# TOWARDS STANDARDISATION IN BREATHOMICS

BY

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*Dedicated to the best mum in the world…*

# Abstract

Exhaled breath VOCs analysis is safe and non-invasive method of monitoring for human metabolic profiles and has the potential to become diagnostic tool in clinical practise. This thesis first describe in detail the different aspects of exhaled breath VOCs and its use as diagnostic tool in respiratory diseases.

The current exhaled breath analysis work-flow including breath sampling, analysis and data processing is also described. A single exhaled breath sample can contain in excess of 500 different chemical species. There is a wide range of factors that can cause the variability to individual breath profiles. In order to detect small changes in breath profiles, a standardised and reproducible approach to exhaled breath analysis methodology is required. The long term storage of exhaled breath samples using multi-sorbent tubes is investigated, the optimum storage protocol and condition is discussed.

A portable breath sampling system was also developed for remote sampling. The introduction of this new feature enables breath sampling to be carried out outside the designated laboratory with no location restriction. This feature combined with the easy to use and non-invasive original sampling unit designed for subjects with impaired lung function minimise participant stress level and discomfort. It also utilises the custom developed air supply filtration assembly to create a standardised purified breathable air that can minimise the method variability and improve standardisation to breath samples collected.

This methodology is tested in an excise induced bronchoconstriction (EIB) study where two groups of participants: healthy and excise induced bronchoconstriction (EIB) positive undergo high intensity cardiopulmonary exercise testing (CPET). The data from two groups of participants is analysed and three markers which shown correlation with EIB positive participants are determined.

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# Glossary of terms

| | |
|---|---|
| AUC | Area under the curve |
| BCA | Breath collecting apparatus |
| BHT | Butylated hydroxytoluene |
| BMI | Body mass index |
| CI | Chemical ionisation |
| COPD | Chronic obstructive pulmonary disease |
| CPET | Cardiopulmonary exercise testing |
| CPX | Cardiopulmonary exercise |
| DC | Direct current |
| EBC | Exhaled breath condensate |
| EI | Electron ionisation |
| EIB | Exercise-induced bronchoconstriction |
| EVH | Eucapnic voluntary hyperpnoea |
| FEV1 | Forced expiratory volume in 1 second |
| FVC | Forced vital capacity |
| GC-FID | Gas chromatography - flame ionization detector |
| GC-MS | Gas chromatography – mass spectrometry |
| IMS | Ion mobility spectrometry |
| LAS | laser absorption spectroscopy |
| LCL | Lower control limit |
| MHR | Maximum heart rate |
| MVA | Multivariate analysis |
| MVV | Maximal voluntary ventilation |
| NIST | National Institute of Standards and Technology |

| | |
|---|---|
| NO | Nitric oxide |
| OPLS-DA | Orthogonal partial least square discriminant analysis |
| PC | Principal component |
| PCA | Principal component analysis |
| PEF | Peak expiratory flow rate |
| PTFE | Polytetrafluoroethylene |
| PTR-MS | Proton transfer reaction mass spectrometry |
| RF | Radio frequency |
| RI | Retention index |
| ROS | Reactive oxygen species |
| RSD | Relative standard deviation |
| SIFT-MS | Selected ion flow tube mass spectrometry |
| SPME | Solid-phase microextraction |
| TD | Thermal desorption |
| UCL | Upper control limit |
| VC | Vital capacity |
| $V_{CO2}$ | Carbon dioxide output |
| $V_E$ | Minute ventilation |
| $V_{O2}$ | Oxygen uptake |
| VOC | Volatile organic compounds |

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# CHAPTER 1. AN INTRODUCTION TO BREATH VOCS AND CURRENT EXHALED BREATH ANALYSIS METHODOLOGY

## 1.1. The composition of exhaled breath VOCs

Exhaled human breath is produced when inhaled ambient air is exhaled through nasal or oral cavities. The composition of exhaled breath changes from the inhaled ambient air mainly due to the intake of oxygen into the body and removal of carbon dioxide from the body. It is a complex matrix of volatile organic compounds, non-volatile organic compounds and inorganic compounds. The main components of exhaled breath are nitrogen (75% v/v), oxygen (16% v/v) water vapour (5% v/v), carbon dioxide (4% v/v) and argon (1% v/v). Exhaled breath has relative humidity of 100% due to water diffusing from the alveoli and other parts of the airway.

Exhaled breath also contains several hundreds of known volatile organic compounds (VOCs) in trace amounts which can variate from parts per million (ppmv) down to parts per trillion (pptv). These volatiles metabolites are present in the bloodstream and diffuse into exhaled breath via alveolar capillary, thus compounds are usually metabolites from protein breakdown and are good indicators of human biochemical mechanisms. Common occurring volatiles metabolites such as ammonia have been studied extensively over an extended time using SIFT-MS (1) (2); Ammonia was shown to a major volatile metabolite in the body at 833 ppb, other common volatiles measured were acetone at 477 ppb, methanol at 461 ppb, ethanol at 112 ppb, isoprene at 106 ppb, propanol at 18 ppb and acetaldehyde at 22 ppb. Other less common VOCs are present in ppb or ppt concentrations. Most of these common human volatiles compounds have been linked to metabolic pathways in the body. Majority of ammonia is produced in the oral cavity and is also produced in the body as protein breakdown product (3) (4). After consumption of foods and drinks, the level of methanol usually increases by an order of magnitude. Methanol and ethanol are possibly generated as anaerobic fermentation products of sugar contained in food and drinks by gut bacteria (5). Acetone is a product of decarboxylation of acetoacetate and the dehydrogenation of isopropanol (6). Isoprene is generated through cholesterol synthesis. Propanol is a product of the enzyme-mediated reduction of acetone (3). Acetaldehyde is generated endogenous ethanol metabolism and might be linked to carcinogenic effect of alcohol, but also can be produced from sugar cellular activity.

