, probably because the control of respiratory functions changes from the involuntary control centre to the voluntary one. Such an alteration also affects dead volume space, which can also vary a lot in the same person[123, 124, 125]. Thus for accurate breath sampling on multiple breaths, the volume of dead space for every single action needs to be calculated.

In addition it is important to hold the entire system at 40 °C to avoid water condensation. The schematic diagram in Figure 5.2 represents the sampling system.

The subject breathes into the sampler through a mouthpiece and an antibacterial filter, connected to the carbon dioxide sensor and to the mass flow. (Figure 5.4 a)

Carbon dioxide concentration is measured by a system based on *Wavelength Modulation Spectroscopy (WMS)*, which is a very efficient optical technique in gas determination. The CO_2 analyzer (laser diode with wavelength emissions at 2004 nm) and the flow meter measure in real time (20-50 measures per second) the CO_2 breath concentration and the flow respectively, in order to accurately draw the curve of CO_2 concentrations as a function of the expired volume.

Data are obtained on a computer using an acquisition card. The typical CO_2 waveform of concentration graphic versus time is shown in Figure 5.3.

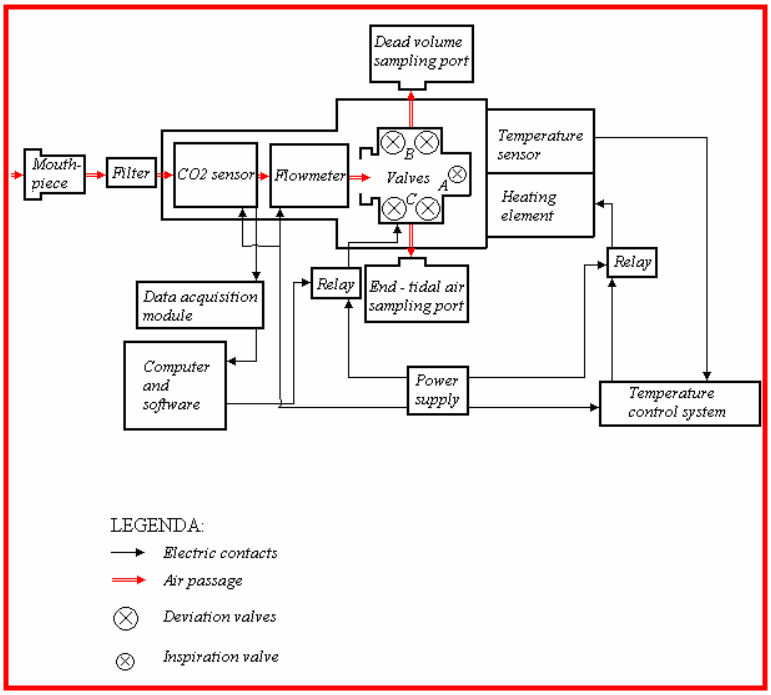

Figure 5.2 *Schematic diagram of the Breath Sampler*[42]

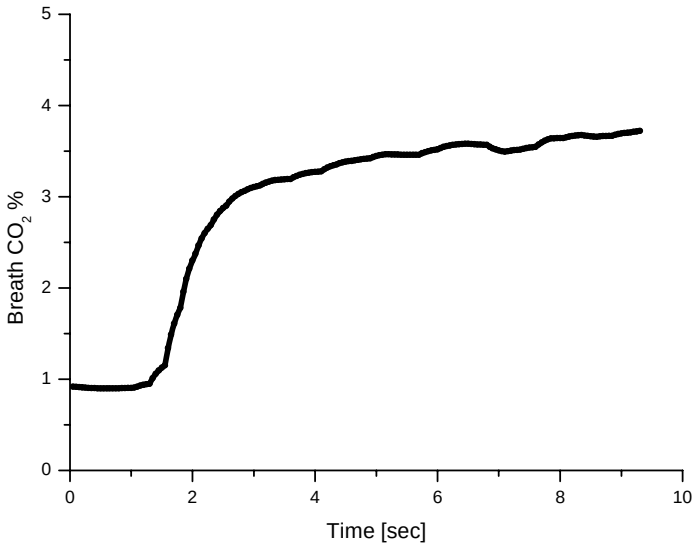

Figure 5.3 *CO_2 % trend in time during expiration*

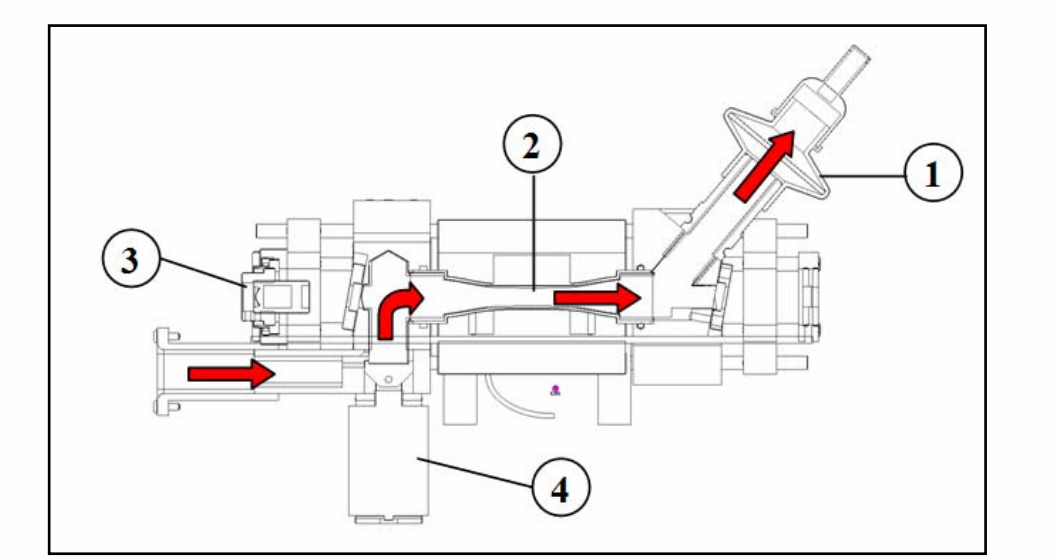

Figure 5.4 (a) *Schematic drawing of Breath Sampler: the arrows show the air flow direction during inspiration. (1) antibacterial filter, (2) flow sensor, (3) laser diode, (4) entrance valve of ambient air*[42]

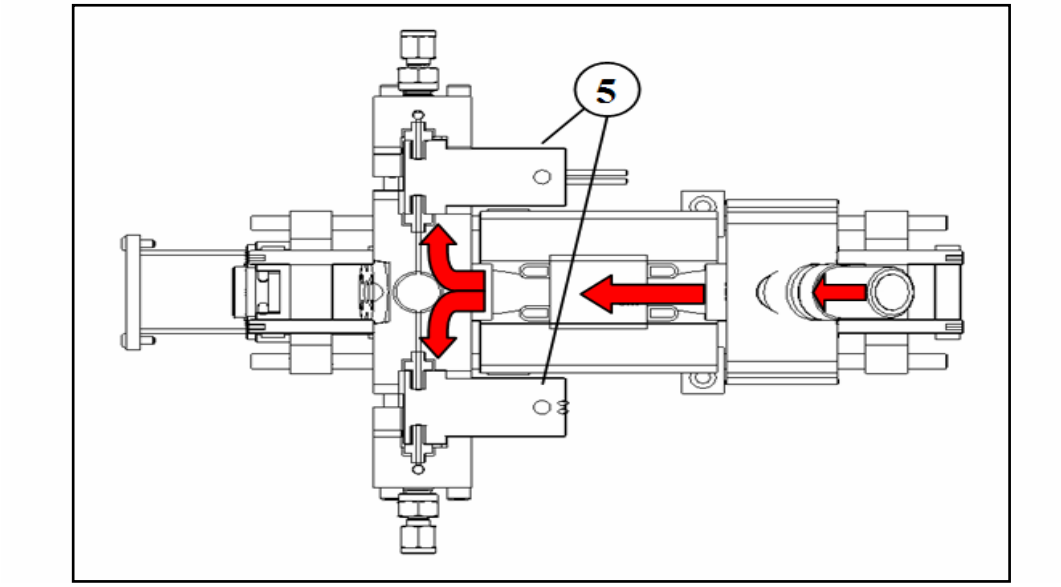

Figure 5.4 (b) *Schematic drawing of Breath Sampler: the arrows show the air flow direction during expiration. (5) bag connectors; (A, B, C) the valves open during dead volume, end-tidal collection and inspiration*[42]

During subject respiration the sampler can be in three positions: (Figure 5.4 b)

1. Inspiration: Valve C open, valves A and B closed;
2. Dead volume collection: Valve A open, valves B and C closed;
3. End-tidal collection: Valve B open, valves A and C closed

The solenoid valve C opens to enable ambient air to enter the instrument during inspiration, valves A and B open during expiration and control the collection of dead volume air or end-tidal air respectively.

