DEVELOPMENT OF A SAMPLING SYSTEM FOR THE COLLECTION OF EXHALED BREATH CONDENSATES AND ITS SUBSEQUENT ANALYSIS USING 2-DIMENSIONAL GAS CHROMATOGRAPHY

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DEVELOPMENT OF A SAMPLING SYSTEM FOR THE COLLECTION OF EXHALED BREATH CONDENSATES AND ITS SUBSEQUENT ANALYSIS USING 2-DIMENSIONAL GAS CHROMATOGRAPHY

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SUMMARY

Exhaled human breath contains thousands of compounds that provide information about the physiological state of the human body, yet it is not used as extensively as blood or urine samples for medical diagnostics. Some of the major reasons for this include the lack of a standard method of sample collection, control of variability, reliable detection at trace level and quantification. Breath analysis has recently attracted renewed interest in medical diagnostics and for monitoring therapeutic progress because it is a less invasive method of sample collection than is currently used in treatment and it can be sampled as often as required. Breath analysis can be performed in surgery or in intensive care and real time analysis is possible on a stationary cycle or during sleep.

The objective of this research is to develop a novel sample collection system that is capable of capturing both the EBC as well as the VOC present in exhaled human breath. Currently, methods of simultaneously capturing both fractions of exhaled breath are not well established and current work focuses on the capture and analysis of either one fraction. Furthermore, the focus is currently on the targeted analysis of specific compounds to monitor ailments such as asthma, cancer etc. In this work, both fractions are successfully collected and analyzed using gas chromatography - mass spectrometry (GC-MS) as well as two-dimensional gas chromatography (GCxGC). This provides analysis of volatile organic compounds as well as proteins and higher molecular weight components present in breath. This broader analysis might find use in medical diagnostics, for detection of markers indicating oxidative stress, detection of lung cancer, monitoring plasma glucose and many more medical applications.

CHAPTER 1: INTRODUCTION AND BACKGROUND

The first step in most clinical diagnostics involves the collection and analysis of blood and urine samples of a patient. These body fluids are known to contain information that enables appropriate diagnosis of a medical condition. The main focus of diagnostics research over the past few decades has been on blood and urine analysis. More recently however, there has been a greater emphasis on non-invasive diagnostic methods especially in the areas of neo-natal care [1] and for the critically ill [2].

The analysis of exhaled breath is one such non-invasive technique [3] that has not yet been fully explored and exploited for clinical diagnostics. The compounds present in exhaled human breath can be broadly divided into two categories – the higher molecular weight compounds present as aerosol particles which can be condensed and collected as exhaled breath condensate (EBC) and the lower molecular weight volatile organic compounds (VOC). The routine blood and urine analysis reveals only the larger, higher molecular weight compounds such as proteins and amino acids, whereas the VOCs are generally lost in sampling or analysis. However, it has been found that these volatile compounds occur at trace levels (parts per billion or parts by trillion volume) in exhaled breath [4]. The quantitative measurement of many of these compounds can give us great insight and information for clinical diagnosis [5]. It is preferable for patients as blood sampling can be painful and collection of urine samples is embarrassing for many people. Breath samples closely reflect the arterial concentration of biomarkers and may avoid the need for collection of arterial blood samples, which is difficult. In cases where multiple

blood samples are required, breath analysis is advantageous. Breath is also a less complicated mixture as compared to serum or blood. Preparation of breath samples for analysis is far more trivial as compared to blood or urine.

Despite all the above-mentioned advantages, breath analysis is still not being used in mainstream clinical diagnosis. This is mainly due to a lack of a standard sampling procedure and difficulty in quantitation. Although a number of compounds have been found in exhaled breath, the origin of some these biomarkers are yet to be established. The instrumentation currently in use for analysis is either too expensive or impractical to use in a clinical setting. The following sections contain a brief review of breath as a clinical sample, different methods that have been used for sample collection as well as instrumentation that have been and are currently in use to analyze the breath sample.

1.1 Breath as a clinical sample

Breath has been identified as a biomarker for various ailments since ancient times. The ancient Chinese and Greek physicians were able to diagnose diseases from the odor of patients' breath. In 1971, Pauling et al. first reported the presence of over 200 volatile organic compounds in human breath using gas chromatography [6]. In figure 1 we see the various peaks of a chromatogram each representing a compound present in human breath. Over the next few decades, great progress was made in terms of investigating the physiological meaning of the various compounds seen in breath as well as the correlations between the compounds and the internal processes in the human body [7]. As technology developed and new instrumentation was made available, the ability to separate, analyze and identify the various volatiles improved.

Exhaled human breath mainly consists of nitrogen, oxygen, carbon dioxide, water vapor and a small fraction of volatile trace components. Table 1.1 shows the percentage composition of human breath. The trace compounds could be endogenous or exogenous. Endogenous compounds are those that are produced inside the human body as a result of metabolism or other physiological processes.

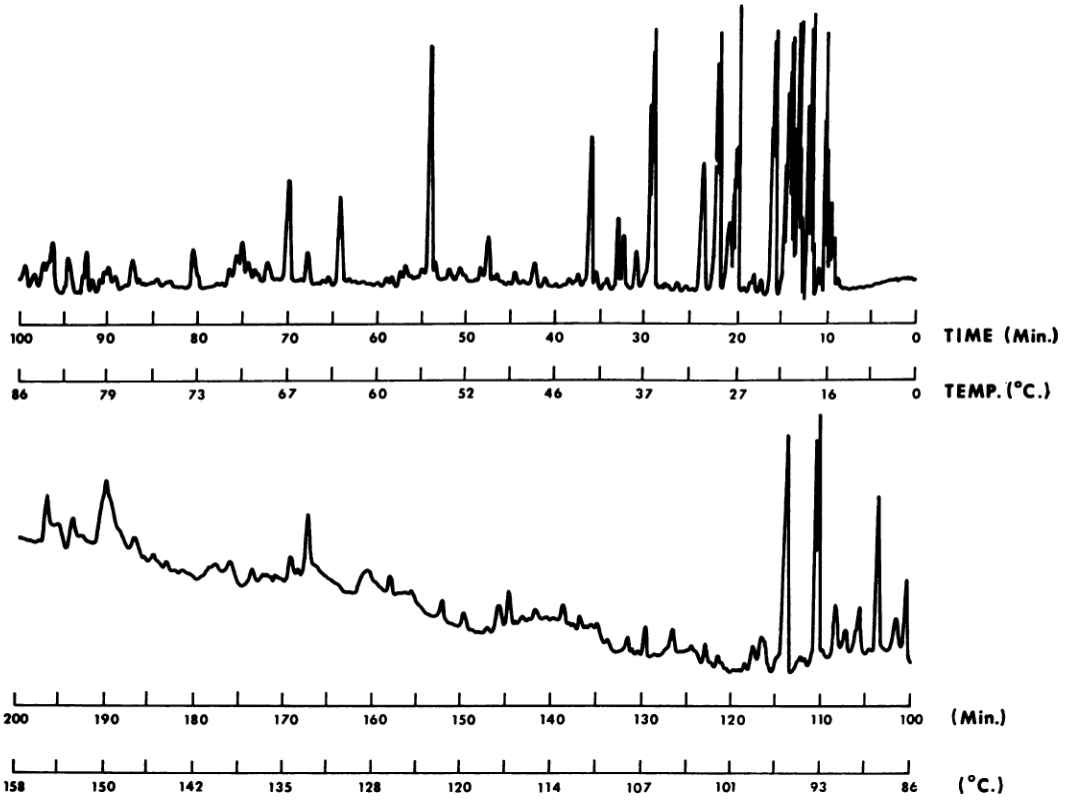


Figure 1.1: An exhaled breath chromatogram [6]

Exogenous compounds are those, which are present in the surroundings and absorbed by the body as contaminants. Analysis of the endogenous compounds provides information about the physiological processes of the body whereas the exogenous compounds reveal information about the quality of the environment and the ability of the body to absorb and expel these contaminants [8]. The endogenous compounds include inorganic gases such

as NO and CO, VOCs such as ethane, pentane, acetone, isoprene and non-volatile substances such as isoprostanes, glucose and cytokines which can also be found in EBC [4].

Table 1.1: Concentration range of various compounds in human breath [9]

Concentration (v/v)	Molecule
percentage (%)	oxygen, water, carbon dioxide
parts-per-million (ppm)	acetone, carbon monoxide, methane, hydrogen
parts-per-billion (ppb)	formaldehyde, acetaldehyde, isoprene, 1-pentane, ethane, ethylene, other hydrocarbons, nitric oxide, carbon disulfide, methanol, carbonyl sulfide, methanethiol, ammonia, methylamine, dimethyl sulfide

Table 1.2: Physiological origins of various biomarkers [9]

Compound	Physiological basis
acetaldehyde	ethanol metabolism
acetone	decarboxylation of acetoacetate
ammonia	protein metabolism
carbon disulfide	gut bacteria
carbon monoxide	production catalyzed by heme oxygenase
carbonyl sulfide	gut bacteria
ethane	lipid peroxidation
ethanol	gut bacteria
ethylene	lipid peroxidation
hydrocarbons	lipid peroxidation/metabolism
hydrogen	gut bacteria
isoprene	cholesterol biosynthesis
methane	gut bacteria
methanethiol	methionine metabolism
methanol	metabolism of fruit
methylamine	protein metabolism
nitric oxide	production catalyzed by nitric oxide synthase
pentane	lipid peroxidation

In order to understand the physiological meaning of exhaled breath, it is important to know the biochemical pathways of generation of the various compounds. The physiological origins of some biomarkers found in breath are given in table 1.2. The various VOCs that are found in human breath can be classified into 5 main categories based on their chemical composition and structure - Saturated hydrocarbons, unsaturated hydrocarbons, oxygen containing, sulphur containing and nitrogen containing compounds.

Saturated hydrocarbons such as ethane, pentane and malondialdehyde are generated from ω3 and ω6 fatty acids during lipid peroxidation [10]. Methylated hydrocarbons have also been reported as lipid peroxidation markers [11], however the biochemical pathways and the meaning of these compounds have not been sufficiently investigated [12]. These hydrocarbons, which are stable end products of lipid peroxidation, have a low solubility in blood. Hence they are ejected into exhaled breath within a few minutes of their formation in tissues. Thus exhaled concentration of ethane and pentane acts as a marker of the degree of oxidative damage in the body [13].

One of the most abundant unsaturated hydrocarbons found in exhaled human breath is isoprene. Its origin lies along the mevalonic pathway of cholesterol synthesis. Experimental evidence initially indicated that the presence of isoprene in breath is related to the oxidative damage of the fluid linings of the lungs and the body [14], [15]. However it has been found that isoprene concentration increases by nearly 5 times during physical exertion [16] or even something as simple as a few leg contractions. The concentration of breath isoprene is also found to vary with age as it is significantly lower in children [17].

Acetone, an oxygen containing compound, is one of the most abundant compounds found in human breath. It falls under the category of ketones along with acetoacetate and β-hydroxybutyrate and is produced due to decarboxylation of acetoacetate and through dehydrogenation of isopropanol [18]. It has been found that breath acetone levels are increased in patients suffering from uncontrollable diabetes mellitus [19]. Acetaldehyde, another oxygen containing compound is produced by the oxidation of endogenous ethanol whereas ethanol itself is produced by the bacteria present in the intestines [20].

Sulphur containing compounds are generated by the incomplete metabolism of methaionine in the transaminative pathway [21]. The concentration of sulphur containing compounds is very low in human blood during normal conditions, but any impairment in the functioning of the liver causes the concentration to rise. The characteristic odour of sulphur is found in the exhaled breath of people suffering from cirrhosis of the liver due to the presence of ethyl mercaptane and dimethyl sulphide [22].

Nitric oxide (NO) has been identified as a key compound in numerous physiological processes and a marker of airway inflammation over the last few decades. Increased NO content has been observed in asthmatic patients [23]–[25] and this has been attributed to the activation of nitric oxide synthase 2A by the damage to the epithelial cells lining the airways and by inflammation of airways. Hence NO is used as a means to distinguish between asthmatic and non-asthmatic patients [26], [27]. Ammonia is another nitrogen containing compound that would appear in breath if its conversion to urea was restricted due to impairment of the kidney. Higher concentration of ammonia was observed in the breath of uremic patients when compared to that of normal patients [28].

1.2 Sampling and pre-concentration

A significant challenge in the clinical implementation of breath analysis involves implementing a standardized method for collecting and analyzing breath samples from patients. Many factors such as the volume of breath collected, the quality of the surrounding air, the method of collection and the method of storage introduce variability in the amount of analyte present in the sample.

There are two basic approaches used when it comes to breath sampling. Mixed expiratory sampling involves the collection of the total breath, which includes the dead space air present in the lungs. The dead space air is basically the volume of breath that does not take part in gas exchange. It usually occupies the airway volumes and consists primarily of carbon dioxide. It accounts for a third of the total tidal breathing volume. Alveolar sampling is another method of collection where only the fraction of breath involved in gaseous exchange is collected. Alveolar breath has fewer contaminants [29] and better represents the actual concentration of analytes present in the human body. The concentration of endogenous compounds is also much higher as compared to mixed expiratory sampling as the dead space gas does not dilute it. In order to distinguish between endogenous and exogenous compounds, different approaches such as background air subtraction or correction of exhaled concentrations by calculating alveolar gradients have been employed [30]. Attempts have also been made to try and control the ambient air by making volunteers breathe pure air for a fixed time before sampling [13].

As exhaled breath contains both EBCs and VOCs, different collection techniques have been implemented depending on the type of sample being collected. Most devices that

have been designed for the collection of EBC involve cooling the exhaled breath in order to condense and collect the liquid condensate. This is typically achieved by breathing

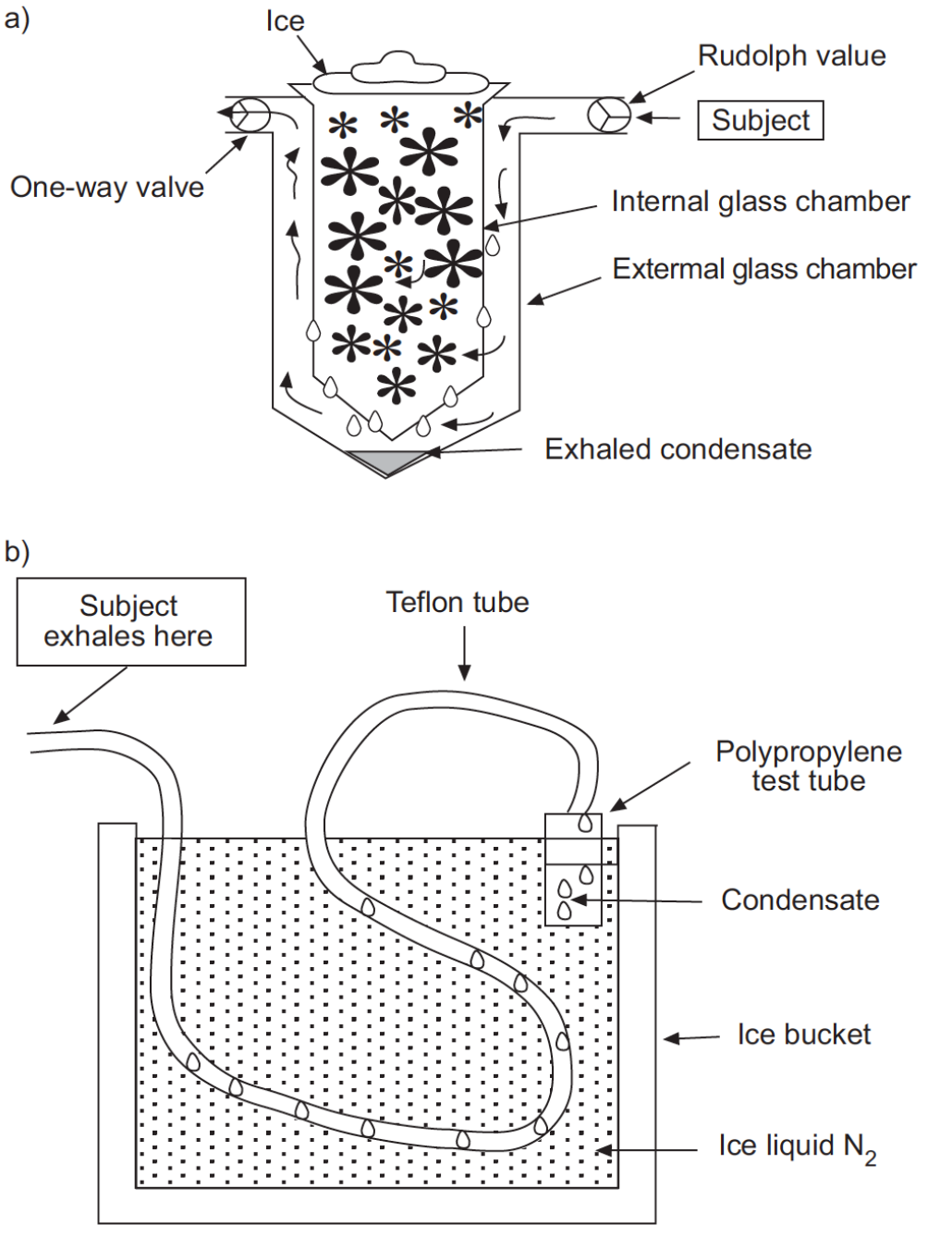


Figure 1.2:(a) EBC collection system using a cold finger [33] (b) EBC collection with tube immersed in cold bath [34]

through a tube, which is immersed in a cold bath containing dry ice or some such coolant. Various materials such as Teflon, polypropylene and Tygon have been used for the tubing [31], [32] whereas different containers such as a double-walled glass chamber [33] with a cold finger or an immersion ice bath [34] have been used as the condensing chamber. The VOCs on the other hand, are generally collected by directly breathing into an inert Tedlar bag or a stainless steel canister [35]. In some cases, the VOCs were captured by exhaling onto adsorptive materials [36], by breathing onto a SPME fiber [37] or by cryofocussation [38].

A preconcentration step is generally carried out before analysis of the breath sample. Since the concentration of the VOCs in exhaled breath is in the ppm-ppv range, direct analysis may not yield good results. Preconcentration by sorbent traps include adsorbing the volatiles onto activated carbon, Tenax, Silica Gel and other such sorbents. Once the breath is passed through the trap, the sorbents are then heated to release the adsorbed compounds. Solid Phase Microextraction (SPME) works in a similar manner, where a fiber coated with either a polar, non-polar or semi-polar compound, acts as the adsorbing material. When the SPME is introduced into a breath filled Tedlar bag, the compounds present tend to adsorb onto the fiber based on their affinity for the coating. Hence different coatings are used to capture different compounds of interest. Like sorbent traps, the SPME fiber is then heated in order to desorb the compounds. A disadvantage of sorbent traps and SPME is that they suffer from memory effects ie. Not all the compounds are completely desorbed sometimes and this could lead to carry over of compounds. Also, SPME fibers are not equally selective towards all the compounds present in a Tedlar bag and tend to adsorb some compounds more than others [39]. This could lead to errors while performing quantitative measurements.

1.3 Instrumentation for breath analysis

Since exhaled breath is a complex mixture of non-volatiles and volatiles in both liquid and gaseous form, chromatography is a popular technique used to separate the mixture into its various constituents. Liquid chromatography techniques are applied to the EBC portion whereas the VOCs are separated via gas chromatography. The gas chromatograph (GC) is usually coupled to a detector that enables the identification and quantification of the constituent compounds. Some of the most important instruments used for this are the flame ionization detector (GC-FID) [40], mass spectrometer (GC-MS) [35], selected ion flow tube mass spectrometry (SIFT-MS) [41], proton transfer reaction mass spectrometry (PTR-MS) [42] and laser absorption spectroscopic techniques [43]. The principle of operation of these instruments is beyond the scope of this thesis, however a brief overview of the GC is presented, as it was the primary separation tool used during the course of this work.

The major working components of the GC can be classified into 3 categories – the injector, the separating column and the detector.

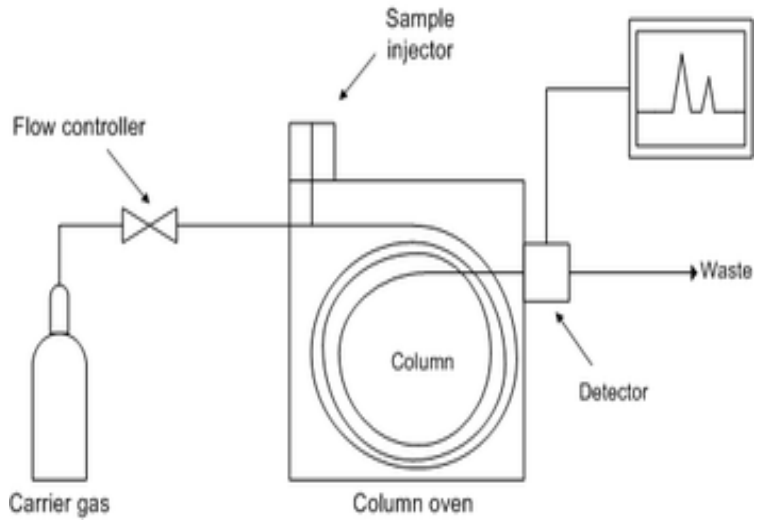


Figure 1.3: Components of a gas chromatography system

The complex breath sample is introduced at the injector in either liquid or gaseous form. The heater at the injector vaporizes the sample, which is then carried along the length of the chromatographic column by the carrier gas or the mobile phase. The carrier gas is usually an inert/unreactive gas such as helium or nitrogen. The chromatographic column is coated on the inside by a liquid or a polymer layer known as the stationary phase. The coating is usually polar, semi-polar or non-polar in nature, and depending on its chemical properties, it interacts in a different manner with the various constituents of the sample. Hence, this causes the gaseous constituents to elute at different times, depending on the interaction with the stationary phase. Thus the mixture of compounds introduced at the injector is separated as it leaves the column. For a given analytical condition the elution times are compound specific and can be used for identification of the eluting components.

The detector attached to the end of the column is used to quantify the amount of analyte passing through the column. The most basic detector, which is the flame ionization detector, pyrolyzes the eluted compound into its constituent ions, which then go on to strike a detector plate generating a current. The magnitude of current is

proportional to the concentration of ions and hence the concentration of the analyte. More sophisticated detectors such as the mass spectrometer (MS) are able to identify the compound by breaking it up into its constituent masses and then analyzing it using a mass analyser.

Recent developments in instrumentation are shifting the focus toward two-dimensional gas chromatography [44]. The 2-D GC has increased resolving power as the analyte is separated using two different stationary phases as opposed to the single stationary phase used in traditional 1-D GC. A shorter secondary phase is attached at the end of the first column and the analyte exiting the first column is collected for a fixed period of time known as the modulation period and is then introduced into the secondary column for further separation. Thus the final chromatogram is a 2-D layout of the separation and is able to bring out compounds that would typically co-elute in a 1-D chromatogram.

CHAPTER 2: EXPERIMENTAL APPARATUS

2.1 Introduction

Although breath analysis exhibits great potential in non-invasive medical diagnostics, its actual clinical implementation has been somewhat limited. One of the major factors is the absence of a standard method or protocol for collection of breath samples. The various methods of collection currently in use have been briefly discussed in the previous chapter, with most groups content to deal with the non-volatiles and VOCs separately. The preferred method for collection of the EBCs has been to make the patient exhale through a tube immersed in an ice bath for a fixed period of time. The water vapor present in the breath condenses on the walls of the tube from where it is collected and analyzed. Groups that were interested in the exhaled breath VOCs, directly collected the patients' breath in Tedlar bags, canisters and by blowing over SPME fibers. In both cases, no attempt is made to isolate the analyte of interest completely from the other fraction. For example, while collecting breath samples in tedlar bags, the presence of moisture in the breath is ignored. Also, due to the focus on a single fraction of breath, very little information exists about the significance of VOCs dissolved in the EBC as well as the broader spectrum analysis of both fractions.

The objective of this research is to develop a breath-sampling device that is able to separate and capture both the EBC and the VOC fractions present in exhaled breath. The patient is made to breathe for a few minutes into the system and two different cold baths or 'stages' are used to separate the analytes based on their condensation temperatures. The less volatile compounds are dissolved in the water vapor and condense at a higher temperature in the first stage whereas the volatiles present in the gaseous phase condense

in the colder, second stage of the system. The samples are then extracted from the system with the help of a syringe (EBC) and a Tedlar bag (VOC).

Significant attention is paid to ensure that the pressure drop across the sampling system stays within a reasonable limit in order to ensure the patient stays comfortable during the course of sampling. The temperatures of the two zones are selected in order to ensure maximum efficiency during the capture of anlytes from the breath. A longer tube length in the sampling system provides greater cooling of the breath but this is at the expense of the pressure drop across the system. The length of the tube is selected after calculating both the amount of cooling achieved as well the pressure drop incurred and finding a solution that satisfies both needs. The detailed design of the system is presented in the following sections; with emphasis on achieving a good yield of the analyte while ensuring the patient is comfortable through the sampling procedure.

2.2 Design of sampling system

Human breath is at a temperature of $37^{O}C$ and has a relative humidity of 95% when it is expelled from the upper respiratory tract. This implies that a large fraction of exhaled breath consists of water vapor as it is almost fully saturated. The non-volatile EBCs are carried by the water vapor whereas the remaining fraction of the breath consists of CO_2, O_2 and the various VOCs. By separating the water vapor from the breath, we can isolate the EBCs for collection from the rest of the breath matrix. As the amount of water vapor present in air is a function of its temperature, it is possible to remove the water vapor by cooling to air to a particular temperature. The patient is made to breathe through a tube immersed in a cold bath and as the breath passes through the tube, it is cooled and the water vapor present condenses onto the walls of the tube. In order to determine what

temperature the breath must be cooled to remove the maximum amount of water vapor, some basic calculations related to human physiology are made. It is found that the tidal volume or the volume of air inhaled or exhaled by the lungs during normal breathing is reported to be roughly 0.5 Liters for a healthy adult. Since the density of air is 1.225 Kg/m^3, the mass of exhaled air per breath is close to 0.6 grams. The specific humidity or the humidity ratio is defined as the ratio of the mass of water vapor to the total mass of air at a particular temperature and this reduces as temperature is reduced. Hence, by looking up the specific humidity of air at different temperatures, it is calculated that in order to remove 95% of the water vapor from breath, it must be cooled from $37^{O}C$ to $-10^{O}C$. It was also calculated that in order to obtain a removal efficiency of 99.5%, it is necessary to cool the air to $-40^{O}C$. Due to the restrictions on the type of cooler available, it was decided that a removal efficiency of 95% was adequate for our purpose.

The tubing for the system is made up of two different sections - the section that is immersed in the cold bath and where the heat transfer takes place is made of stainless steel whereas the remaining section is made of tygon tubing. This is done in order to provide a highly conductive section for the region where heat transfer takes place and insulate the other areas by using tygon. The length of the conductive section needed to cool the breath to the required temperature can be calculated using the following equation [45]

$$\frac{T_s - T_x}{T_s - T_i} = \exp\left(\frac{-PLh}{\dot{m}c_p}\right) \quad (1)$$

where T_s is the bath temperature, T_i is the initial temperature of exhaled breath, P is the perimeter of the tube, L is the tube length, $\dot{m}$ is the mass flow rate and h is the heat

transfer coefficient which can be calculated by using the Nusselt number for transition flows. After fixing the bath temperature at -40^{O}C, the temperature distribution across a 1 meter tube for different diameters was calculated and plotted on Matlab (Mathworks, Natick,MA) (fig 2.1). The pressure drop across the first stage is given by [45],

$$\Delta P = \frac{f \delta u_m^2 (x_2 - x_1)}{2D} \quad (2)$$

where δ is the density of air, u_m is the mean velocity, D is the diameter of the tube and f is the friction factor. The friction factor for fully developed laminar flow is given by 64/Re where Re is the Reynolds number of the flow. The pressure drop across the same configuration of tubes was also calculated and plotted (fig 2.2). It is clear that shorter tube lengths are required for cooling when the diameters are less, however the increase in the pressure drop is significant. It is to be noted that a normal human being generates approximately 1mm hg (133 Pa) pressure during exhalation. In order to have a comfortable experience while breathing into the system, the pressure drop across it must be close to normal human exhalation pressure. The 6 mm diameter configuration was found to fit the need of the experiment well, as for a short tube length (25 cm) and low pressure drop, the required exit temperature is obtained.

The first stage bath consists of an insulated container filled with a liquid having low freezing point such as isopropyl alcohol. A NESLAB (Thermo Fischer, Waltham MA) chiller is used with a temperature feedback 'cryotrol' to maintain the cold bath at the required temperature. The probe of the chiller is immersed into the bath along with the feedback probe to maintain the bath at a constant temperature of -40C. T-type thermocouples are inserted at the beginning and end of the first stage in order to track the temperature of the inlet and outlet breath.

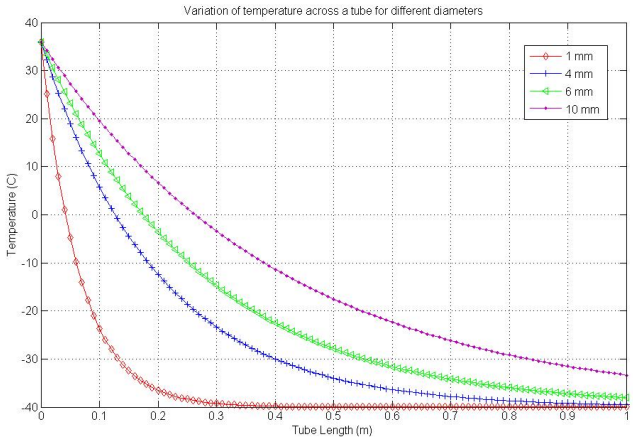
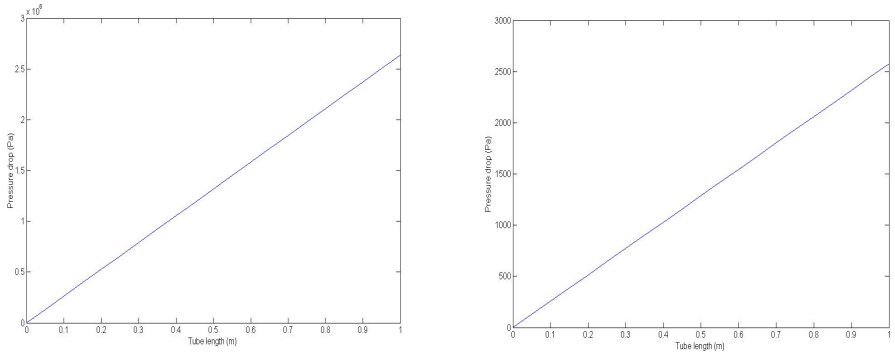


Figure 2.1: Variation of temperature along tube length for different tube diameters

a) b)



c) d)

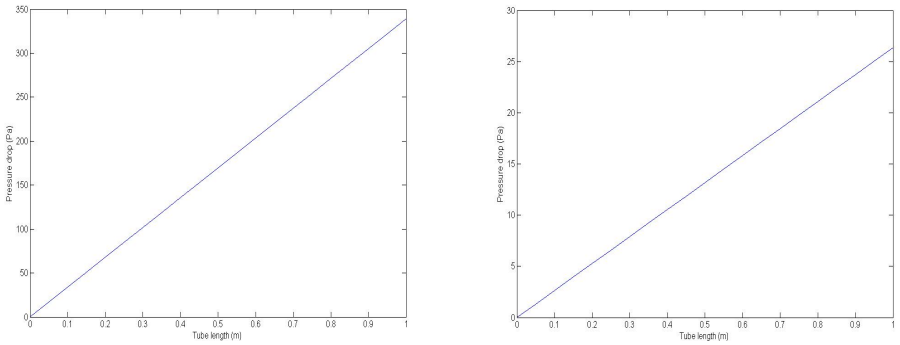


Figure 2.2: Variation of pressure along tube length for diameters of a) 1 mm b) 4 mm c) 6 mm and d) 10 mm

A slightly different design approach is followed while building the second cooling stage of the system. The air entering the second stage is now at -10^{O}C and is almost completely dry. The breath now consists primarily of carbon dioxide, oxygen and trace level VOCs. The exit temperature of the air stream is calculated based on the removal efficiency desired. The first step is to determine the VOC concentration at the outlet of the second stage for a given removal efficiency. This is calculated by first determining the partial pressure of the VOC at the outlet, P_{voc}. Assuming that ideal gas laws apply [46],

$$P_{VOC} = 760 \frac{moles\ VOC\ in\ outlet\ stream}{Moles\ VOC\ in\ inlet\ stream - Moles\ VOC\ removed} \quad (3)$$

where P_{voc} = Partial pressure of the VOC in exit stream (mm Hg).

And the condenser is assumed to operate at a constant pressure of one atmosphere. This equation can be further simplified and written as,

$$P_{VOC} = 760 \left[\frac{y_{VOC,in}(1-\eta)}{1-(\eta * y_{VOC,in})}\right] \quad (5)$$

$y_{VOC,in}$ = Volume fraction of VOC in inlet stream.

η = Removal efficiency (moles of VOC removed/moles VOC in inlet). We set a removal efficiency of 90%.

At the outlet of the second stage, the VOC in the gas stream is assumed to be in equilibrium with the condensate in the tube. At equilibrium, the partial pressure of the

VOC in the gas is equal to its vapor pressure at that temperature. Therefore by determining the temperature for this condition, it is possible to specify the outlet temperature for the second stage. This temperature can be calculated from the Antoine equation [47] that defines the relationship between vapor pressure and temperature for a particular compound as

$$\log P_{VOC} = A - \frac{B}{T_{con}+C} \quad (6)$$

where T_{con} is the unknown condensation temperature and A, B, C are constants specific to the VOC that are determined for the NIST web book. In cases such as ours where the gas mixture is complex and consists of a number of VOCs, the outlet temperature can be estimated by taking a weighted average of the temperatures necessary to condense each VOC in the gas stream at a concentration equal to the total VOC concentration. Eight commonly occurring VOCs were considered and the outlet temperature of each VOC was calculated (table 2.1). Based on these numbers, the exit outlet temperature of the second stage of the condenser was set at -120^{O}C. Although it is possible to ensure a greater recovery of VOCs by setting the exit temperature equal to the lowest VOC recovery temperate (in this case, it would be -147^{O}C for CO_2), doing so would also cause a large amount of solvent (CO_2) to condense onto the tube. Hence the final breath sample in the bag is a lot more dilute. In order to avoid this, we take the average condensation temperatures of the VOCs.

Table 2.1: Selected compound set and their individual outlet temperatures

Compound of interest	**Outlet temperature for 90% recovery**
Carbon dioxide	-147.76^{O}C
Isoprene	-102.13^{O}C
Methanol	-105.34^{O}C
Ethanol	-115.6^{O}C
Dimethyl Sulfide	-128.51^{O}C
Chloroform	-124.85^{O}C
Benzene	-133.5^{O}C
Toluene	-102.31^{O}C

The second stage is set up similar to the first stage with the tubes running into an insulated bath. Since the required outlet temperature is below -100^{O}C, liquid nitrogen (bp:-196^{O}C) is used for the cooling bath. Equation 1 is once again used to determine the appropriate tube length required for the cooling. Figure 2.3 shows the variation of temperature along a tube for different tube diameters. It is seen that the selection of the 6mm diameter tube provides the required outlet temperature at a length of approximately 27 cm. The combined pressure drop of the first stage and the second stage tubing is approximately equal to the pressure generated by the lungs during breathing. Hence this particular combination of tube length and diameter is calculated to be the best fit for the breath sampling device.