The system is controlled by a dedicated software that enables the operator to calibrate flow and CO_2 sensors, to select different collection modalities, and lastly to visualize and save exit data (CO_2 concentration, expiration flow and volume values), with a graphic interface.

There are three different ways to close valve A and at the same time open valve B:

1. *CO_2 value modality*: attainment of a set-point value for the absolute concentration of CO_2;
2. *CO_2 fraction modality:* attainment of a user-defined percentage of end-tidal CO_2 concentration, obtained from the product between $CO_{2\ MAX}$ and α, where α is a parameter chosen by the operator, and represents a fraction between 0 and 1 of the CO_2 maximum value recorded in the last respiration cycle;
3. *Fowler method modality:* attainment of a specific value of expired volume calculated according to the Fowler method from relevant data from the last respiration cycle, corresponding to anatomic dead space volume[49].

Fowler's manual estimation of anatomic dead space relies on a tedious graphical procedure prone to inaccuracies and errors.

More recently, an algorithm was proposed for use with real-time computer-assisted determinations[126], but this approach turned out to be too time consuming for breath-by-breath calculations. We have developed an algorithm that implements Fowler's method in only few steps.

The difference between the areas of shaded regions A and B is calculated by subtracting the area of the baseline rectangle *abcd* from the area of the region *abce* below the curve of CO_2 concentration versus expired volume and subtracting the area of the rectangle *hdfe* and adding the area of the triangle *gef* to the previous result. (Figure 5.5)

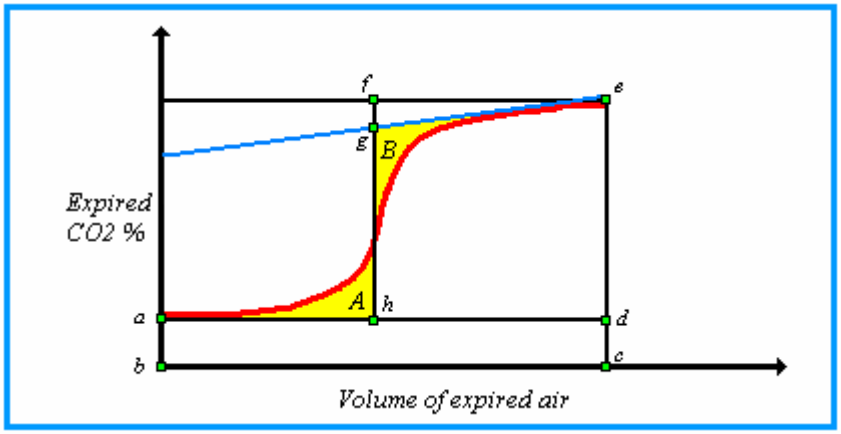

Figure 5.5 *Graphic illustration of the algorithm for the implementation of Fowler's method.*

The efficiency of the algorithm used to calculate the anatomic dead space volume was determined comparing the results from a number of single breaths with the values obtained manually calculating the two areas A and B using the graphic method.

In Fowler's manual estimation using a graphical approach, the human assessor draws a straight line by fitting the plateau of the curve of CO_2 concentration versus expired volume; then he/she moves the vertical line back and forth, limiting the shaded regions *A* and *B* until they have the same area. (Figure 5.5)[42]

There is a margin of arbitrariness in the drawing of the straight line, which leads to a spread in results obtained by different assessors (or even by the same assessor in repeated estimates).

Table 5.1 *Comparison of anatomical dead space estimations in data of single breaths obtained from five test subjects: proposed algorithm, graphical method (average ± standard deviation), absolute and percentage deviations. The standard deviation accounts for the variability due to manual estimations of four assessors on the same data.*

	Dead SpaceVolume (ml)			
Subject ID	**Algorithm**	**Manual**	**Absolute deviation (ml)**	**Percentage deviation (%)**
1	232	237 ± 8,5	5	2
2	144	140 ± 3,5	-4	3
3	151	140 ± 3,5	-11	8
4	121	125 ± 5	4	3
5	179	170 ± 8	-9	5

As shown in Table 5.1 the two data sets were in good agreement and did not present significant differences.

5.1.2 Preliminary measurements on the Breath Sampler performance

Firstly the flow meter and the CO_2 sensor were calibrated. The flow meter was calibrated using a primary flow calibrator, while the CO_2 sensor was calibrated using a certified 5% v/v CO_2 gaseous mixture in nitrogen.

The calibration procedure was conducted as follows:

- Purified air (without CO_2) was flowed through the sampler and the signal value was set to zero;
- The 5% v/v CO_2 gaseous mixture was flowed through the sampler and the signal was set to 5%.

The profiles of CO_2, flow and expired volume curves versus time were then observed for consecutive breaths. (Figures 5.6 a, b, c)