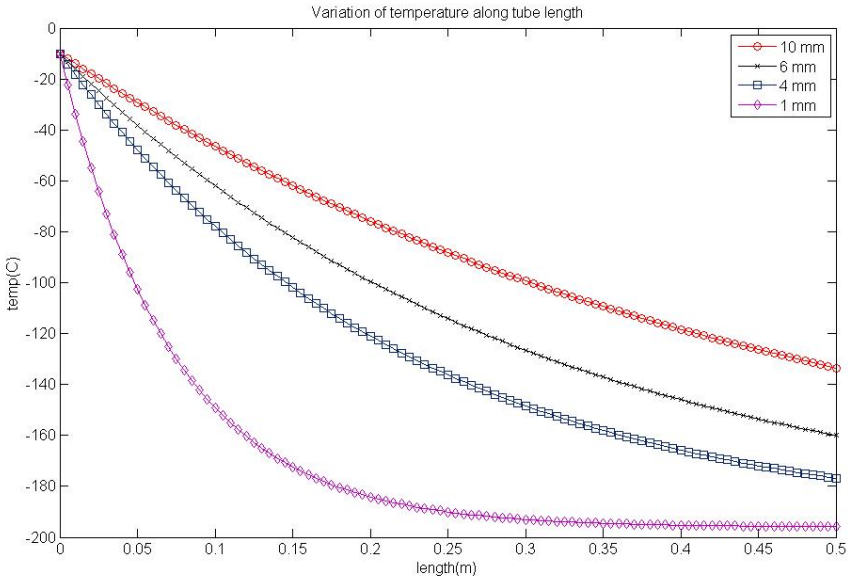


Figure 2.3: Variation of temperature along tube length for the second stage

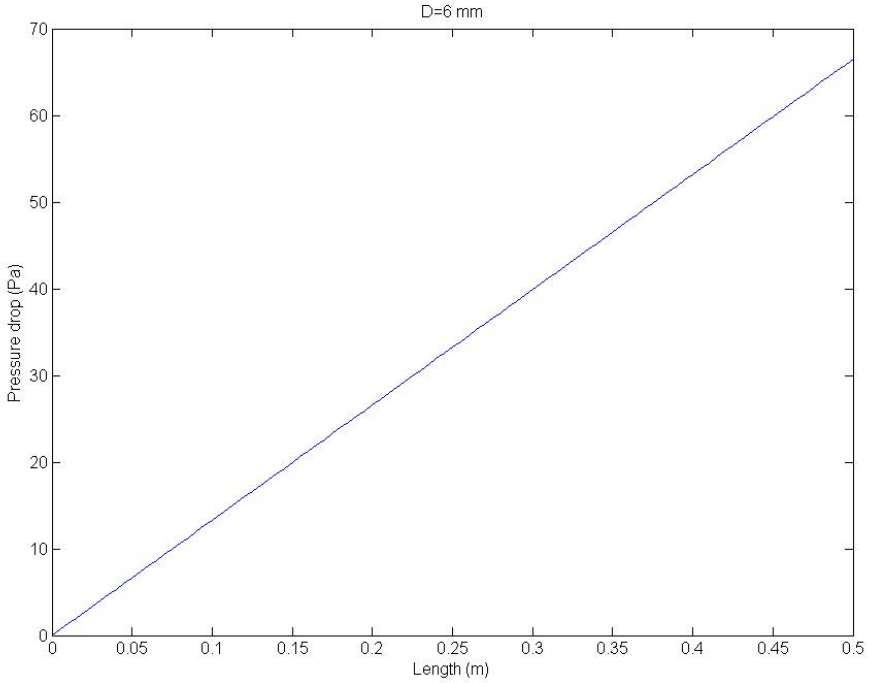


Figure 2.4: Pressure drop across second stage for 6mm diameter tube

The complete breath sampling set up is shown in figure 2.5. Commercially available 'Spirette' mouthpieces (Medline, GA) were used for the patient to breathe into the system. Two-way valves separate the two stages such that the collection of sample from one stage does not affect the other. A syringe is used to collect the liquid EBCs from the first stage whereas a Tedlar bag is the preferred method of collection of the gaseous VOCs. The technique employed to collect the two fractions is discussed in detail in the next chapter.

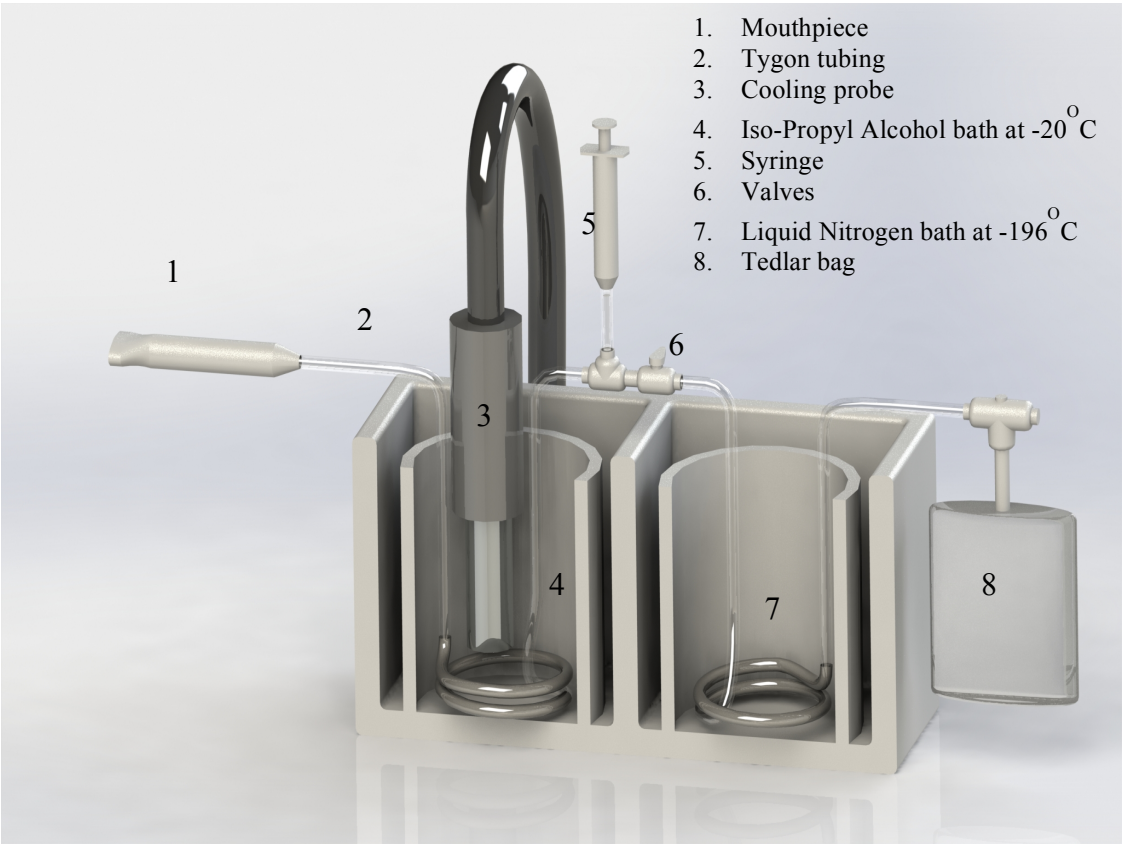


Figure 2.5:Variation of temperature along tube length for different tube diameters

2.3 Two-channel breath sampling system

As an extension to the breath sampling system described in the previous section, a two-channel sampling system was implemented in order to separate the dead space breath and alveolar breath. The objective of this is to determine which of the compounds seen in exhaled breath are endogenous and which are exogenous. It also gives us further insight into the biological pathways of some of the exhaled breath compounds.

The construction of the two-channel device is very similar to the single channel device described in the previous section. The most significant difference is that an additional second channel or set of tubes is connected to the exit of the mouthpiece. The design and construction of this second channel is the same as the first channel in terms of length of tube, temperature of cold bath etc. Two solenoid valves (Mcmaster Carr) are placed between the mouthpiece and the two channels in order to divert the exhaled breath into the appropriate set of tubes and a pressure sensor (10 inch AllSensors corp.) is placed at the mouthpiece in order to track the pressure of exhaled breath. Due to the presence of an additional set of tubes, solenoid valves etc, the pressure drop in the two-channel breath sampling system was greater than that seen in the single channel system. A number of different sensors (2.5 inch and 5 inch) were trialed before settling on the 10-inch sensor.

The pressure sensor and the solenoid valve are controlled via Labview on a PC. The solenoid is powered by a 24V DC source and has a single output state- when powered, the solenoid is at high or ON and when the circuit is incomplete the solenoid is low or OFF. Using two such solenoids, it is possible to divert the flow of air into the appropriate set of tubes. The pressure sensor is provided with a 4.5-5.5V DC input and its output varies from 0.25-5V, where 0.25V indicates atmospheric or base pressure. When the

volunteer breathes into the mouthpiece, the pressure read by the sensor begins to increase and consequently, the output voltage is increased. The blue line in figure 2.6 shows the variation of the pressure sensor output as a person breathes into the mouthpiece. In order to separate the breath into two parts, the average duration of each breath is first calculated to be approximately 8 seconds. Next, the gradient of the pressure sensor output is calculated, and if it is above a value of 0.5V (which would indicate that the person has started to exhale), it is used as a trigger for the solenoid, which switches on and diverts the breath into the first channel. After 4 seconds, the first solenoid is switched off and the second solenoid is switched on which diverts the breath to the second channel. The second solenoid is switched off after 4 seconds at which point, both the solenoids are in the off state. Again on the next breath, the positive gradient of pressure is detected and the first solenoid is turned on. In this manner, the valve is continuously toggled on and off to separate the breath into two different sets of tubes. The yellow line in figure 2.6 shows the toggling for the first solenoid. As can be seen, the solenoid is actuated at the beginning of every exhalation expect on one instance. The time of 4 seconds is chosen on the basis that it takes approximately 8 seconds for one complete exhalation, hence half of it is collected as dead space and the second half of the breath is collected as the alveolar component. The rest of the breath collection procedure such as condensation and collection of analytes is the same as the method employed for the single channel system.

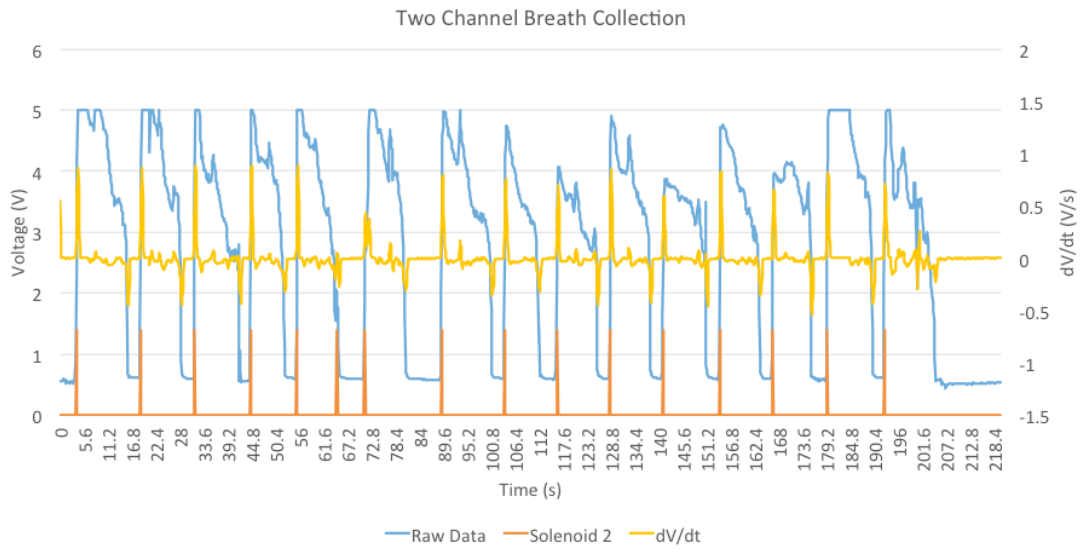


Figure 2.6: Pressure curve of exhaled breath for the two-channel setup

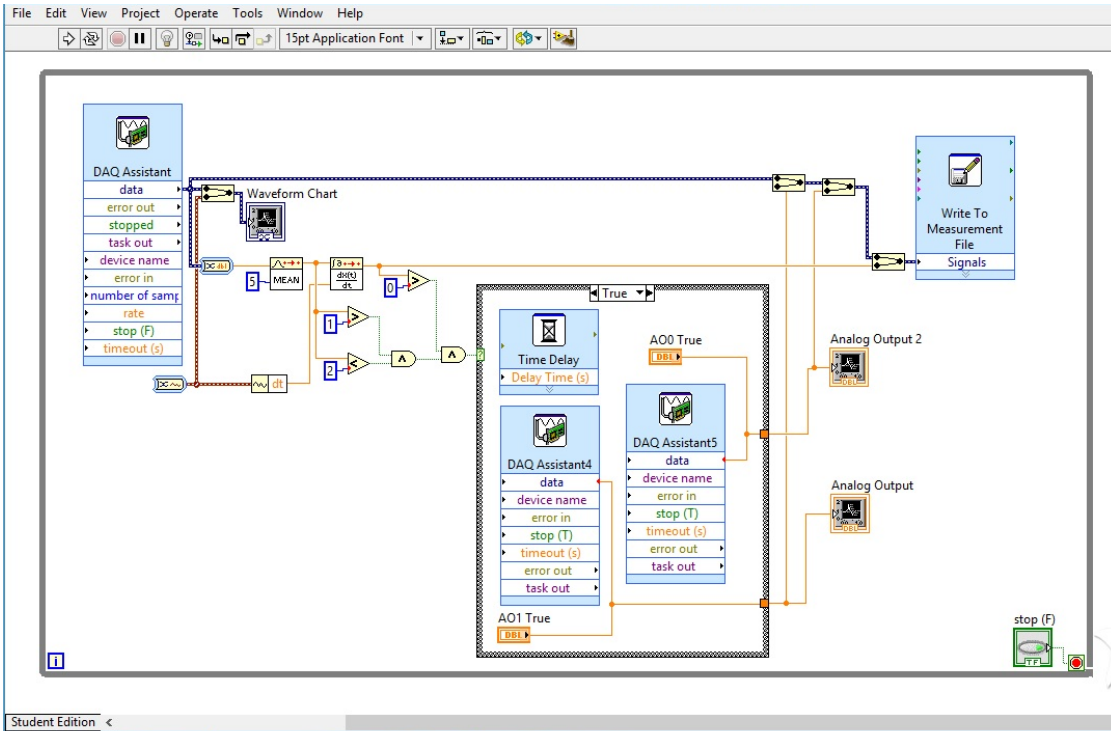


Figure 2.7: Labview schematic for 2-channel control

CHAPTER 3: EXPERIMENTAL PROCEDURE

3.1 Collection of samples

In order to collect breath samples, volunteers were made to breathe through the system described in chapter 2 for 3 minutes. Special emphasis was placed on inhaling through the nose and exhaling through the mouth so that the volunteer does not accidently inhale the contents of the system. A spirometer was attached to the mouthpiece in order to track the breath volume generated for different volunteers. It was found that the average volume of breath exhaled in three minutes was roughly 22 liters. Thermocouples were placed at the exits of the first stage and second stage in order to ensure that the required outlet temperatures were achieved. A typical temperature profile during breath collection can be seen in figure 3.1. The blue line represents the outlet temperature of breath at the end of the first stage and the red line represents the temperature of the breath exiting the second stage. It is seen that the target temperatures are achieved and the variation over the sampling period is minimum.

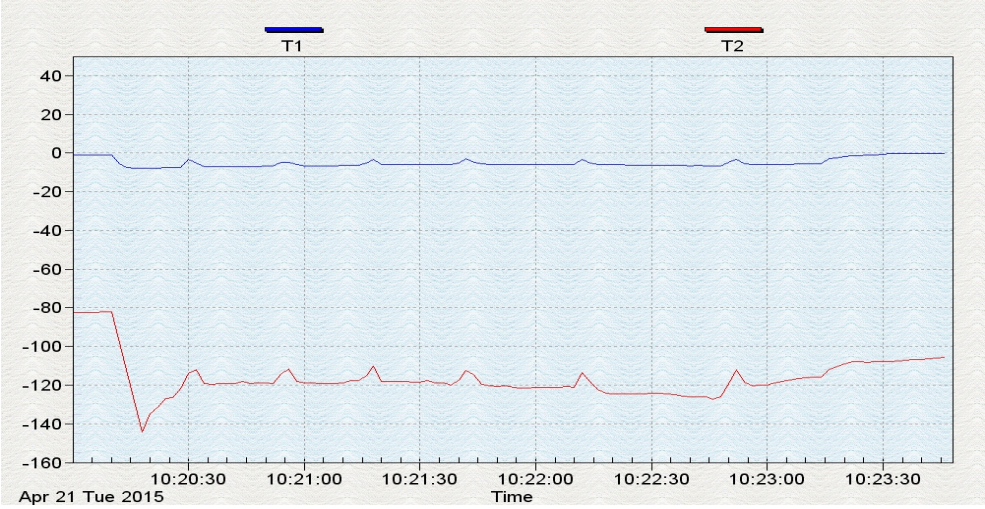


Figure 3.1: Temperature of breath at the exit of first stage (T1) and second stage (T2) during the course of collection

At the end of the sampling period, the valves of the system were closed and a Tedlar bag (Zefon int.) was connected to the outlet of the system. When the second stage is removed from the liquid nitrogen bath, the condensed VOCs inside the tube vaporize and build up pressure. This pressure difference forces the VOCs from the tube into the Tedlar bag. A syringe is connected to the outlet of the first stage and is used to collect the liquid condensate trapped in the tube. One end of the first stage tubing is capped whereas the other end is connected to the syringe. When suction is created, the liquid condensate is forced into the syringe. At the end of each collection, a purge cycle is employed where the tubing is flushed with iso-propyl alcohol in order to get rid of the contaminants. This ensures that there is no cross contamination between different breath samples.

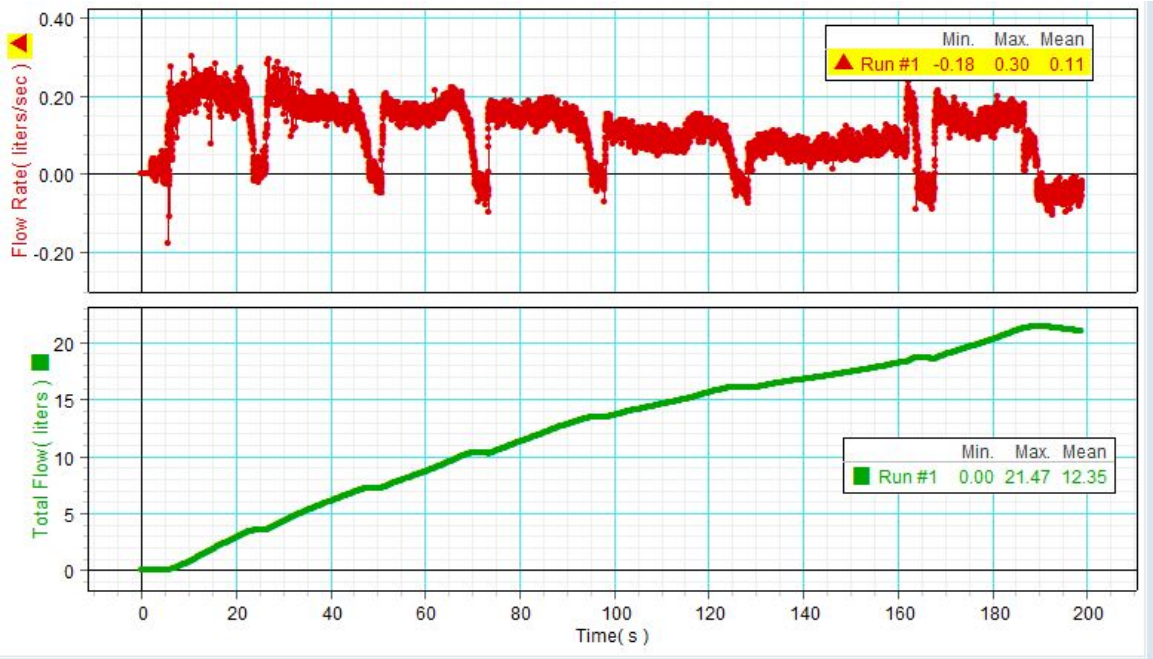


Figure 3.2: Flow rate and total volume of breath exhaled by a volunteer as read by a spirometer

3.2 Pre-concentration

Various methods of pre-concentration such as the use of carbon sieves, sorbent traps etc. have been used on VOCs. In our experiments, the direct injection of the VOCs from the Tedlar bag into the GC using gas tight syringes was first attempted. Due to the poor strength of signal, it was decided that SPME would be the preferred method of pre-concentration. As mentioned earlier, a SPME fiber is very similar to an inside-out GC column, where the fiber is coated with an adsorptive polymer. The SPME fiber is directly introduced into the Tedlar bag and left there to sample the VOCs for a specific period of time after which, it is introduced into the injection port of the GC where the analytes are desorbed into the column. There are two main factors that need to be considered while using SPME for sampling – one of them being the type of polymer coating that is used and the other being the actual sampling time. Three different SPME fibers were used for the sampling of the VOCs to determine which polymer was best suited for our application - PDMS-Carboxen, PDMS-DVB and PDMS-DVB-Carboxen fibers were each introduced into the Tedlar bag for 45 minutes before being injected into the GC-FID. All the fibers were first pre-conditioned at 230^{O}C in the injector of the GC to remove all the impurities in the fibers. Care was taken to ensure that they were directly moved to the Tedlar bag after pre-conditioning in order to avoid picking up anything from the atmospheric air. Figure 3.2 shows the comparison of the 3 fibers when they were run on a 30m long HP-5 column in a GC-FID. Since the PDMS-Carboxen fiber showed the strongest signals among the three fibers, it was decided as the fiber of choice for the experiments. Once the choice of fiber was decided, the Tedlar bag was sampled for different time intervals to determine the ideal sampling time. In figure 3.3, we see that the sampling time of 45 minutes displays the greatest signal strength.

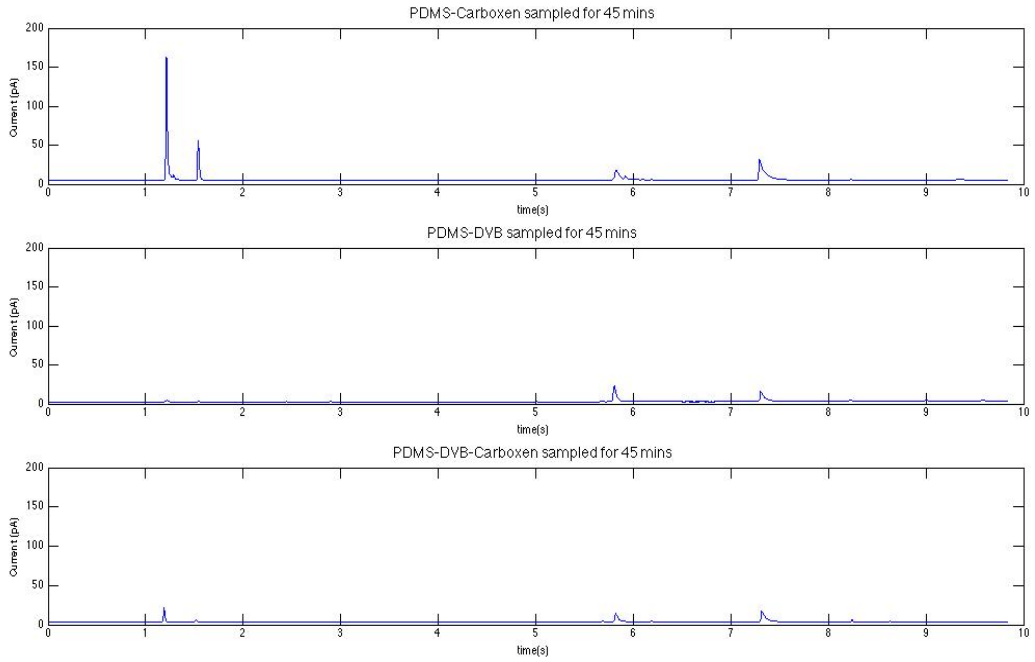


Figure 3.3: Comparison of different SPME fibers

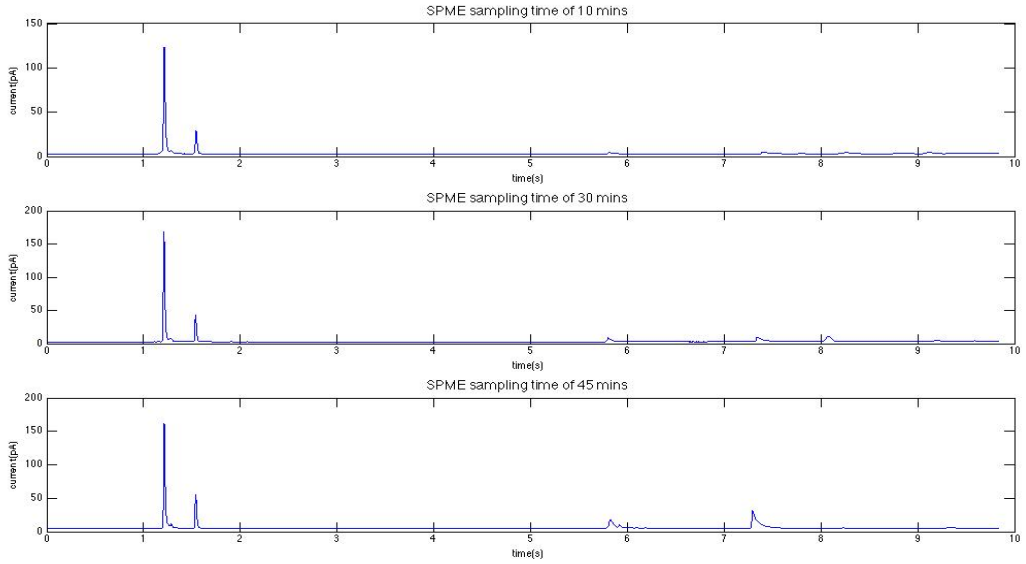


Figure 3.4: Comparison of different sampling times

3.3 Analysis of samples

The analysis of the breath samples was first done on the GC-FID. A 30m HP-5 column was used in the GC and the operating conditions were as follows. A pulsed-splitless injection at an injector temperature of 230^{O}C, initial oven temperature of 40^{O}C, a temperature ramp of 15^{O}C/min and a final temperature of 200^{O}C was used. Although the FID was able to detect some compounds, the need for compound identification and better separation led us to explore the option of a GC-MS. With the mass spectrometer, it was possible to identify the various peaks in the chromatogram based on their signature masses. The built-in library provided with the software matches the peak to a compound and provides a similarity match for reference. The GC-MS provided much more clarity on the compounds present in breath and their relative concentration.

Yet the biggest problem remained that the number of peaks on the chromatogram did not match up to the expected number of compounds in human breath. In order to get a better idea of the location of commonly occurring compounds in human breath, a set of standards were purchased and injected one by one into the GC-MS. Since a known compound was injected into the GC, identifying its elution time was trivial. In the same manner, all the standard compounds were individually injected into the GC and their elution time was noted. It was interesting to note that nearly 20 of the standard compounds co-eluted within 10 seconds of each other. As a result of this co-elution, many compounds of interest could not be identified on the chromatogram as compounds of higher concentration eluting at the same time masked them.

A simple solution to the above problem involved using a column that would cause the compounds to elute more slowly. After reviewing the products available in the market, it

was decided to go with the 30m poraplot Q column (Agilent Technologies, CA). The column was able to slow down the elution of all the compounds, thereby providing a much greater degree of separation with running time being the trade off. It also had no retention affinity for carbon dioxide, which was one of the compounds of high concentration, causing it to elute right at the beginning such that it did not co-elute and mask the trace compounds. However, due to the slowness of the poraplot column, the higher molecular weight EBCs virtually never eluted. This made it an impractical solution in situations where the VOCs and EBCs were to be analysed on the same column.

2- dimensional GC or GCxGC offered solutions to the above mentioned drawbacks of analyzing both the VOCs and EBCs on the same platform. 2-D GC, as the name suggests contains two different dimensions for the separation column – in our case, a 30m first dimension column and a 2m second dimension column. The properties of the 2-D GC is given below:

- GCxGC/TOF MS (Pegasus 4D, LECO, St Joseph, MI)
- First dimension GC column: Rtx-Wax (30m length, 250 mm column i.d.; Restek Corp., Bellefonte, PA)
- Second dimension GC column: Rtx-5 (2m length, 180 mm column i.d.; Restek Corp. Bellefonte, PA)
- Carrier Gas: Helium (99.999% purity; Nexair, Atlanta, GA)
- Data processing was done on software (Chromatof) native to the instrument

The same set of standard compounds that were run on the GC-MS were now run on the 2-D GC in order to determine the location of the commonly occurring compounds on

the chromatogram. The 2-D GC displayed superior separation quality, where the compounds that were previous co-eluting were now separated on the second dimension of the GC. The TOF MS attached to the system enabled the identification of these standards by providing the first and second retention time along with the signature masses and similarity hits.

Previously reported work on breath analysis focus on the total number of compounds seen in breath when analysed by GC. A key factor that is often overlooked is the presence of markers originating from exogenous sources. Such sources would include the background room air, analytes not fully desorbed from the SPME fiber, siloxanes and other impurities present in the GC column as well as contaminants in the Tedlar bag. In an attempt to be as comprehensive as possible while isolating biomarkers present in exhaled breath, samples of all the above mentioned exogenous sources were analysed to get an idea of these background compounds. A blank SPME fiber was run on the GC before every set of runs to determine any carryover in the fiber. SPME fibers were also placed in the room where the experiments were run and also in a Tedlar bag filled with ultra high purity helium in order to determine the compounds arising from these sources. These chromatograms were later compared with actual breath chromatograms to try and distinguish the endogenous compounds present in the breath from the compounds arising from these external sources.

The next step was to analyse the actual breath samples provided by the volunteers. 3 healthy, non-smoking volunteers were selected to provide breath samples for the experiment. One of the volunteers provided 3 breath samples in a day – in the morning, afternoon and the evening and also provided breath samples on 3 consecutive days in the

morning. One single breath sample was collected from the other two volunteers in order to compare breath samples across three different persons. After each collection, the system was thoroughly rinsed with IPA in order to get rid of any residual impurities. Each of the VOC samples was sampled for 45 minutes before being analysed in the 2-D GC. The EBC samples collected from the volunteers were first derivitized by a TMAH derivitization protocol before being injected into the GC with a syringe. The various samples were run on the GC and the analysis of the data is presented in the next section.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 1-D Gas Chromatography

A chromatogram of a breath sample run on a 1-D GC-FID is shown in figure 4.1. It is observed that a number of compounds elute right at the beginning followed by a period of inactivity. This is followed by the elution of two tailing peaks and a peak of low signal strength right at the end. In order to validate the effectiveness of the sampling system, a volunteer was made to breathe directly into a bag, which was then sampled using the same PDMS-Carboxen SPME fiber and run on the GC-FID. The chromatogram of the breath sample run through the system is compared with that of the direct breath in figure 4.2. It is clear that the signal strength in the case of the sample run through the system in more than an order of magnitude higher than the other case. This validates the fact that the sampling system is effective at preconcentrating the VOCs present in the breath sample.

A striking feature of the 1-D chromatogram shown in figure 4.1 is the lack of distinct peaks. Although the number of compounds reported in human breath exceeds a hundred in literature, there are only three major peaks in the 1-D chromatogram. A closer inspection of the first peak reveals a couple of shoulder peaks, but they still do not add up in terms of the number of peaks expected in human breath. In order to get a better idea of the compounds present, the GC was connected to a MS detector and the peaks were identified. The largest peak in the first elution was identified as carbon dioxide whereas the shoulder peaks were identified as acetone and ethanol. Since a large fraction of

exhaled breath consists of carbon dioxide, it is easy to see that it floods the chromatogram and potentially elutes over VOCs having low concentration.

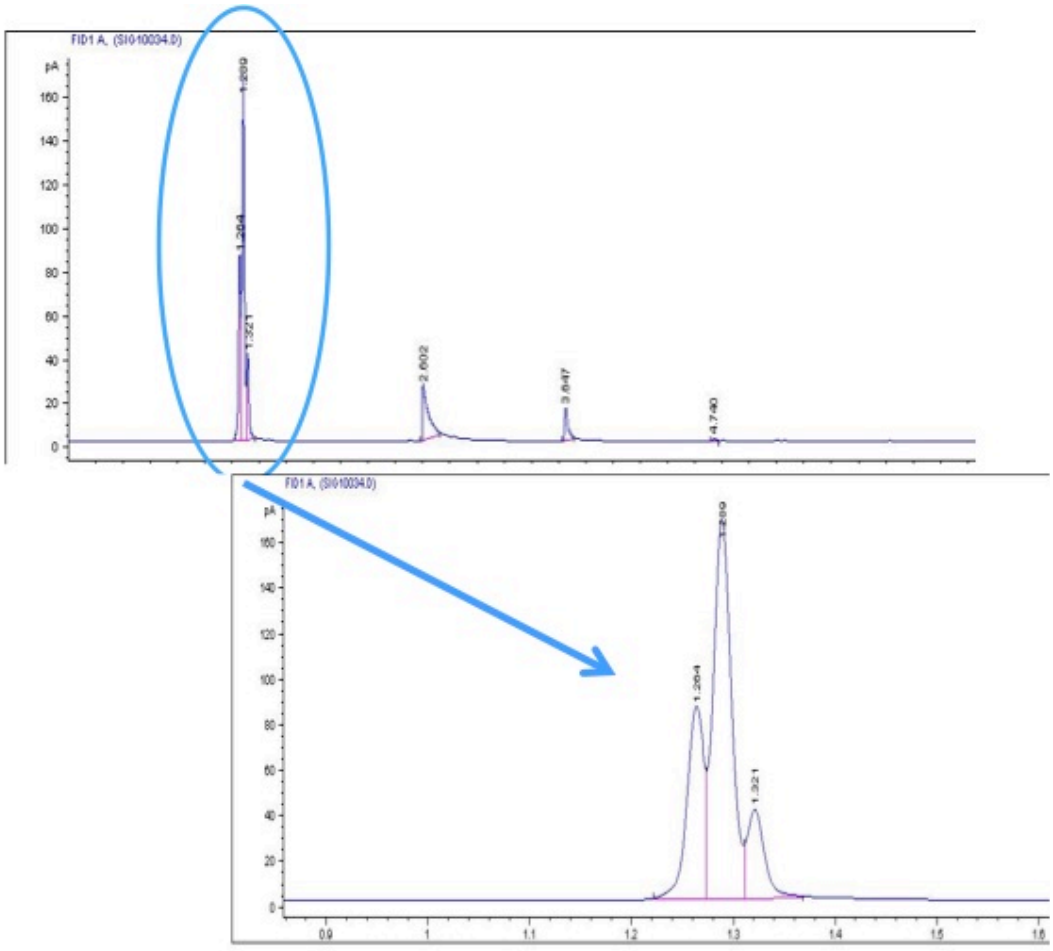


Figure 4.1: A GC-FID chromatogram of exhaled breath

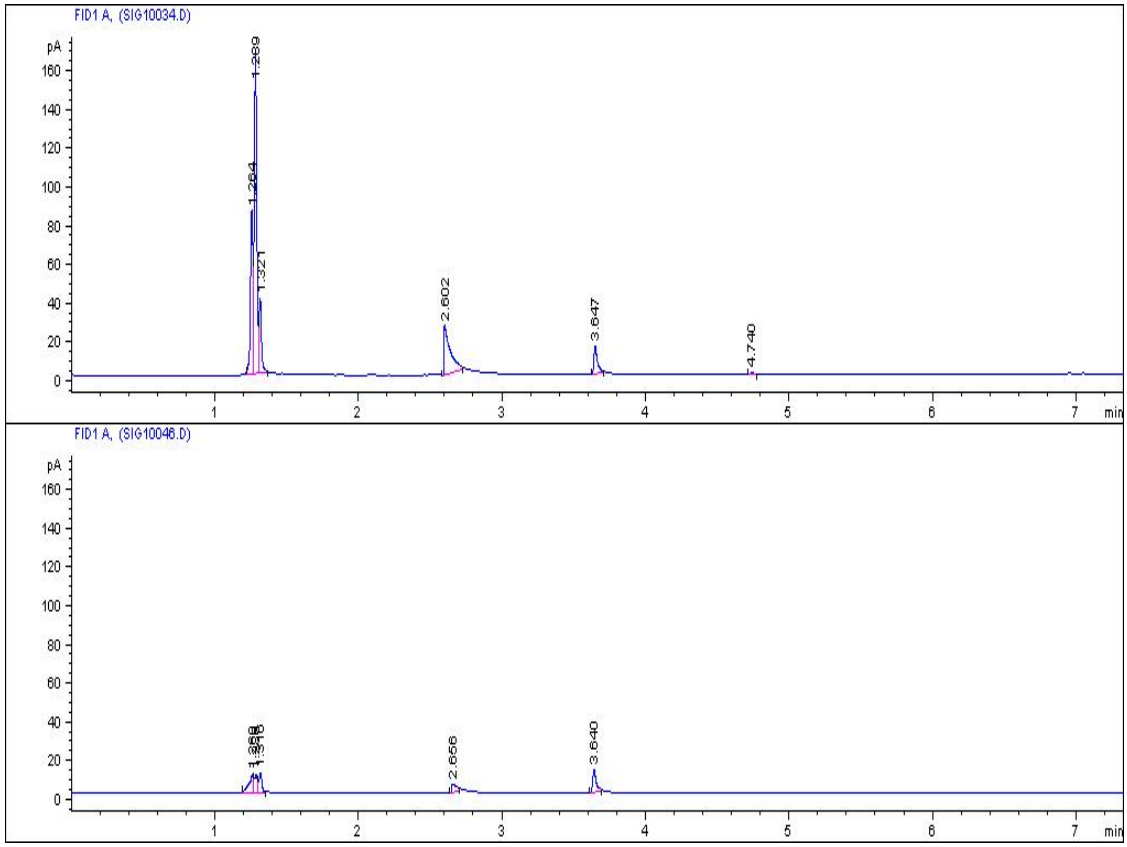


Figure 4.2: A comparison of exhaled breath concentrated by system (top) vs direct breath (bottom)

The same holds with Acetone and Ethanol, which are compounds found in higher concentration in exhaled human breath. Hence it is possible that the trace level VOCs were captured by the breath sampling system and the SPME fiber, yet the sheer concentration of carbon dioxide and other VOCs of large concentration caused them to be overshadowed on the chromatogram.