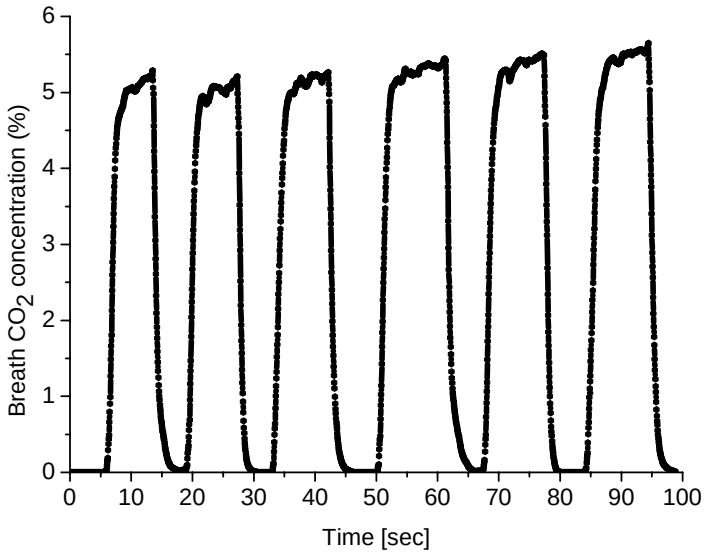

Figure 5.6 (a) *CO_2 percentage trend in time for consecutive breaths*

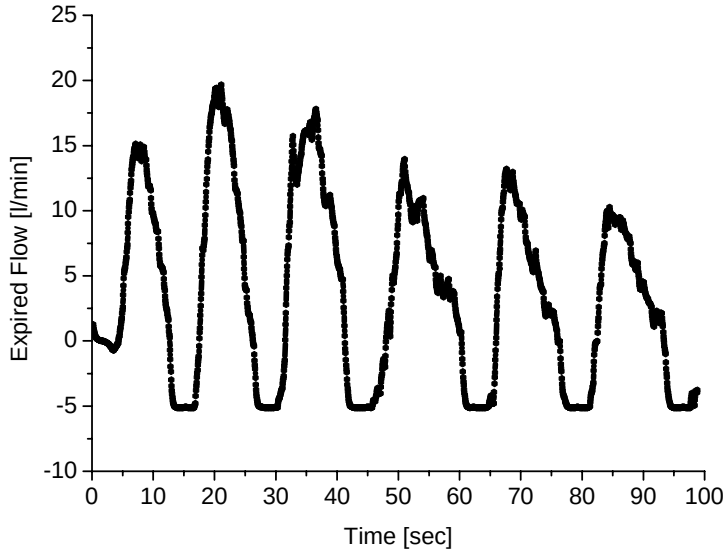

Figure 5.6 (b) *Flow trend in time for consecutive breaths*

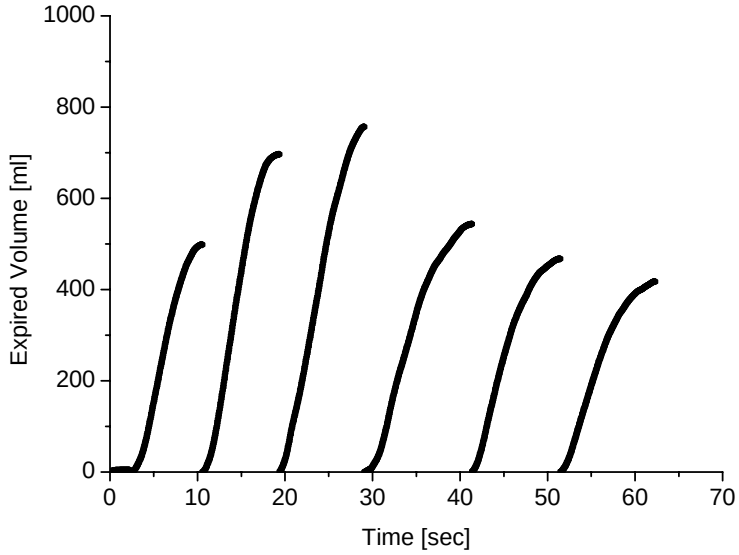

Figure 5.6 (c) *Volume trend in time for consecutive breaths*

The data shows that during spontaneous respiration the flow has an irregular trend and that a slight increase in CO_2 concentration corresponds to a decrease in ventilation in the last three respiration cycles. (Figure 5.6 c)

Better regularity in respiration could be obtained by giving the subject some feedback to help them control respiration frequency and volume44. For this reason, in the latest version of the software a metronomic signal to synchronize respiration and a graphic visualizer of volume in time were added, in order to maintain constant tidal volume.

Any sample contamination by the instrument material was evaluated by comparing the chemical composition of purified air, with the same air passed through the sampler, both collected in Nalophan bags. (Figure 5.7)

The samples were transferred from the bag to the desorption tube packed with Tenax GR and analyzed by TD/GC-MS. (MTD 3, Table 2.11)

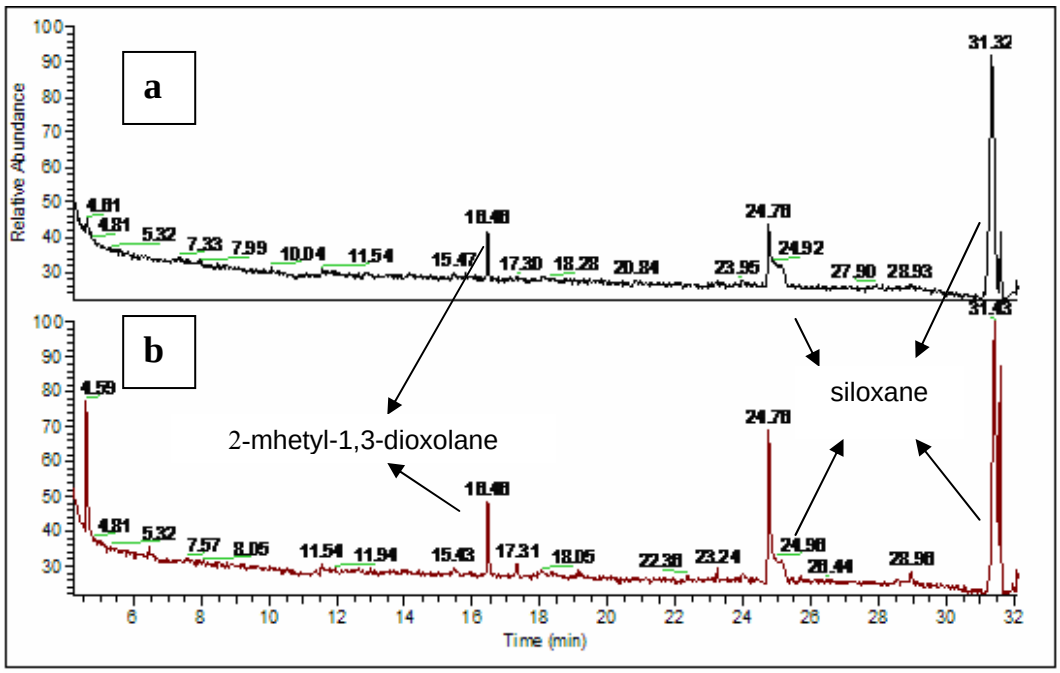

Figure 5.7 *Comparison between the chromatogram of purified air (a) and the chromatogram of purified air passed through the Breath Sampler (b) and collected in a Nalophan bag*

There were no substantial differences in the two chromatograms, the major signals being at RT=16.46 min, corresponding to 2-methyl-1.3-dioxolane, a compound released from the Nalophan bag and to RT= 24.78 min and RT=31.32 min, which corresponds to siloxane from chromatographic column purging.

Signals at the retention time of the characteristic compounds present in breath were not observed. This means that the Breath Sampler does not release compounds at measurable concentrations at least in terms of the experimental condition used.

5.1.3 Breath collection by the Breath Sampler

Tests to sample different fractions of breath were carried out in a healthy subject. In order to simplify and accelerate the experimental work, only isoprene and acetone were considered.

The subject breathed spontaneously into the Breath Sampler and the two breath fractions were collected in two different Nalophan bags. The *"CO_2 fraction modality"* was used, setting up α= 0.8. One bag was filled with dead volume and the other one with the end-tidal. A third bag was filled by the subject breathing directly into the bag, in this way a mixed breath sample was obtained.

A comparison between the chromatograms obtained is reported in Figures 5.7a and 5.7b.

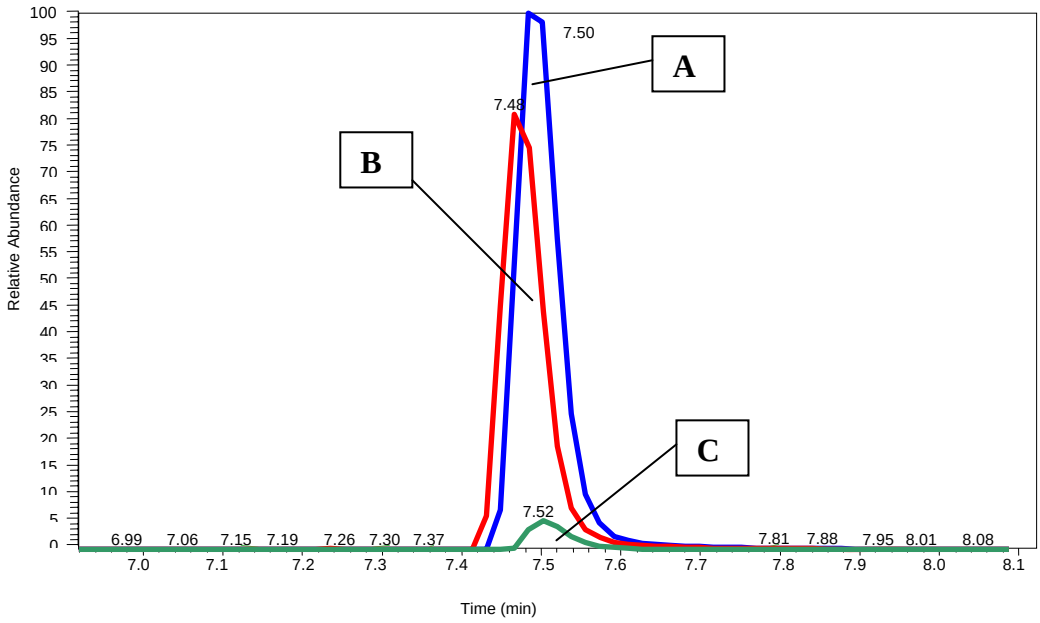

Figure 5.7 (a) *Chromatographic peak of acetone (EI, m/z=58) obtained sampling different fractions of breath: A) relates to the end-tidal portion; B) relates to the mixed breath sample; C) relates to the dead volume fraction.*