In order to confirm the presence of trace VOCs, a set of standards (table 4.1) was purchased and injected one at a time into a GC-MS. The RTX-5 column was retained and the GC conditions were the same as mentioned in the previous chapter. Although the

detector used in this case is the MS, the retention time and the retention order of the compounds will remain the same as it is only dependent on the GC conditions and the column used. The standards were diluted in Hexane before injection. This was done in order to avoid injecting a high concentration of the standard and flooding the column. If the concentration is low, the peaks are sharp and peak broadening and tailing is avoided.

From table 4.1, we can see the retention times of the 46 standards along with the similarity match on the GC and the signature mass detected for each compound. The time corresponding to the peak apex was recorded while noting the retention time of each standard. As predicted, it can be seen that approximately 15 other compounds elute within the first ten seconds of the first peak elution. In general, the time between eluting compounds is not more than a few seconds and there are distinct regions of peak concentration. For example, there is a large group of compounds that elute within the first minute of the run. From there on the congestion of compounds is slightly less and the elution times are more spaced out. There is a small group of compounds eluting together right at the very end as well, but a majority of the compounds are grouped at the beginning. This result clearly explains the lack of compounds seen when an actual breath sample was run on the GC-FID. The chemical properties of the RTX-5 column are such that the affinity towards the target compounds is very low. Due to this, the analytes are not separated very much by the column before eluting. Also when a compound of high concentration such as carbon dioxide elutes at around the same time, due to the broad nature of the concentrated peak, the lower concentration VOCs are hidden behind the

Table 4.1: List of standards with retention time on 1-D GC

	Compound	Retention time (s)	MS Similarity match	Signature m/z
1	Acetaldehyde	26.1015	880	44
2	Methanol	26.174	882	29
3	Acrolein	28.7418	947	56
4	Pentane	29.033	940	57
5	Acetone	29.1021	858	58
6	Propanal	29.2636	920	58
7	Furan	29.3158	869	68
8	Ethanol	29.4268	909	45
9	Isoprene	29.6693	926	67
10	Acetonitrile	29.735	864	41
11	Carbon Disulfide	33.4208	906	76
12	2-Methyl Propanal	34.2442	889	72
13	Cyclopentane	34.9134	822	69
14	3-Methyl-pentane	34.98	944	71
15	Methacrolein	35.6233	895	70
16	2-Methyl-pentane	36.8935	929	71
17	2,3-Butanedione	38.0119	868	43
18	Hexane	39.419	952	86
19	2-Butanone	40.3322	839	72
20	Methyl Vinyl Ketone	40.7424	882	70
21	2-Methylfuran	41.3123	935	82
22	1-Propanol	42.485	908	59
23	Methylcyclopentane	45.0089	935	69
24	Ethyl Acetate	45.2254	864	88
25	Cyclohexane	53.4848	947	84
26	Benzene	55.2569	888	78
27	Hydroxyacetone	56.9033	845	74
28	2-Pentanone	64.2898	870	86
29	1-Butanol	65.2746	906	56
30	Pentanal	68.9218	893	86
31	2,4,4-Trimethyl-1-pentene	73.0464	893	97
32	2,5-Dimethylfuran	73.7824	901	96
33	Toluene	115.957	867	91
34	Hexanal	149.966	878	56
35	Ethylbenzene	242.713	826	91
36	Styrene	305.665	786	104
37	o-Xylene	310.629	702	57
38	Nonane	336.381	843	128
39	Benzaldehyde	573.38	796	106
40	Decane	578.755	923	142
41	6-Methyl-5-hepten-2-one	590.326	889	108
42	Butyrolactone	633.947	906	42
43	Acetophenone	708.933	952	105
44	2-Propanol	920.965	934	43
45	Dimethyl Disulfide	693.546	923	62
46	2-Pentene	805.345	940	56

peak. As a result, the chromatograph displays three large, broad peaks and very little else in the region of interest.

In order to obtain better separation of the compounds, a Poraplot Q (Agilent) column was installed on the GC-MS and the standards were run using the same conditions as before. The entire set of standards was combined and diluted before injection. In the case of the Poraplot, the system was run for an hour. It was seen that a few compounds eluted almost immediately at the beginning of the run. After this there is a period of inactivity before the next compound elutes at around the 800-second mark. From there on, there is a slow, distinct elution of the standards with little or no overlap. This is a significant improvement over the previous column, where a large number of compounds co-eluted. Further MS investigation revealed the compound that eluted at the beginning to be carbon dioxide. The chemistry of the column is such that the carbon dioxide has little or no retention and hence is naturally separated from the other compounds of interest. This mitigates the problem previously faced in the RTX-5 where the carbon dioxide was completely masking the other compounds. Hence a clean, well-separated spectrum of breath is obtained without the interference of spurious compounds.

Although the Poraplot does a great job separating the VOCs, the decision to move to the 2-D GC was taken due to the amount of time taken for the separation. Since breath analysis involves both the EBC and VOC samples, the amount of time taken to separate the EBC samples must be considered. Due to the high molecular weight and non-volatile nature of the EBCs, a larger run time and a higher temperature ramp is needed for effective separation. The maximum temperature the column can be heated as specified by the manufacturer is $250^{O}C$. This is a lot lower when compared to columns such as the

RTX-5 and RTX-50, which can be heated up to 300^{O}C. Hence if the objective is to analyse both the VOCs and EBCs on the same system, the Poraplot column is not a practical option. This led to trials with the 2-D GC.

4.2 2-D gas chromatography

A default column set of ZB-5 for the first dimension and ZB-50 for the second dimension was chosen for the first set of experiments on the 2-D GC. The set of standard compounds were injected one at a time in order to determine the retention time for each of the compounds. Figure 4.3 is a peak apex plot of the standards on the default column set. It can be seen that the same compounds that were all co-eluting on the 1-D GC, are now separated based on their retention time in the second dimension. However, there is scope for improvement as the retention times of the compounds are very low and they are still concentrated at the beginning of the run.

In order to achieve optimum separation of compounds on the 2-D GC, a column set selection, which is beyond the scope of this thesis, was performed in order to select the best combination of columns for the first and the second dimension. It was decided to go with the reversed orthogonality set of RTX-wax for the first dimension and ZB-50 for the second dimension. The RTX-wax slows down and separates the compounds on the first dimension whereas the RTX-50 provides an additional level of separation. A 2-D chromatogram of exhaled breath is presented in figure 4.3. The GC conditions are reported in the previous chapter. A 1-D chromatogram on the RTX-5 column is also shown in order to compare the separating power of the 2-D GC with the conventional 1-D GC.

It is immediately apparent that the 2-D chromatogram is much richer in terms of the number of compounds separated. The compounds that were previously hidden behind the larger peaks in figure A are well separated along the second dimension in figure B. Since the operating conditions for both the 1-D and the 2-D GC are the same, the location of the large peaks in both the chromatograms remains the same, but the smaller peaks appearing in the 2-D GC are not seen on the 1-D GC. A convolution time of 7 seconds is chosen for the second dimension in order to prevent peak folding.

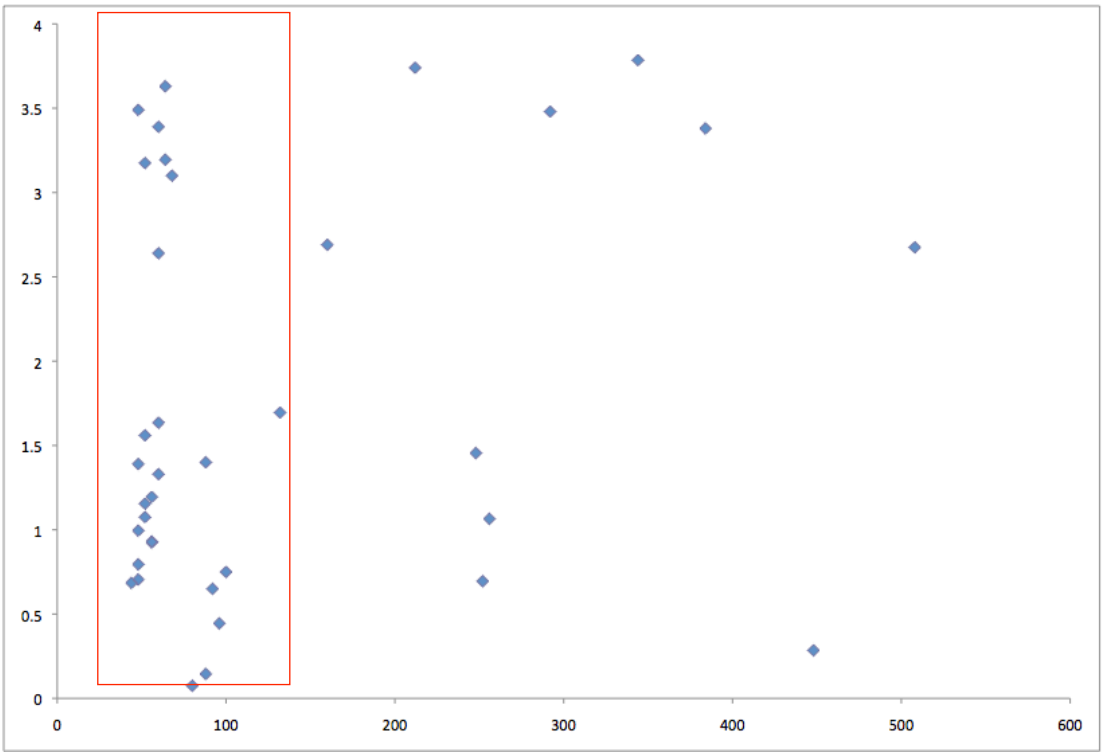


Figure 4.3: Peak apex plot of standards on ZB-5/ZB-50 column. Marked in red is the separating power of the 2-D GC

4.3 2-D GC data analysis

The 2-D chromatogram shown in figure 4.4 was then analysed by the CromaTOF software to identify the various compounds present in the breath sample. By using the

data from the Time-of-flight mass spectrometer, the software is able to assign a compound name and CAS number to each of the identified peaks based on their signature ions. The user can specify the height and width threshold for the software to identify a peak. The software returned 5639 compounds with various similarity hits for the breath sample. It is important to note the way the software identifies the various compounds. Based on the threshold parameters entered for height and width, the software identifies a particular area on the chromatogram as a compound. If the width of the peak is too large, the software breaks the peak into multiple sections and identifies each of them as a separate compound i.e. if the peak concentration is large or there is trailing, the software will break the large peak into multiple smaller peaks and assign an identity to each of these small peaks. Due to this, it is possible that many of the compounds occurring at higher concentration are identified as peaks at multiple locations. Hence it is safe to say that the software generates a list of identified peaks rather than compounds. An example of such a plot is shown in figure 4.5.

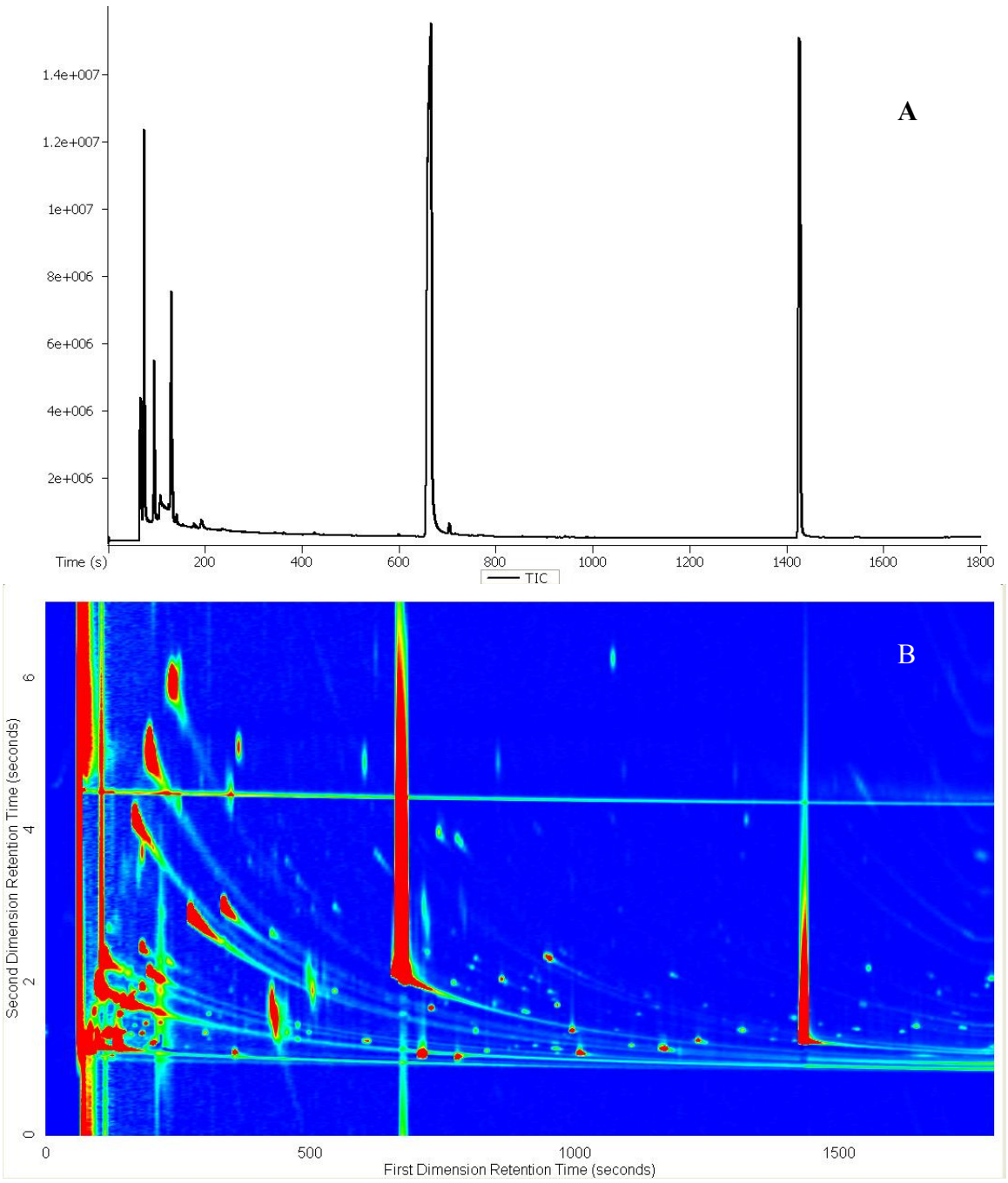


Figure 4.4: A) Conventional GC/MS chromatogram of exhaled breath B) GCxGC/MS contour plot of same sample

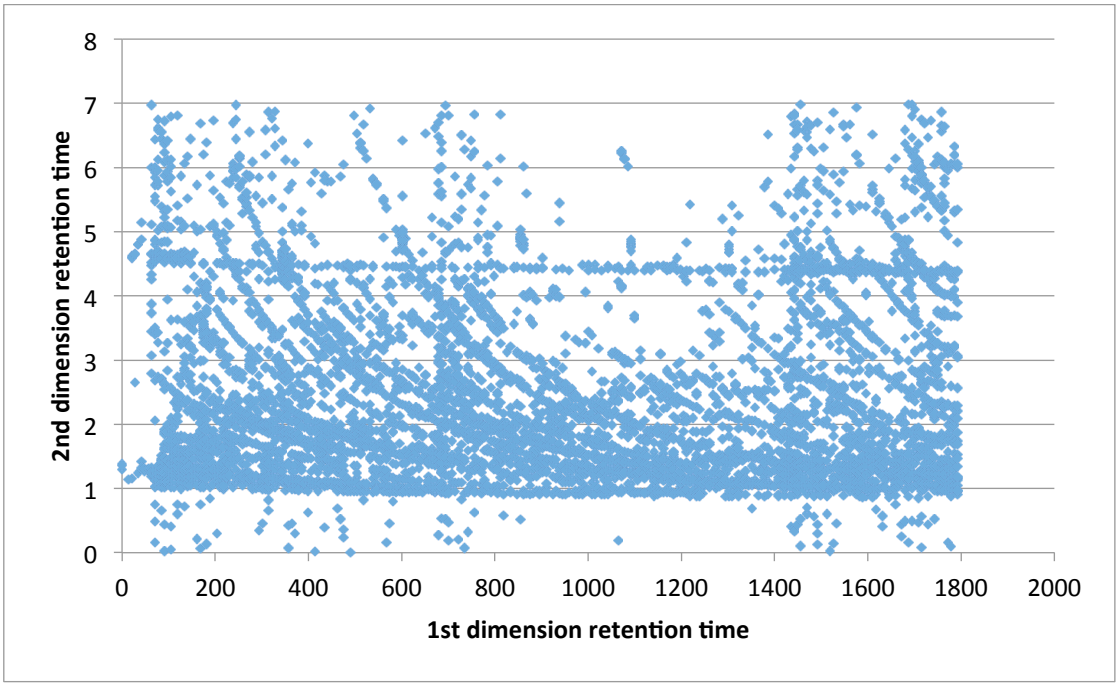


Figure 4.5: Plot of peaks identified by the ChromaTOF software

In order to get a better idea of the actual number of compounds present in human breath, it is necessary to translate the total number of peaks identified by the software into a set of actual compounds. For this, what is known as a 'peak combine' was performed. Basically, the data was sorted by CAS number followed by compound name. If the same compound appeared more than once at different locations for a particular sample, the corresponding peaks were all grouped together and represented as a single peak. Amongst all these peaks, the one having the largest area is chosen as the representative peak. For example, if 12 different peaks occurring at different retention times were assigned the CAS number for acetone, the peak having the largest area is used to represent the compound and the rest of the peaks are removed from the chromatogram. This method of combining peaks to compounds is a better representation of the total number of compounds present in a sample. A peak combine performed on the raw

chromatogram in figure 4.5 is shown in figure 4.6. This process considerably reduced the chromatogram from 5639 or so peaks to about 2025 compounds.

The chromatogram obtained after the peak combine represents the compounds present in exhaled breath, Tedlar bag, SPME and the GC-column. In order to make it truly representative of the endogenous compounds present in exhale breath, a further reduction was performed. As mentioned in the previous chapter, the contents of the Tedlar bag and SPME were analysed by GC. The compounds occurring due to these two sources were compared with the reduced chromatogram obtained from peak reduction and the commonly occurring compounds were removed (figure 4.7). In order to remove only the significant compounds occurring in the bag and the SPME, the areas of the occurring compounds were first filtered and only those compounds that had an area greater than 10000 units were compared to the breath sample. Next, the compounds common to just the Tedlar bag/SPME and the breath sample were compared according to their relative areas. The compounds that appeared in a higher concentration in just the tedlar bags were removed and the remaining compounds were retained. This way, approximately 40 compounds that were definitely contaminants form the Tedlar bag and SPME were removed from the chromatogram. Finally, the siloxanes occurring in the reduced chromatogram were also removed (figure 4.8) as they are compounds typically seen due to column bleed and rarely occur in exhaled breath. All compounds having a

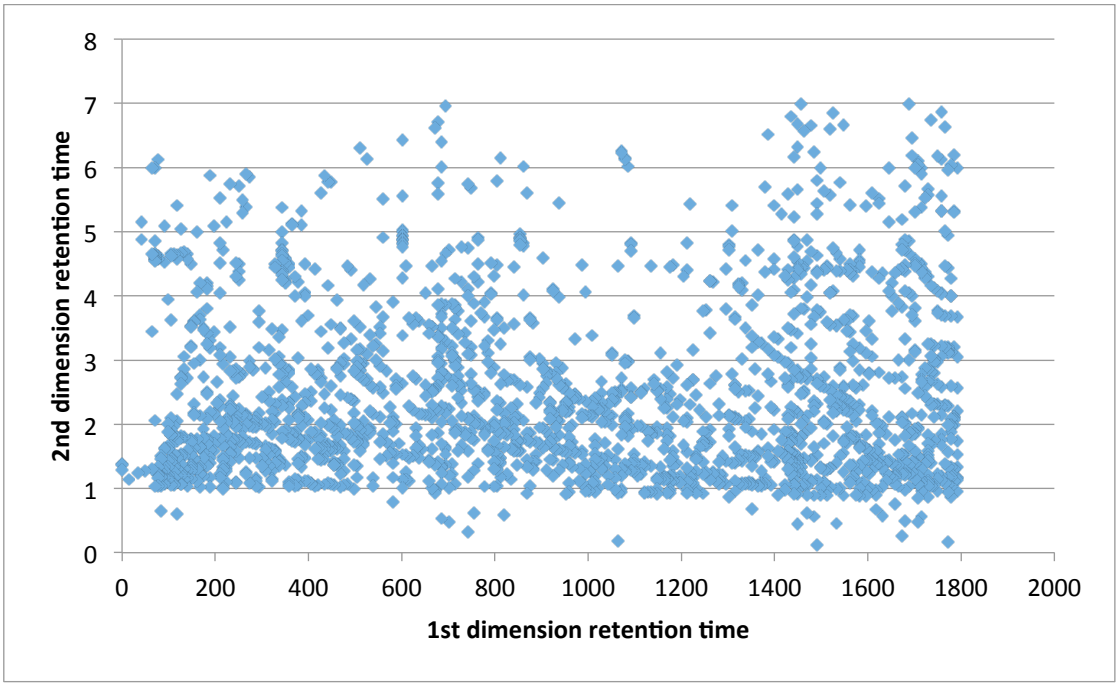


Figure 4.6: Peak combine applied to raw chromatogram data

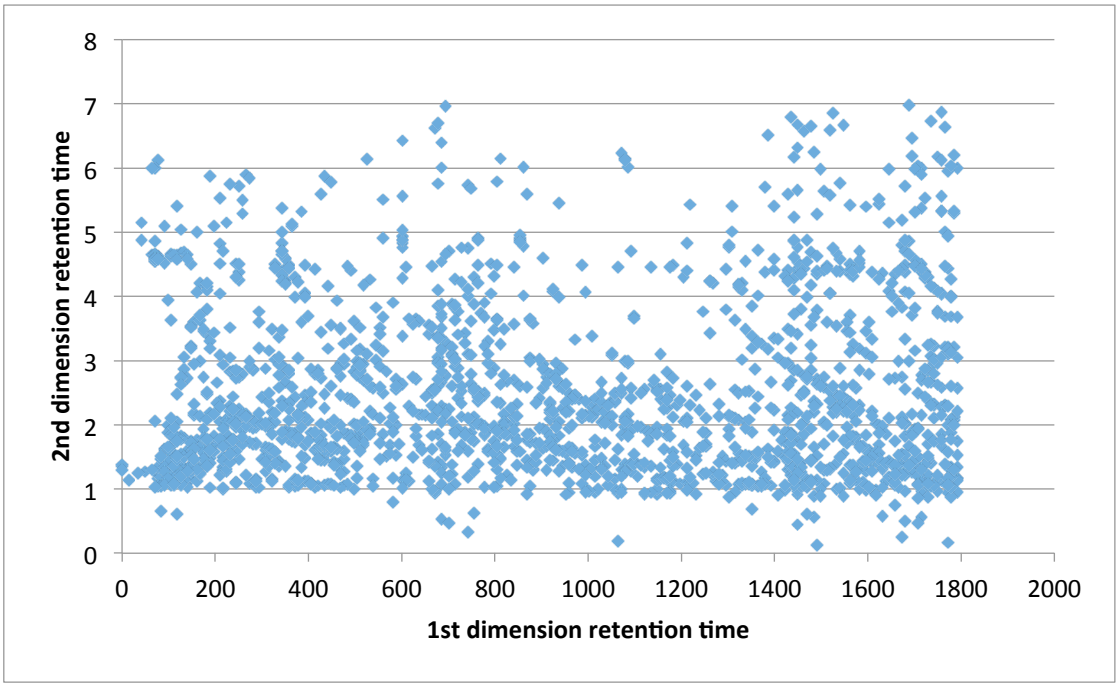


Figure 4.7: Chromatogram after removal of bag compounds

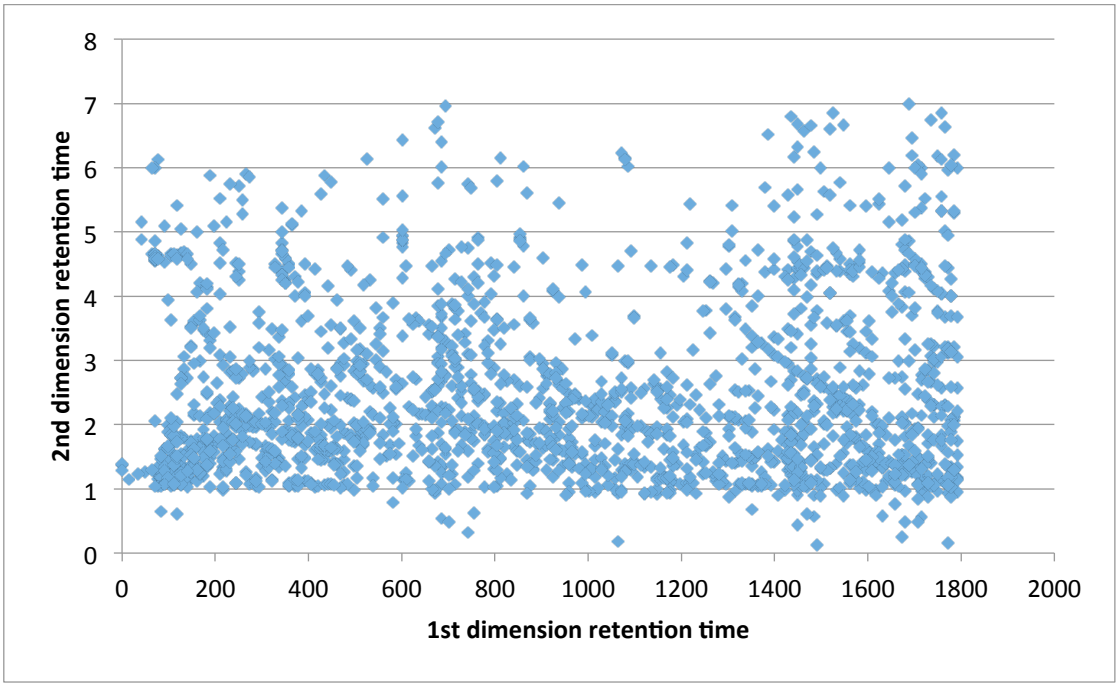


Figure 4.8: Chromatogram after removal of siloxanes

peak area less than 10,000 were removed as well. It was found that the similarity match for these compounds was really low and the software was unable to identify the compounds with a fair degree of certainty. Hence these compounds were disregarded as unknowns.

The final chromatogram after all the reductions is shown in figure 4.9 and has exactly 664 unique compounds. As can be seen, this data is clear and a better representation of the endogenous breath compounds. A search for the purchased standard compounds revealed that 42 of the 46 compounds were present in the final breath chromatogram. The standards have been marked with red dots in figure 4.10. Thus it is safe to conclude, that apart from the compounds reported as confirmed in literature, there are many other

compounds of interest present in exhaled breath, which could potentially be investigated further.

It is safe to say, the data was filtered at every step and utmost care was taken to ensure that the final chromatogram is as close to the true representation of exhaled breath. By following this method, false positives arising from external factors such as the Tedlar bag and SPME fibers, as well as internal sources such as column bleed and noise have been eliminated to a large extent. The reversed orthogonality column does a good job of separating the compounds on both the first and second dimension and a good spread is seen. Although the large cluster of data from the original chromatogram has been reduced to a more digestible form using the data reduction techniques, there is significant scope for improvement in the manner of data reduction.

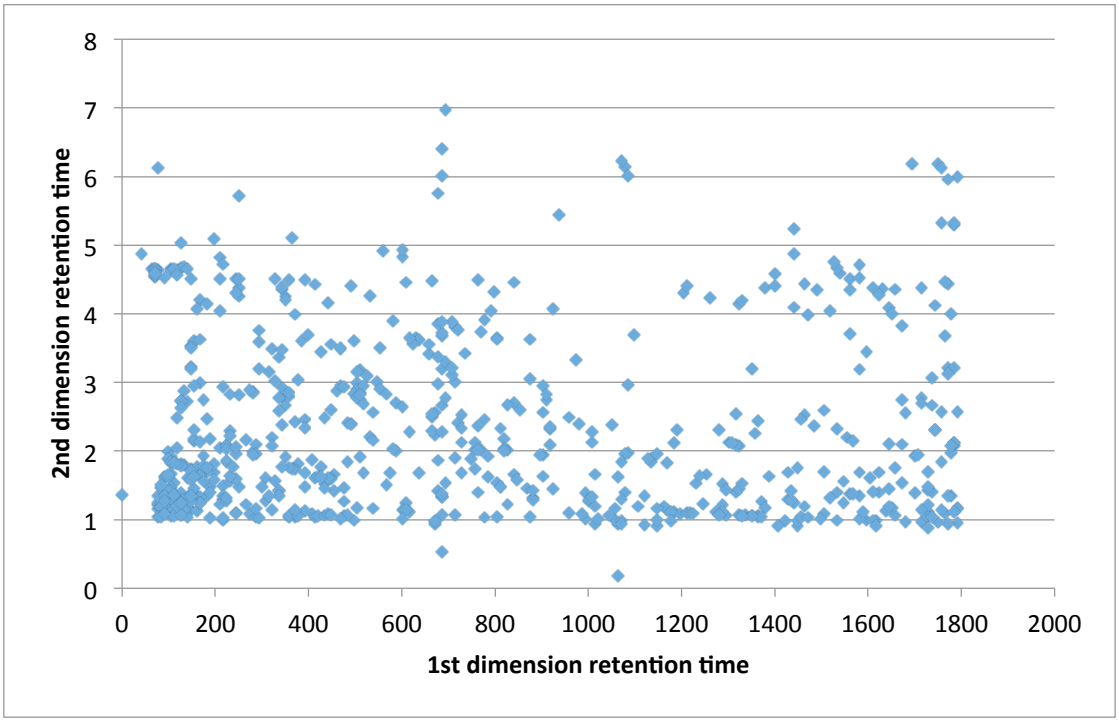


Figure 4.9: Final chromatogram

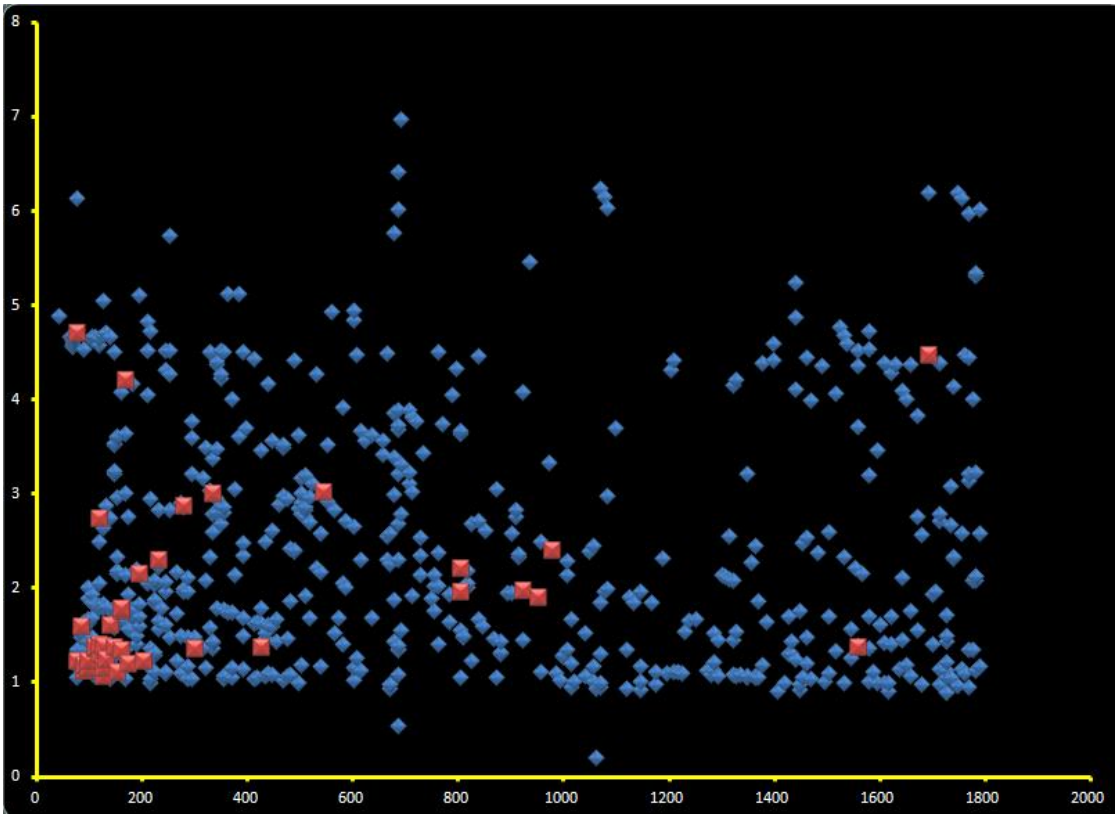


Figure 4.10: Peak apex plot of EBC sample with standards from table 4.2 highlighted in red

4.4 Comparison between breath samples

The data reduction technique was applied to each of the three breath samples collected from the same volunteer. The samples were collected on the same day, in the morning, afternoon and evening and stored in Tedlar bags. The samples were run on the 2-D GC, and the data obtained was processed by the method previously described.

The final compounds present in each of the breath samples were then compared with each other in order to see how many compounds were common between the samples. It was found that out of the 664 compounds that were determined to be uniquely present in one

breath sample, 98 compounds were present in at least two of the samples. Furthermore, only 18 compounds were found to be present in all the three samples.