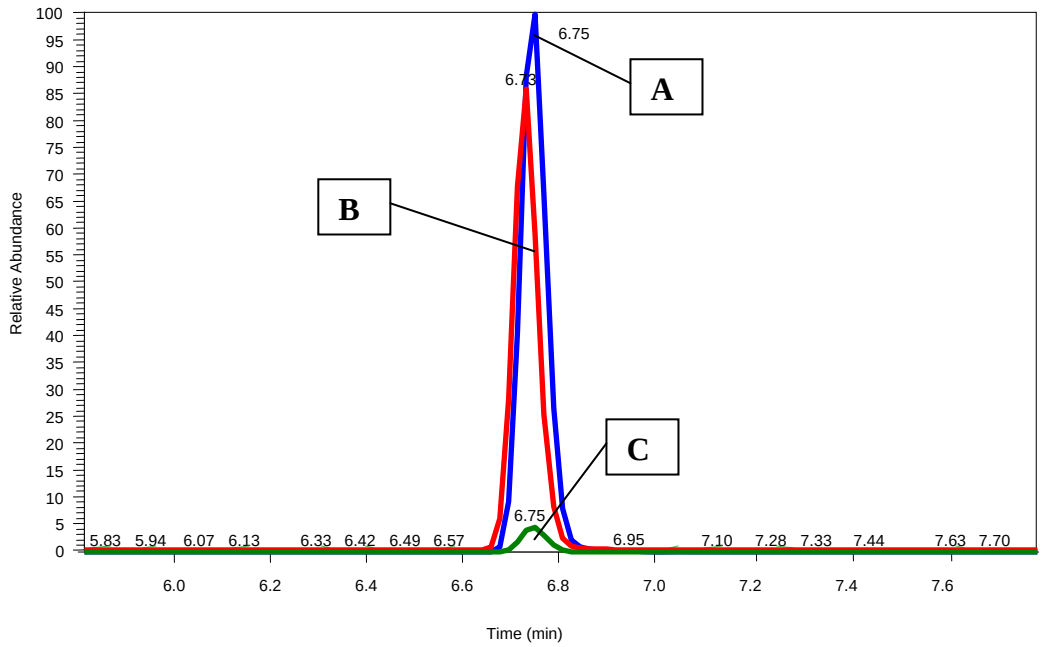

Figure 5.7 (b) *Chromatographic peak of isoprene (EI, m/z=67) obtained sampling different fractions of breath: A) relates to the end-tidal portion; B) relates to the mixed breath sample; C) relates to the dead volume fraction.*

These preliminary results showed that the amount of acetone and isoprene in end-tidal sample was considerably higher than the amount in the dead volume sample and was nearly equal to the mixed breath sample. In the end-tidal sample there was a 20 % higher amount of acetone compared to the mixed breath sample and a 70 % higher amount compared to the dead volume sample. Instead the increases in isoprene on the other hand were 29 % and 80 % respectively.

This demonstrates the capacity of the instrument to separate the two breath fractions. In this case the end-tidal content of acetone and isoprene is similar to the mixed breath sample content, due to the low presence of these two compounds in the dead volume. However it is not possible to know this information a priori, therefore the best solution is to sample the end-tidal separately, also to reduce ambient air contamination.

In order to work out if different ways of breathing could change breath composition, systematic measurements were made.

A volunteer was asked to breathe in three different modalities, independently, at close intervals in a time span of one hour:

A) Spontaneously breathing;

B) Controlled breathing: 3 sec of inspiration and 3 sec of expiration;[126]

C) Deep breathing: an attempt to completely empty the lung for each breath.

The $\%CO_2$ breath trend in time and the $CO_2\%$ maximum value reached for each breath, for the three different breathing modalities are shown in the figures below.