Table 4.2: Standards identified in breath sample

Serial No.	Name	CAS	Unique Mass	RT 1	RT 2
1	3-Methylpentane	96-14-0	41	77	1.22
2	Acetaldehyde	75-07-0	29	77	4.7
3	Carbon disulfide	75-15-0	76	84	1.28
4	Isoprene	78-79-5	68	91	1.12
5	2-Methylpentane	107-83-5	43	91	1.12
6	Dimethyl sulfide	75-18-3	62	91	1.18
7	Furan	110-00-9	68	98	1.145
8	Acetone	67-64-1	43	98	1.22
9	2-Methylfuran	534-22-5	39	112	1.35
10	2-Methyl-propanal	78-84-2	72	112	1.37
11	Methacrolein	78-85-3	70	119	1.3
12	Ethyl acetate	141-78-6	88	119	1.405
13	Nonane	111-84-2	128	119	2.735
14	Methanol	67-56-1	29	126	1.06
15	Propanal	123-38-6	27	126	1.145
16	2-Butanone	78-93-3	72	126	1.385
17	Isopropyl alcohol	67-63-0	59	133	1.24
18	Benzene	71-43-2	78	140	1.605
19	Methyl vinyl ketone	78-94-4	55	147	1.37
20	Cyclohexane	110-82-7	84	154	1.1
21	2,3-Butanedione	431-03-8	43	161	1.335
22	2-Pentanone	107-87-9	86	161	1.75
23	Pentanal	110-62-3	44	161	1.77
24	Decane	124-18-5	142	168	4.2
25	Acetonitrile	75-05-8	41	175	1.195
26	Toluene	108-88-3	91	196	2.15
27	1-Propanol	71-23-8	59	203	1.225
28	Hexanal	66-25-1	44	231	2.29
29	Ethylbenzene	100-41-4	91	280	2.865
30	1-Butanol	71-36-3	31	301	1.35
31	o-Xylene	95-47-6	106	336	299
32	Styrene	100-42-5	104	427	2.66
33	Pentane	109-66-0	72	490	1.1
34	1-Hydroxy-2-propanone	116-09-6	43	497	1.36
35	6-Methyl-5-hepten-2-one	110-93-0	108	546	3.01
36	Methylcyclopentane	96-37-7	56	805	1.955
37	Benzaldehyde	100-52-7	106	805	2.215
38	Hexane	110-54-3	86	924	1.975
39	Butyrolactone	96-48-0	42	952	1.89
40	Acetophenone	98-86-2	105	980	2.395
41	Acrolein	107-02-8	56	1561	1.375
42	Cyclopentane	287-92-3	42	1694	4.475

In conclusion, a breath-sampling device capable of capturing both the liquid fraction as well as the volatile compounds presents in exhaled breath was constructed. This device was further extended to a two-channel breath sampling system that is capable of separating the dead-space air from the alveolar air in exhaled breath. The captured samples, which were stored in tedlar bags, were then extracted using SPME and analysed using gas chromatography. The analysis was performed using various configurations on both 1-D and 2-D GC and the results were documented and reported. Due to its superior resolution and separation, the 2-D GC was the instrument of choice for breath analysis. The data from the GC was further analysed and a unique data reduction technique was applied on the raw data obtained from the GC. This technique enabled us to refine the 5000 odd peaks obtained by the GC software to a more realistic 664 compounds by eliminating background contamination as well as noise from the system. The observations from the data reduction experiments further emphasize the need to develop a standardized method for breath collection as well as analysis. Finally a comparison was performed on samples collected at different times of the day from the same volunteer and it was found that 18 compounds were present in breath at all times of the day.

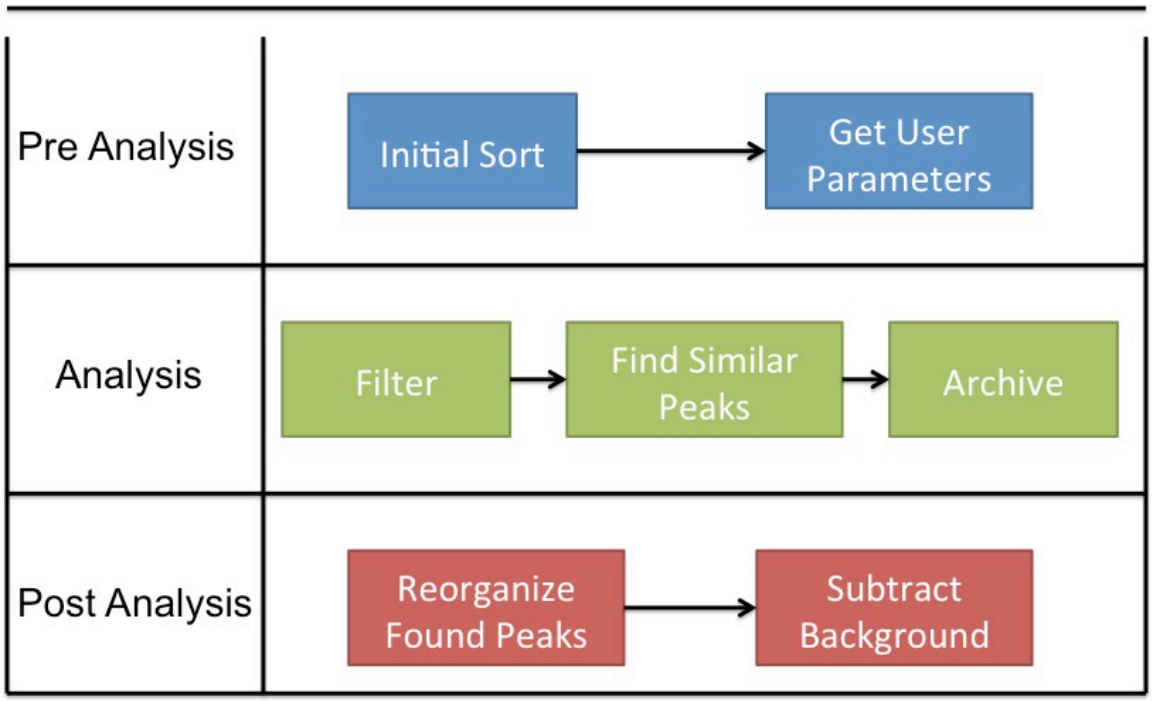


Figure 4.11: Chromatogram data processing algorithm highlighting the different steps during data reduction

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Exhaled breath analysis has garnered significant interest and attention as a means of non-invasive medical diagnostics. However, the lack of a standard method of sample collection, control of variability, reliable detection at trace level and quantification of breath samples has held back exhaled breath analysis when compared to more conventional techniques such as blood and urine analysis.

In this work, a novel breath sample collection system that is capable of capturing both the volatile and non-volatile fractions of exhaled breath is designed and constructed. The collection system makes use of two distinct temperature zones to condense and capture the non-volatile and volatile fractions in liquid and gaseous states. The breath flows through stainless steel tubes immersed in these cold baths, and as the temperature of the breath decreases, it condenses on the walls of the tube. The liquid portion is collected in syringes whereas the gaseous VOCs are stored in Tedlar bags. The design of the sampling system is further extended to separate the dead-space breath from the alveolar breath. This is done by introducing a second 'channel' or a set of tubes to separate the flow of breath into two different collection regions. The flow of the air into the two channels is regulated by means of a pressure sensor and a solenoid valve. Based on the pressure, the first half of the exhaled breath (dead-space breath) is directed through one channel and the second half (alveolar breath) is directed through another channel.

The collected breath samples were sampled from the Tedlar bags by means of SPME. Different coatings and sampling times were compared before settling on a PDMS-

Carboxen fiber with a sampling time of 45 minutes. The samples were then analyzed by gas chromatography. Both 1-D and 2-D gas chromatography was used to separate the compounds in exhaled breath. Many different column configurations were tried on the 1-D FID GC, but the separation power of the single dimension column was limited. Although the PoraplotQ column displayed good separation, the long run time was not favorable for this particular application. The 2-D GC was able to provide a chromatogram of higher resolution as well as greater separation. The overlapping of compounds seen on the 1-D GC was absent on the 2-D GC. Coupled with a time of flight (TOF) mass spectrometer, more than 5000 peaks were identified on the 2-D GC chromatogram, which was the instrument of choice for the analysis of exhaled breath.

In order to separate the endogenous breath compounds from exogenous compounds, a unique data processing tool was applied to the raw data obtained from the commercial GC software. The two main features of this tool were the combination of peaks into compounds and the subtraction of background compounds. The peaks identified by the GC software were combined into compounds based on their CAS number and chemical formula. These compounds were further filtered by removing the ones that were also present in the SPME blank and Tedlar bag. The noise level compounds were also subtracted based on the assumption that the probability of correct identification was low due to the weak signal strength. This way, we were left with a reduced set of compounds, which were a more accurate representation of the actual compounds present in exhaled breath. A comparison of three samples of breath collected from the same person at different times of the day revealed that out of a maximum of 664 compounds present at most in any sample, 98 compounds were present in at most 2 samples and only 18

compounds were present in all 3 samples. The compounds found in all three samples are listed in table 5.1.

Table 5.1: List of compounds common to all 3 breath samples

Compound	RT 1	RT2	Formula	CAS number
Eucalyptol	364	5.09	C10H18O	470-82-6
Unknown 1677	1078	6.12	C13H17NO2	185957-97-5
Phenol, 2,4-di-t-butyl-6-nitro-	1519	4.045	C14H21NO3	20039-94-5
4-(1-Hydroperoxy-2,2-dimethyl-6-methylene-cyclohexyl)-pent-3-en-2-one	1554	2.2	C14H22O3	125284-20-0
Butylated Hydroxytoluene	1323	4.145	C15H24O	128-37-0
Pentadecanoic acid	1764	3.68	C15H30O2	1002-84-2
Guanidine carbonate	1463	1.19	C3H12N6O3	593-85-1
2-Propenenitrile	168	1.235	C3H3N	107-13-1
Propane, 1-(methylsulfinyl)-	1085	1.98	C4H10OS	14094-08-7
Propane, 1-(methylthio)-	133	1.795	C4H10S	3877-15-4
Pyrazine	378	1.81	C4H4N2	290-37-9
2-Butanone, 3-hydroxy-	476	1.46	C4H8O2	513-86-0
p-Dithiane-2,5-diol	189	1.48	C4H8O2S2	40018-26-6
Butane, 2-(methylthio)-	154	2.18	C5H12S	10359-64-5
Benzonitrile	917	2.085	C7H5N	100-47-0
Hexane, 2,3,4-trimethyl-	98	1.995	C9H20	921-47-1
Methane, dibromochloro-	483	1.85	CHBr2Cl	124-48-1
Methane, bromodichloro-	308	1.6	CHBrCl2	75-27-4

5.2 Recommendations

In this work, a two-stage system for breath collection was designed and implemented. If such a system is to be used in a clinical setting, the two most important factors that must be considered in the design is the portability of the system and the ease for patients. In its current form, the prototype system consists of lengthy tubing and requires a

constant supply of liquid nitrogen for the cooling of the second stage. Miniaturizing the system by reducing the length of tubing as well as finding an more portable alternative to the current cooling method is something that could be considered. Reducing the pressure drop across the system such that it makes for easier patient breathing can be looked into as well. A standardized method of sample collection in terms of breath volume collected will definitely play a big role in quantitation of the compounds

Tedlar bags and SPME were the methods of collection and sampling used in this work. Although Tedlar bags are convenient in terms of handling and storage, they introduce a significant number of exogenous compounds that could be avoided during analysis. The use of polished canisters and adsorbent traps has been reported in literature. The sampling on SPME can be avoided as well by maybe directly introducing the breath sample into the analysis system. As SPME is based on surface adsorption, the analytes having higher concentration tend to occupy more of the surface sites as opposed to the low concentration analytes. This makes quantitation using SPME very tricky and challenging.

In terms of analysis, 2D GC proved to be better than 1D GC thanks to its superior separating power. However, 2D GC with a TOF mass spectrometer is an expensive instrument and not very portable. Furthermore, selecting the correct combination of column sets for breath analysis is something worth investigating. The separation of the breath compounds is heavily dependent on the columns used, and selecting the wrong column set doesn't really provide the 2D GC with any significant advantage over the 1D GC. Also, a study on the analytes endogenous to the GC column is worth performing as a blank run on the GC still spits out many compounds and sometimes carry overs from the

previous analyses. Reducing this would definitely help filter the final list of exhaled breath compounds.

The data processing done in this work is unique in the area of breath analysis as it has not been done by anyone else previously as per our knowledge. A number of previous articles on exhaled breath claim a large number of detected exhaled compounds, however there is very little work done in trying to determine whether these compounds are actually from the human body or other external sources. As we have found out, a number of compounds seen by the GC are in fact contaminants from sources such as the SPME fiber and the Tedlar bag. Further work must be done to determine the effect of one's surroundings during the breath collection process. For instance, if the patient's breath is being collected in a clinical setting, it is possible that the levels of common clinical disinfectants such as alcohol could be greater in the exhaled breath. It is definitely beneficial to look more into accurately eliminating the exogenous compounds from the endogenous compounds. Table 5.1 lists all the compounds found common to the three breath samples, however, the list doesn't include some of the common compounds cited in literature such as acetone, isoprene etc. This indicates that a lot more work needs to go into developing the data processing algorithm to ensure accurate data reduction. The two-channel breath collection device that has been introduced in this work also needs to be followed up on, in terms of optimizing the switching of channels as well as analysis of the dead space and alveolar breath.

APPENDIX A: DATA PROCESSING ON BREATH SAMPLES

In this appendix, the data-sorting tool developed has been applied to breath samples collected from two other volunteers. Figure A.1 shows the raw data of volunteer 1 as processed by the Chromatof software. The peaks are then grouped into distinct compounds in figure A.2. The exogenous compounds arising from the Tedlar bag and from column bleed are then removed and represented in figure A.3. Finally the noise level compounds are removed and the final chromatogram is show in figure A.4.

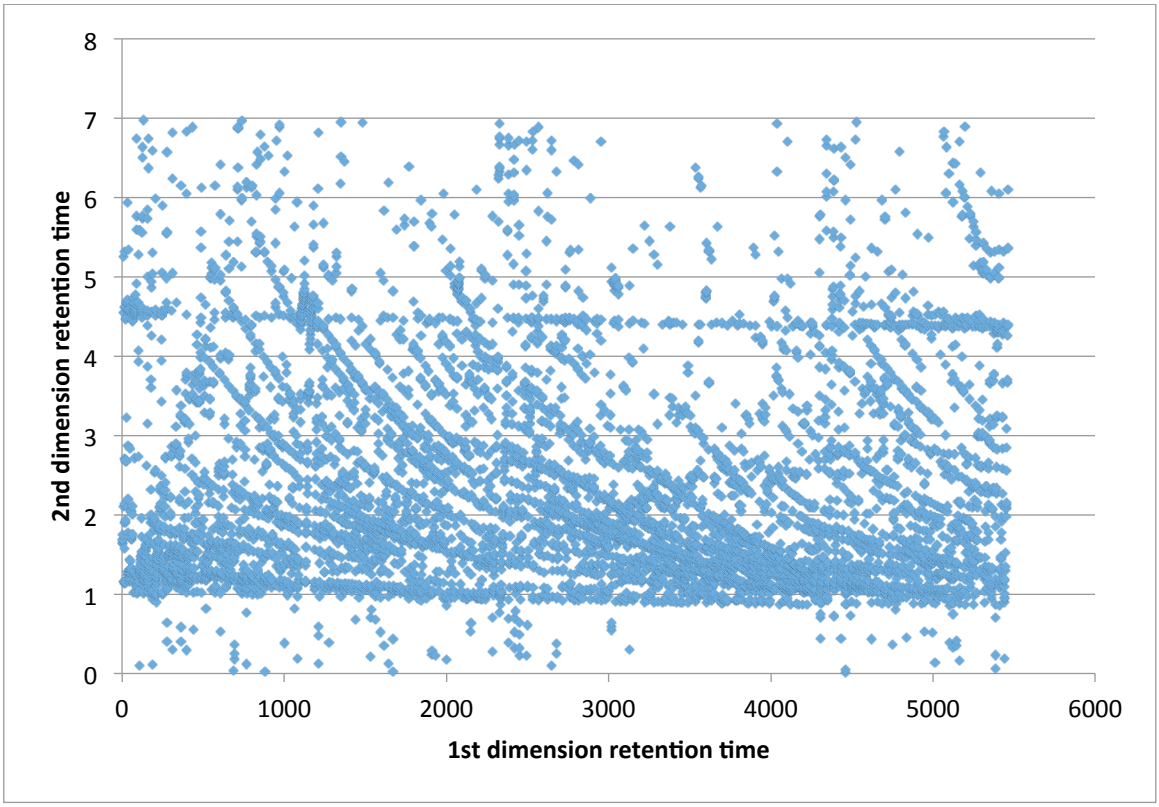


Figure A.1: Raw breath chromatogram from volunteer 1

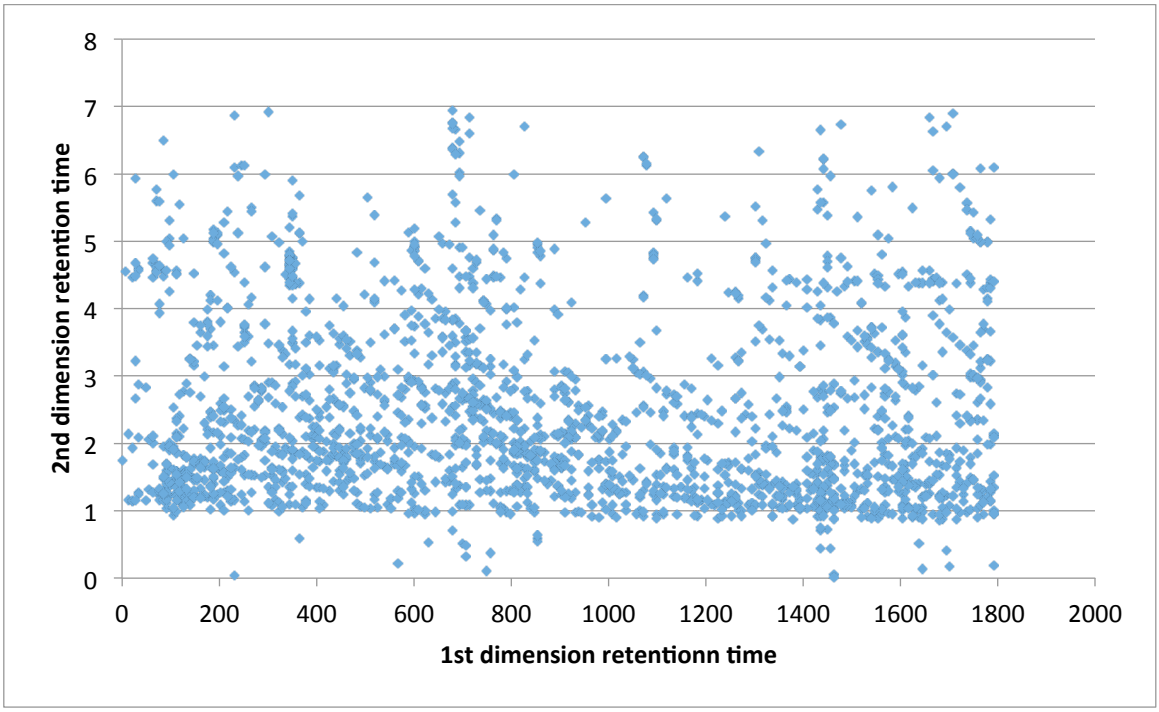


Figure A.2: Peak combine (volunteer 1)