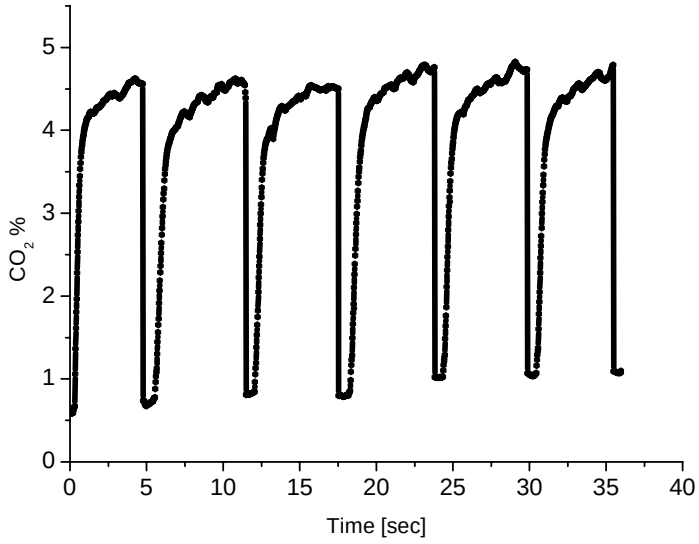

Figure 5.8 (a) *$\%CO_2$ trend in time: Breathing modality A*

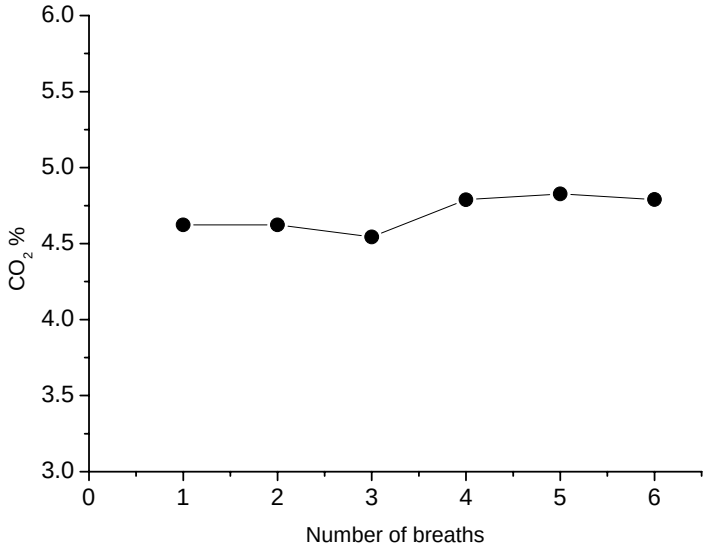

Figure 5.8 (b) *$\%CO_2$ maximum value for each breaths: Breathing modality A*

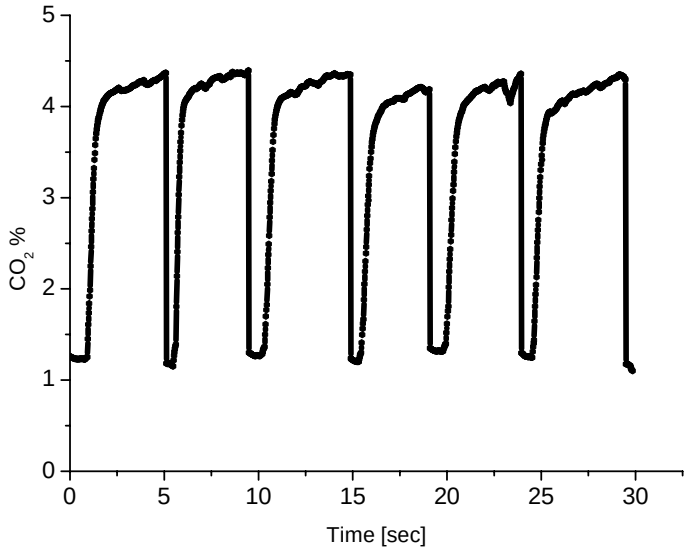

Figure 5.9 (a) *% CO_2 trend in time: breathing modality B*

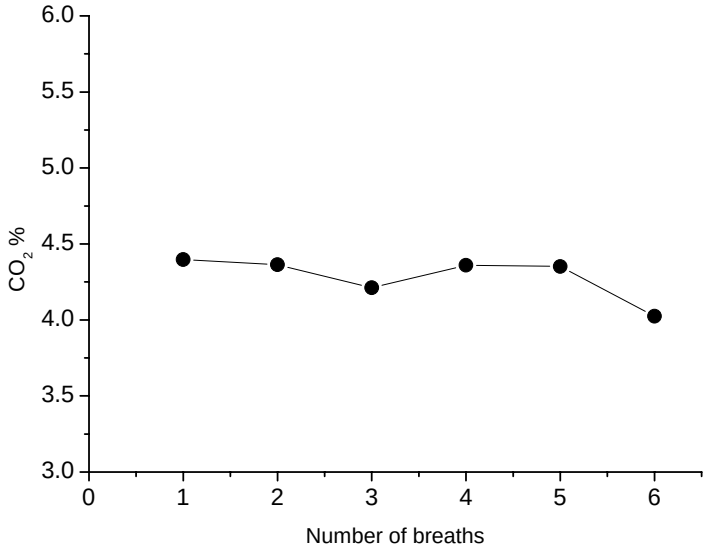

Figure 5.9 (b) *%CO_2 maximum value for each breathes: breathing modality B*

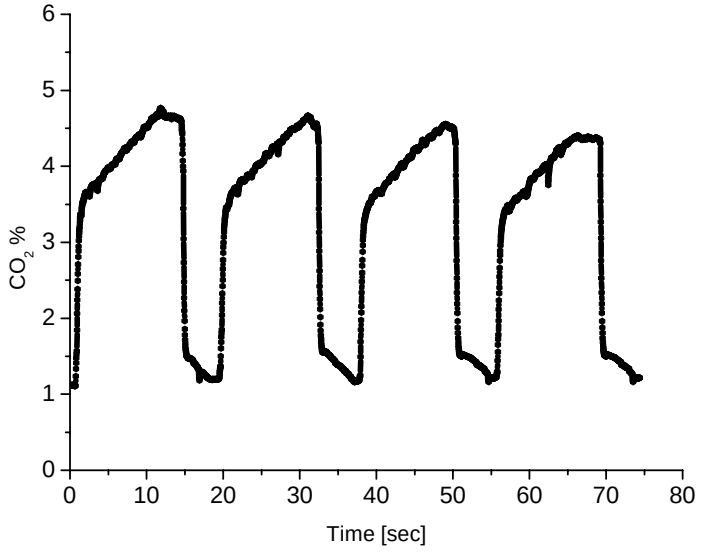

Figure 5.10 (a) *%CO_2 trend in time: breathing modality C*

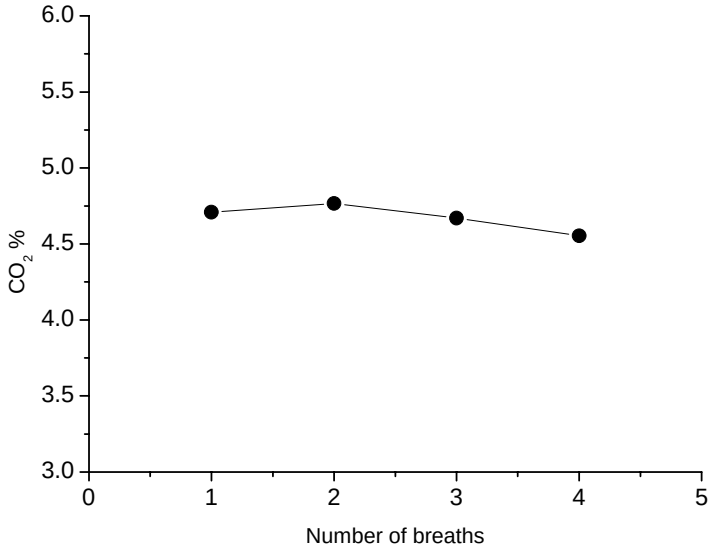

Figure 5.10 (b) *% CO_2 maximum value for each breathes: breathing modality C*

For the compounds considered the three different breathing modalities do not affect CO_2 breath content, as is highlighted by the mean breath CO_2 value and the relative %RSD.

The data in Table 5.2 show the concentration of the compounds present in breath samples obtained with the different breathing modalities.

Table 5.2 *Comparison between the compound concentrations in breath samples, obtained breathing in three different modalities*

	Concentration [ng/l]				
Compounds	***A modality***	***B modality***	***C modality***	***Mean***	***%RSD***
Ethanol	40	30	30	30	7%
Isoprene	360	430	500	430	16%
Acetone	600	800	1000	800	20%
DMS	28	35	27	30	14%
Isopropanol	11	12	10	11	9%
n-propanol	5	5	5	5	13%
Pentane	3.0	4.0	3.0	3.0	16%
Hexane	5.0	5.0	4.0	5.0	10%
CO_2 % bag content	4.2%	3.7%	3.9%	3.9%	7%

The breathing modality does not seem to affect breath compound content, as the data are in agreement with a mean RSD% equal to 13 %.

These results are in contrast with those reported in the literature[126], and also with our expectations.

This may be due to several factors regarding the Breath Sampler prototype. From the CO_2 breath profile it is possible to see that the CO_2 value between breaths does not return to zero. This is probably due to the fact that part of the internal volume of the sampler is poorly ventilated. Laser instability was another of the problems we encountered; sometimes during breath sampling the CO_2 signal was lost and the valves started to open and close randomly. In this case there was no certainty with regard to the composition of the collected sample.

From these preliminary results it is clear that some problems need to be solved in order to collect different breath fractions.

Another of the major problems encountered, which caused the exclusion of most of the measurements made, was ambient air contamination. It is very important to verify ambient air composition before colleting breath samples; if in the ambient air the concentration of some compounds of interest is higher than in breath samples the interpretation of the results may be wrong.

The discussion on the best solution to this problem still divides the scientific community. Some suggest that each compound should be weighed on the basis of its concentration gradient between breath and ambient air [57]. Others maintain that compounds whose concentration in ambient air is comparable or higher than in breath should not be taken into account in the characterization of subjects [58]. Finally others

suggest that the subjects should be provided with purified air in order to circumvent the problem[59, 60]. In fact, this last solution is also disputable. It has been proved that different compounds have different washout periods ranging from minutes to hours[61], which restricts the usefulness of purified air delivery for the removal of exogenous compounds to a very limited number of cases.

All these considerations confirm the importance of standardizing breath collection and separating dead volume from end tidal.
On the basis of the results obtained a new Breath Sampler is in the planning stage in order to solve the problems encountered. Breath will be collected directly on the desorption tube, a different kind of detector technology will be used and most importantly the internal structure will be modified.

5.2 Towards a different breath sample collection

Recently there has been increasing interest in Exhaled Breath Condensate (EBC) collection. EBC contains several components; the principal component being condensed water vapour.[127] This fluid represents nearly all of the volume (>99%) of fluid collected in EBC.[128] Only a small fraction of the condensate is derived from respiratory droplets that are released by turbulent airflow, and possibly by other currently not completely understood mechanisms. This fraction can be added to the water vapour from anywhere between the alveoli and the mouth.[129] Therefore, in EBC samples not only volatiles are found, but also several other mediators with no volatile characteristics (which can be both hydrophobic and water-soluble molecules) including adenosine, isoprostanes, leukotrienes, peptides, cytokines, protons and various ions[130, 131].

During my PhD I spent two months at the U.S. Environmental Protection Agency (EPA) in Durham (NC, USA) in Dr. Joachim Pleil's laboratory. The aim of my research was to optimize the analytical methodology for measuring polar volatile organic compounds (PVOCs) in EBC samples, in order to detect metabolic responses to environmental stressors. In fact environmental exposures, individual activities, and

disease states can perturb normal metabolic processes and be expressed as a change in the patterns of polar volatile organic compounds present in biological fluids.

A simple procedure to measure PVOCs in aqueous media was developed and the precision and sensitivity of the method were assessed. Lastly different sampling methods were compared and applied to a series of human EBC samples to assess the variance within a small group of volunteers.

5.2.1 Method development

Neat aldehydes and neat alcohol standards, purchased from Accustandard, were used to prepare stock solutions. All standards and blanks were prepared using high purity de-ionized water. Initial experiments were conducted to assess mass spectrometry parameters.

The compounds used to prepare the stock solutions are reported in Table 5.3.

Table 5.3 *Compounds used to prepare stock solutions; 3-hexanol is the internal standard*

Compounds	**Density (mg/μl)**
2-methyl propanal	0.793
butanal	0.802
3-methyl butanal	0.796
pentanal	0.818
1-propanol	0.803
hexanal	0.814
2-methyl 1-propanol	0.802
3-methyl 3-pentanol	0.827
1-butanol	0.810
4-methyl 2-pentanol	0.807
heptanal	0.817
1-pentanol	0.815
octanal	0.821
2-ethyl 1-butanol	0.833
1-hexanol	0.819
1-heptanol	0.823
Average density	0.812

Compounds (IST)	**Density (mg/μl)**
3-hexanol	0.814

The Stock solutions were prepared as follows:

Table 5.4 *Stock solution preparation*

Stock solution	Procedure	Concentration
Stock A	2 µl of each neat compound in 20 ml of water	0.1 µl/ml
Stock B	2 ml of Stock A in 18 ml of water	0.01 µl/ml
IST* Stock A	1 µl of neat 3-hexanol compound in 8 ml of water	0.125 µl/ml
IST* Stock B	2 ml of IST Stock A in 6 ml of water	0.0031 µl/ml

*Internal Standard

Three replicates of a standard solution were run in scan method. Three glass bulbs and caps were prepared by washing in a dishwasher and three Tenax TA tubes were conditioned, purging with Helium (100 ml/min), at 290°C for two hours. Clean bulbs were placed on a slanted rack; ~ 700 mg of NaCl were added, followed by a Tenax TA tube.

1.5 ml of a 200 ppb standard solution prepared from Stock B, was injected into the bulbs.

Samples were allowed to equilibrate with their respective Tenax tubes for 24 hours at room temperature. After 24 hours the samples were analyzed by TD/GC-MS. Chromatographic, mass spectrometric and thermal desorption methods are described in Table 5.5 and 5.5 (a).

Table 5.5 *Chromatographic, mass spectrometric and thermal desorption method*

MTD 4	
Chromatographic method	
Inlet Temperature	250 °C
Oven temperature ramps	40°C for 2 min; 10 °C/min until 250°C for 8 min
Column head Pressure	15 psi
Inlet mode	splitless
Transfer line temperature	280 °C
Column	HP-5MS from Restek (30 m x 0.25 mm x 0.25 µm)
Mass Spectrometric method	
Ion source temperature	250 °C
Scan mode	SIM
Solvent delay	3.5 min

Table 5.5 (a) *Chromatographic, mass spectrometric and thermal desorption method*

Thermal Desorption method	
	Tenax TA
Internal trap	Water Management from Markes
Valve and transfer line temperature	150 °C
Dry purge	3 min at ambient temperature
Tube desorption Temperature	260 °C
Tube desorption Time	10 min
Trap focusing Temperature	0 °C
Trap desorption Temperature	310 °C
Trap desorption Time	3 min

From the chromatograms two ion fragment masses were chosen for each compound and a SIM method was carried out as follows

Table 5.6 *Characteristic ions in SIM mass spectra*

Group No.	**RT**	***ion***						*ion n°*	*Dwell Time*
1	3.50-8.00	42	43	44	58	59	72	6	80
2	8.00-9.30	43	55	56	72	73	74	6	80
3	9.30-10.80	41 69	44 70	45 87	55 96	56	59	10	70
4	10.80-12.50	42	43	55	57	70	84	6	80
5	12.5	43	56	69	70			4	100

Table 5.7 *Compounds present in each group*

Group No.	**Compounds**
1	2-methyl propanal; butanal; 3-methyl butanal; pentanal; 1-propanol
2	hexanal; 2-methyl 1-propanol; 3-methyl 3-pentanol;
3	1-butanol; 4-methyl 2-pentanol; heptanal; 3-hexanol
4	1-pentanol; octanal; 2-ethyl 1-butanol
5	1-hexanol; 1-heptanol

Once the method parameters were defined, the linear concentration range was assessed using multi-point calibration.

In order to prepare the calibration solutions, Stock B was diluted as follows:

Table 5.8 *Calibration solution schedule preparation.*

	Blank	**Step 1**	**Step 2**	**Step 3**	**Step 4**	**Step 5**
Stock B (μl)	0	30	100	100	100	100
Water (ml)	5	30	25	12.5	8	5
Internal Std B (μl)	15	90	75	37.5	24	15
Conc IST (ppb)	93	93	93	93	93	93
*Conc Std (ppb)**	0	10	40	80	125	200

*ρ_{std} was only used for the Stock A calculation. All others were majority water

Calibration results in terms of R squared, calculated on the area counts corrected for the internal standard are reported in Table 5.9.

Table 5.9 *Calibration results in terms of R squared*

Compounds	***Slope***	***Intercept***	**R squared**
2-methyl_propanal	7.63E-04	1.11E-03	0.997
butanal	7.19E-04	3.51E-03	0.985
3-methylbutanal	1.21E-03	7.80E-04	0.996
pentanal	1.37E-03	8.61E-03	0.990
1-propanol	5.07E-04	-2.49E-03	0.959
hexanal	1.15E-03	1.30E-02	0.988
2-methyl-1-propanol	2.82E-03	-7.04E-03	0.994
3-methyl-3-pentanol	8.64E-03	2.48E-02	0.997
1-butanol	3.55E-03	-1.45E-03	0.976
4-methyl-2-pentanol	7.60E-03	3.42E-02	0.993
heptanal	9.24E-04	9.58E-03	0.990
1-pentanol	3.15E-03	-5.68E-03	0.991
octanal	5.34E-04	1.70E-02	0.916
2-ethyl-1-butanol	4.95E-03	1.23E-02	0.995
1-hexanol	6.81E-03	-7.93E-03	0.989
1-heptanol	3.98E-03	-9.79E-03	0.961

The response is linear within the concentration range considered.

In order to evaluate the precision of the analytical procedure, 10 replicates of two different standard solutions were run.

The estimated method precision expressed as a percentage relative standard deviation (%RSD) at two spike levels is reported in the following table.

Table 5.10 *RSD% for 10 replicates at two spike levels*

	% RSD 5ppb	**% RSD 100ppb**
2-methyl_propanal	12%	10%
butanal	17%	16%
3-methylbutanal	21%	17%
pentanal	18%	18%
1-propanol	nd	18%
hexanal	38%	33%
2-methyl-1-propanol	41%	30%
3-methyl-3-pentanol	28%	32%
1-butanol	22%	35%
4-methyl-2-pentanol	41%	34%
heptanal	34%	35%
1-pentanol	35%	25%
octanal	31%	20%
2-ethyl-1-butanol	43%	28%
1-hexanol	38%	32%
1-heptanol	30%	29%
Mean RSD%	**30%**	**26%**

The reproducibility of the method was around 30%, which is good if we consider the low standard solution concentrations, which are representative of the breath condensate compound concentrations.