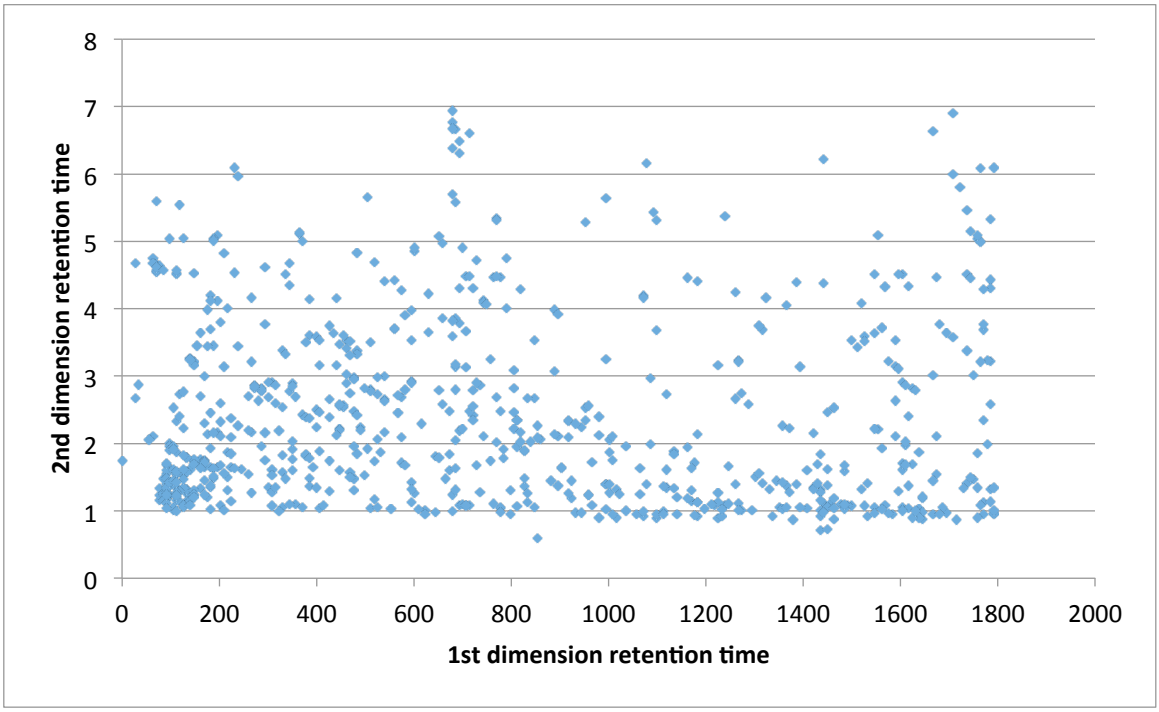


Figure A.3: Chromatogram after removal of siloxanes and noise (volunteer 1)

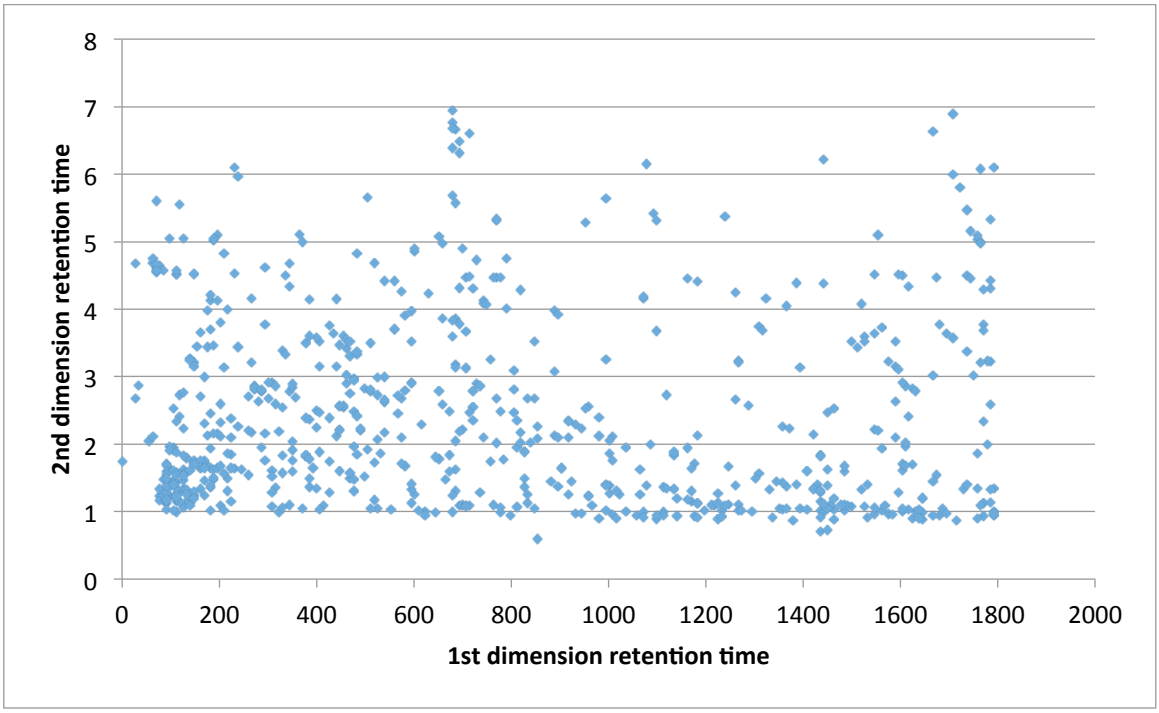


Figure A.4: Final chromatogram (volunteer 1)

Similarly, the same data processing is applied to the breath sample obtained from volunteer 2 and the data is shown below.

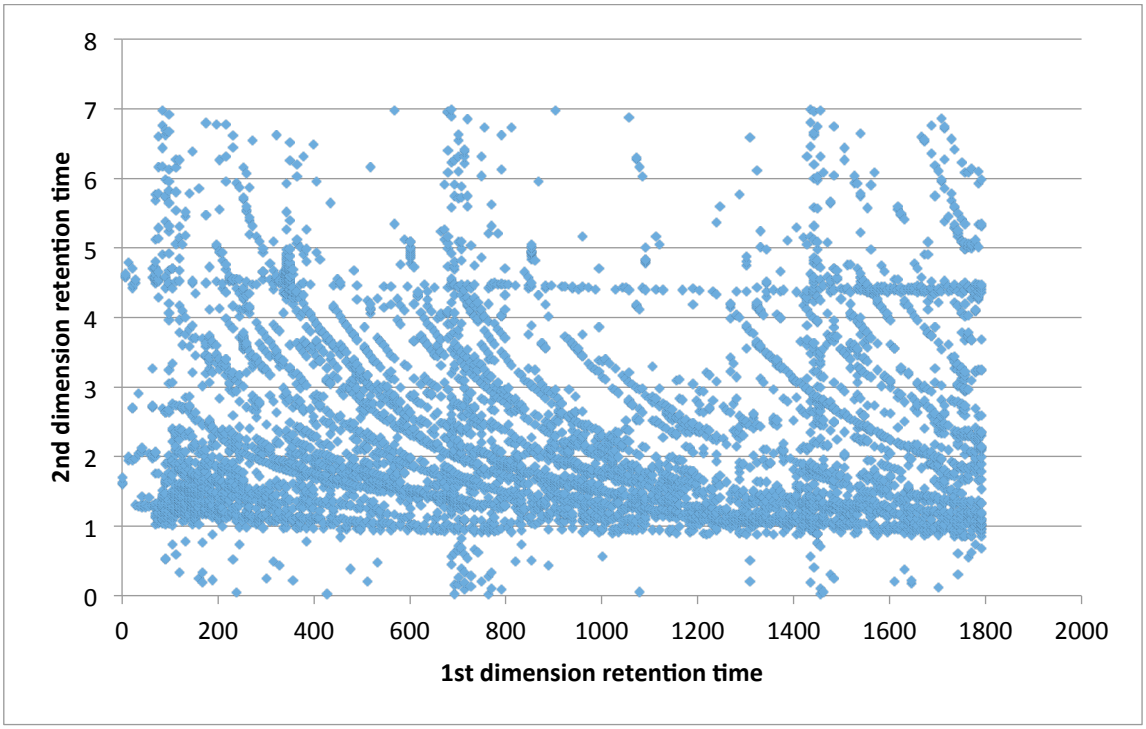


Figure A.5: Raw chromatogram (volunteer 2)

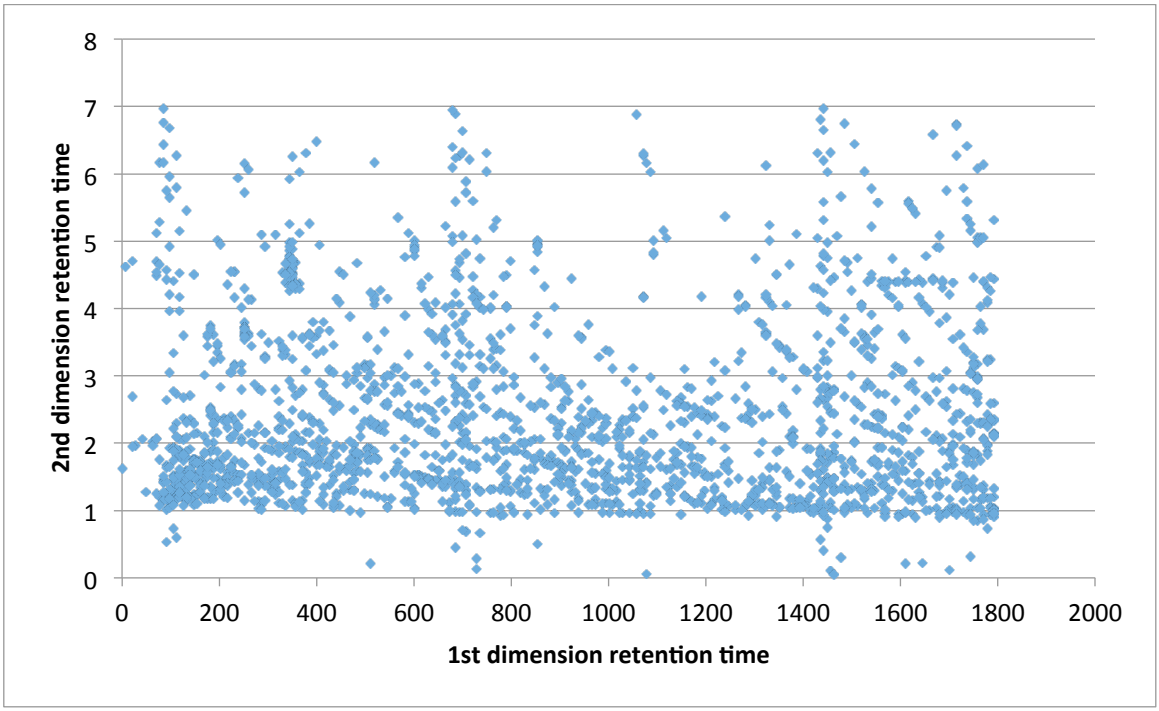


Figure A.6: Peak combine (volunteer 2)

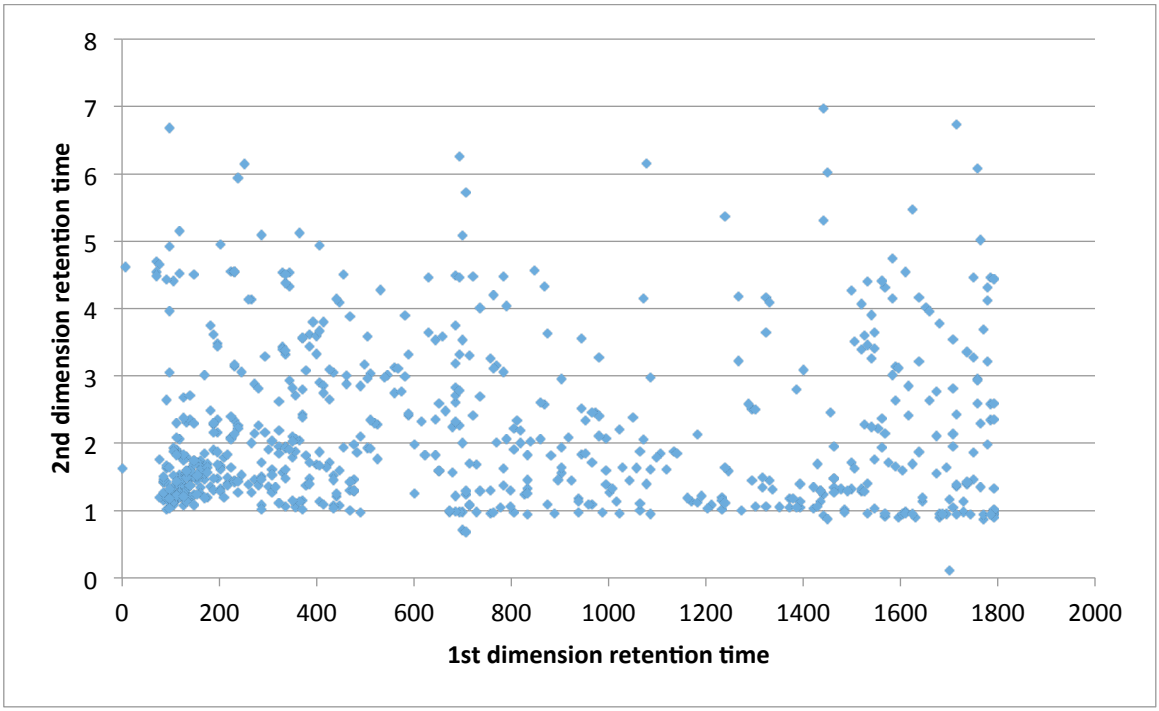


Figure A.7: Chromatogram after removal of siloxanes and noise (volunteer 2)

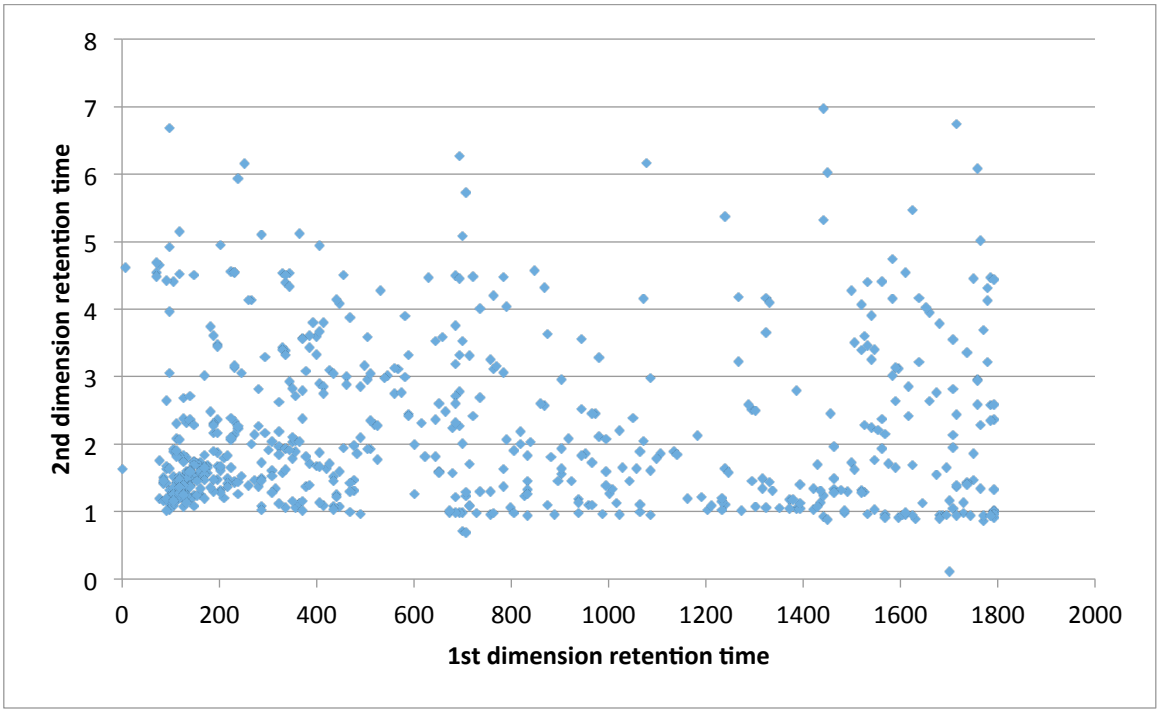


Figure A.8: Final Chromatogram (volunteer 2)

Table A.1: List of standards found in volunteers 1 and 2

Serial no.	Name	CAS number	Found in sample
1	Methyl Alcohol	67-56-1	1,2
2	Acetaldehyde	75-07-0	1,2
3	Ethyl Alcohol	64-17-5	2
4	Propanal	123-38-6	2
5	Acetone	67-64-1	1
6	Isopropyl Alcohol	67-63-0	2
7	Dimethyl Sulfide	75-18-3	1,2
8	2-Pentene	109-68-2	1
9	2,3-Butanedione	431-03-8	2
10	2-Methylfuran	534-22-5	2
11	2-Butanone	78-93-3	1
12	2-Methylpentane	107-83-5	2
13	Benzene	71-43-2	1,2
14	Hexane	110-54-3	1
15	2-Pentanone	107-87-9	1,2
16	Toluene	108-88-3	1,2
17	Hexanal	66-25-1	1,2
18	o-Xylene	95-47-6	2
19	6-Methyl-5-hepten-2-one	110-93-0	1
20	Acetophenone	98-86-2	2

Table A.2: Breath volumes of volunteers measured using spirometer

Subject	Total exhaled volume (liters)	Mean flow rate (liters/sec)
Volunteer 1	21.47	0.11
Volunteer 2	27.71	0.14
Volunteer 3	25.83	0.13

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