5.2.2 Exhaled breath condensate collection device

Two different EBC collection devices were tested in order to assess EBC volume recovery.

1) EBC samples were collected using a modified R-Tube (Respiratory Research, Inc. Charlottesville, VA). The original product was modified and the new one consisted of a non-rebreathing valve and a 22 cm length x 2.6 cm diameter tube (Figure 5.11). An 8 cm diameter co-axial container (Figure 5.12) surrounding the R-Tube was filled with granulated dry ice (-80°C) to chill the R-tube.

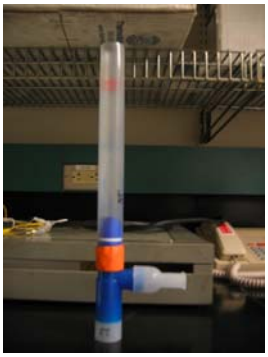

Figure 5.11 *R-Tube*

Figure 5.12 *Modified container to chill the R-Tube*

A seated subject performed the tidal breathing manoeuvre through the device for 10 min. Upon completion, the R-tube was removed from the valve, capped at both ends, extracted from the chilling container and allowed to warm to room temperature. The melted EBC was consolidated from the walls of the R-tube using an internal o-ring plunger and transferred immediately with a calibrated glass syringe to a 4 ml vial, held at -80°C.[132]

2) A second EBC collection device developed in the EPA laboratory was also used. Breathing manoeuvres were the same as for the R-tube, whereas with this device, a 150 cm length x 2 cm diameter Tygon tube immersed in dry ice was used. (Figure 5.13)

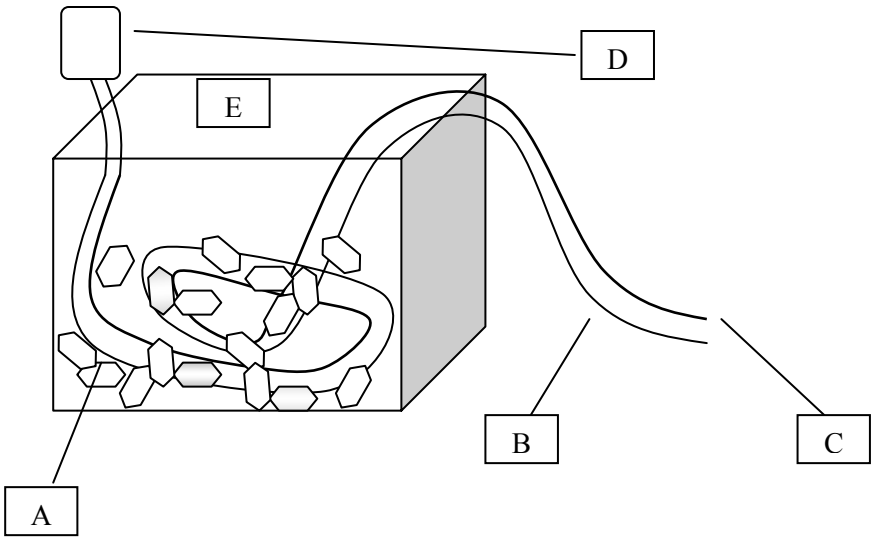

Figure 5.13 *Schematic diagrams of the exhaled breath condensate collecting device: (A) Dry ice; (B) Tygon tube; (C) End of the tube where the subject breathes; (D) One-way valve, filter and Eco Vent; (E) Polistyrol container*

After 10 min of breathing, the tube was capped at both ends, with one end using a 10 ml vial as a termination. The tube was allowed to warm to room temperature and the beaded EBC liquid was driven into the vial using centrifugal force (swinging the tube in an overhead circle for 1 min). The vial was immediately capped and stored in dry ice or in the glacier.

The end of the tube was connected to a non-rebreathing valve followed by a real-time pneumotachograph (EcoVent, Jaeger). EcoVent recorded exhaled breath volume, time exhaled, tidal volume (TV), peak expiratory flow, minute ventilation and breathing frequency (bf)[133, 134].

Since ambient air contains molecules that could influence EBC composition through several possible mechanisms, room air was also collected. (Figura 5.14)

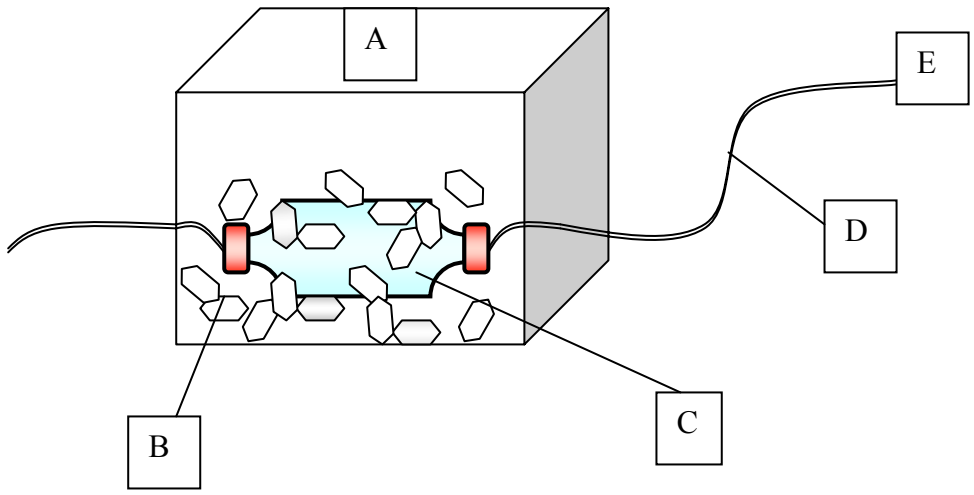

Figure 5.14 *Schematic diagrams of ambient air collecting device: (A) Polistyrol box; (B) Dry ice; (C) Glass bulb; (D) Teflon tube; (E) Pump*

Room air was pulled from a pump (9 L/min for 15 min) through the glass bulb immersed in dry ice in the box. In this way the air condensed in the bulb. After defrosting, the sample was transferred to a vial.

To decide how long the air should be collected, the following calculations were performed: at a temperature of 25°C and a relative humidity of 75%, the air water content was 16 g per m^3 of air.[135] This corresponds to16 ml of H_2O / m^3, so for a sample of 2 ml, it is necessary to collect 0.125 m^3 of air → 125 L.

5.2.3 Human EBC sample collection and analysis

For this preliminary evaluation, 10 samples were collected from volunteers; the idea was to compare the two different collection methods, in order to figure out which was the best. The data showed that there were no differences between the two collection methods regarding breath compound concentrations; the only advantage with the second method was the higher recovery in condensate sample volume. (Table 5.12) This allowed the sample to be split and run in duplicate, in order to work out the reproducibility of the real breath sample.

Table 5.11 *Concentrations of compounds found in breath samples, collected with the two different methods.*

	Collection method number 1				
Compounds	**Concentrations [ppb]**				
	EBC1	**EBC2**	**EBC3**	**EBC4**	**EBC5**
2-methyl propanal	3	1	2	3	2
3-methylbutanal	58	1	1	1	2
1-propanol	54	146	259	279	349
2-methyl-1-propanol	2	4	3	4	3
1-butanol	25	98	42	28	120
heptanal	3	3	4		5
1-pentanol	3		0.1		
octanal	0.4	7	15	15	22
1-hexanol	2	3	4	6	4
1-heptanol	4	1	2	11	2

Table 5.11 (a) *Concentrations of compounds found in breath samples, collected with the two different methods.*

	Collection method number 2				
Compounds	**Concentrations [ppb]**				
	EBC6	**EBC7**	**EBC8**	**EBC9**	**EBC10**
2-methyl propanal	3	3	8	14	4
3-methylbutanal	75	75	66	66	65
1-propanol	190	172	66	124	185
2-methyl-1-propanol	6	32	135	221	23
1-butanol	5	4	5	3	4
heptanal	2			3	
1-pentanol	3	4	4	6	3
octanal	84	1		19	
1-hexanol	2	3	7	2	2
1-heptanol	4	3	4	4	3

Table 5.12 *Breath condensate volume collected with the two methods; it was also possible to measure the exhaled breath volume, only with the second method.*

Sample ID	Exhaled breath volume	Breath condensate volume
EBC 1	nd	1.8 ml
EBC 2	nd	1.7 ml
EBC 3	nd	2.1 ml
EBC 4	nd	1.6 ml
EBC 5	nd	2.3 ml
EBC 6	168.2 L	4.5 ml
EBC 7	175 L	4.2 ml
EBC 8	122.8 L	4.0 ml
EBC 9	125.1 L	4.2 ml
EBC 10	174 L	5.5 ml

It is possible to estimate the concentrations only for a few molecules, (Table 5.11) because most of the compounds are of a very low concentration, near to the zero point of the calibration.

In fact one of the current limitations of EBC measurements is the low concentration of many compounds so that their measurements are limited by the sensitivity of the assays.

Moreover, a comparison of compound concentrations in ambient air and in breath shows that they are similar to each other, which could mean that these compounds are not endogenous. However further measurements are needed to verify these preliminary results. The monitoring of all the parameters necessary to calculate the compounds concentration in ambient air is of utmost importance, in order to compare those present in breath samples.

It is clear that EBC could contain many potential markers. Collection of EBC is a completely non invasive method of breath sampling, collection devices are portable and can be used in a wide range of settings including intensive care units, workplaces and at home. It is now important to optimise their measurements and to study the clinical value of monitoring markers in breath in a variety of specific diseases and establish the reproducibility of these measurements. This is a complex task as each marker needs to be considered individually due to differing solubility, stability, volatility and amounts[130].

Conclusions

The validation of an analytical methods on the one hand and the development of sampling system on the other hand are the most significantly contributions made by this research work. Single molecules or sets and/or patterns of exhaled biomarkers were also investigated in order to establish correlations between the composition of breath and patients' clinical conditions.

The results show the critical aspects that currently limit the use of breath analysis for clinical diagnoses. In fact the direct identification of disease markers presents various problems, such as the high levels of interfering substances and the inter- and intra-individual variability. In addition, the metabolic pathways that give rise to their production are not always known, and the complexity of human physiology makes their determination a challenging task.

Furthermore, depending on the type of breath or the sampling technique used, the results can vary considerably. Therefore, the standardization of methods is important for improved interpretation of data among subjects who are or are not exposed to VOC.

For this reason, many studies must be performed and if the diagnostic potential of breath analysis is to be used in clinical practice, we have to learn more about origins, physiological bases, and exhalation kinetics of the volatile substances.

The research has been aimed at specific pathologies, involving a suitable statistical number of subjects in order to define for each parameters, the ranges of "normality" and the ranges of disease pathologies.

Simplification is essential, because the methods currently employed to collect, concentrate and assay breath samples are too complicated and expensive for general use. A new generation of instruments providing quick readouts could transform breath analysis into an inexpensive and useful screening test for many different diseases and thus increase its diffusion. In our opinion, dedicated infrared spectrometers or mass spectrometers, and sensor-based systems would be the main candidates for such a new

generation. Sensor systems, in particular, should be able to combine high throughput and ease of use with low costs.

Finally the progress will require teamwork amongst device makers, experts in breath analysis, and clinicians.

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Congresses

V Mediterranean Basin Conference on Analytical Chemistry, 24 – 28 Maggio 2005, Silvi Marina, Italia, with the scientific presentation "Chemical characterization of exhaled air samples of patients with diabetes by gaschromatography/mass spectrometry", F.DiFrancesco, M.Onor, S.Tabucchi, M.G.Trivella, A.Ceccarini, R.Fuoco.

XXII Congresso di Chimica Analitica, 11-15 Settembre 2005 , Università degli studi di Cagliari, centro Polaris, Pula, Sardegna with the scientific presentation "Caratterizzazione chimica dell'espirato umano in pazienti affetti da diabete mediante gascromatografia/spettrometria di massa", F.DiFrancesco, M.Onor, S.Tabucchi, M.G.Trivella, A.Ceccarini, R.Fuoco.

XII Hungarian-Italian Symposium on Spectrochemistry, 23-27 Ottobre 2005, Pécs, Ungheria, with the scientific presentation "Advances in clinical application of breath analysis by gas chromatography/mass spectrometry", F.DiFrancesco, M.Onor, S.Tabucchi, M.G.Trivella, A.Ceccarini, R.Fuoco.

International Scientific Meeting *"Breath Analysis in Physiology and Medicine"* , 4-5 Settembre 2006, Praga, with the scientific presentation "Sampling bags for brath analysis", F.DiFrancesco, M.Onor, S.Tabucchi, S.Ghimenti, M.G.Trivella, A.Ceccarini, R.Fuoco.

XXII Congresso Nazionale della Società Chimica Italiana, 10-15 Settembre 2006, Firenze, with the scientific presentation "Caratterizzazione chimica dell'espirato umano in pazienti affetti da diabete mediante gascromatografia/spettrometria di massa", F.DiFrancesco, M.Onor, S.Tabucchi, S.Ghimenti, M.G.Trivella, A.Ceccarini, R.Fuoco.

XX Congresso Nazionale di Chimica Analitica, la Chimica dell'ambiente e degli Alimenti, 16-20 Settembre 2007, San Martino al Cimino (VT), with the scientific presentation "Aspetti metodologici del campionamento dell'espirato", F.DiFrancesco, S.Ghimenti, M.Onor, S.Tabucchi, R.Fuoco.

International Scientific Meeting *Breath Analysis Summit 2007: Clinical Application of Breath Testing* , 1-3 Novembre 2007, Cleveland, with the scientific presentation "Implementation of Fowler's method for alveolar breath sampling", F.DiFrancesco, C.Loccioni, G.Pioggia, M.Ferro, M.Onor, S.Tabucchi, M.Fioravanti, A.Russo, I.Roehrer.

Publications

F. Di Francesco, S. Tabucchi, C. Loccioni, M. Ferro, G. Pioggia, "Development of a CO_2 triggered alveolar air sampler", in Proceedings of the IEEE International Symposium on Industrial Electronics (ISIE'07), Vigo (Spain), 2007.

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Schools and Seminars

"Chromatography and Mass Spectrometry, Bio-Agricultural Applications" course organized by Italian Chemical Society (SCI), (Mass spectrometry Division) in collaboration with Thermo Electron Corporation

Workshop on *"Solid Phase Micro Extraction: 10 years of innovation in preparation of analytical sample"* organized by SUPELCO

"Instrumental Analytical Techniques for Ph.D. students" course organized by University of Pisa, Pisa, 2006

11° Mass Spectrometry Course for Ph.D. Students" organized by Italian Chemical Society (Mass Spectrometry Division), Siena 2007

Workshop on *"Evolution in Mass Spectrometry"*, organized by Thermo Fisher Scientific, Florence 2007

Workshop on *"State of the art in environmental analysis: normative aspects and instrumental solutions"* organized by Perkin Elmer S.p.A., Florence 2007

"Industrial Health and Safety Course", organized by Servizio Prevenzione e Protezione, Pisa 2007

Participation to the European Project: BIOTEX (Bio-sensing Textile to support health management)

Foreign experiences

During the PhD I stayed for two months at the U.S. ENVIRONMENTAL PROTECTION AGENCY (EPA), Research Triangle Park in Durham (North Carolina-USA). In this period I made research work on determination of volatile polar compounds (alcohols and aldehyde) in exhaled breath condensate and on determination of toxic Diesel compounds contained in plasma samples of exposed subjects.