The exhaled breath is saturated with water vapour, apart from the gaseous VOCs, it also contains aerosol particles transported from lower respiratory tract and it reflects the

composition of alveolar extracellular lining fluids. These non-volatile compounds include a wide range of compounds ranging fromorganic molecules such as urea, organic acids, amino acids, peptides, proteins to inorganic ions. It also contains important metabolites such as leukotrienes and isoprostanes which are inflammatory mediators which can bevaluable means of monitoring lung diseases.

## 1.2. The physiology of exhaled breath VOCs

The VOCs in breath are products of metabolic pathways in human body, VOCs produced in remote parts of the human body enters the blood stream at parts of body where it originated. The VOCs are then transported in the blood stream to alveolus to be exhaled into the breath. The alveolus is located at the end of the lung where blood carrying $CO_2$ and VOCs enters the pulmonary arterial capillaries around the alveolus. The interface between the capillary endothelium and type-1 alveolar cells is only 0.1µm thin thus facilitates the rate of gas transfer.

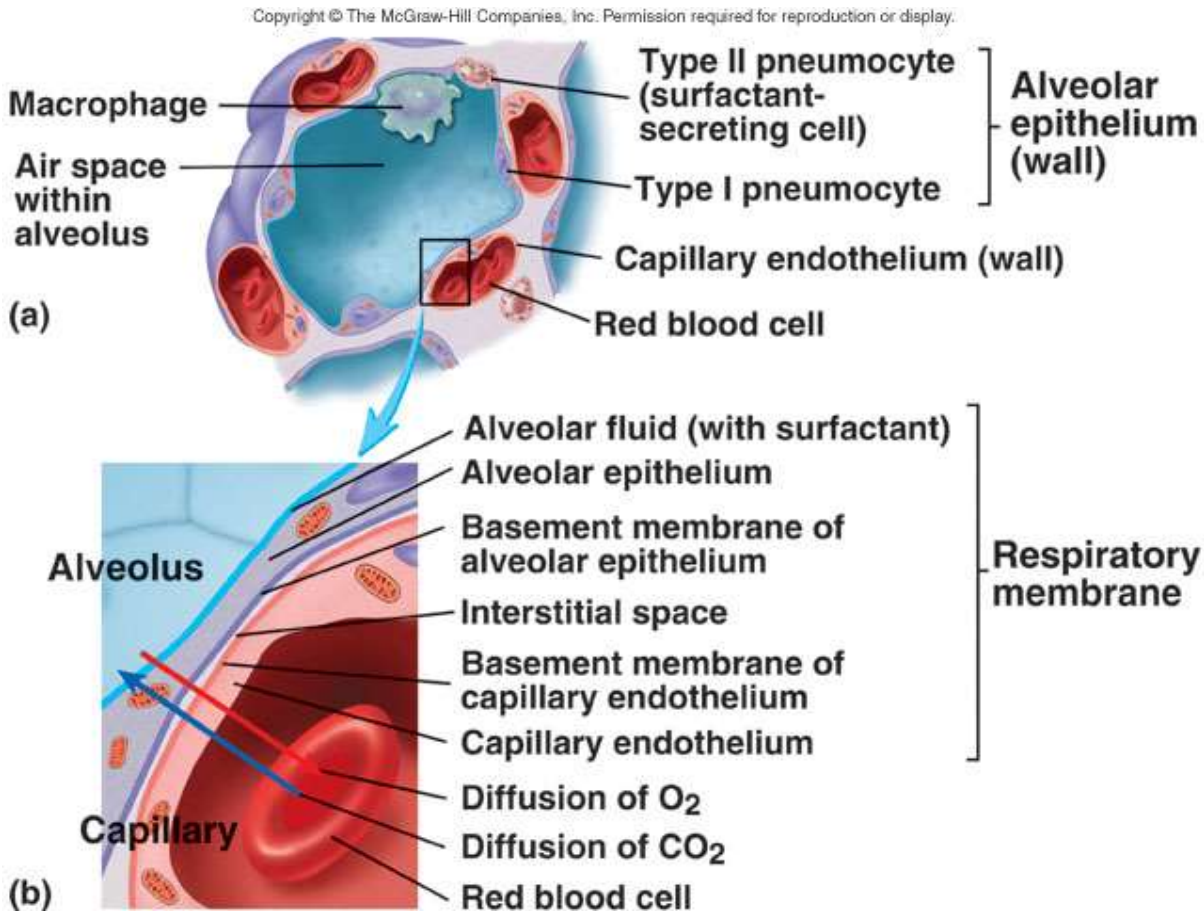


**Figure 1.2. Schematic of respiratory alveolar and capillary membranes (a) Alveolar epithelium (b) Respiratory membrane, oxygen diffuse into alveolus into red blood cell via capillary, while carbon dioxide diffuses into alveolus from the red blood cells into the exhaled breath (7).**

The concentration of a specific VOC in the alveoli is proportional to its concentration in blood. A mathematical model relating concentration between alveoli and blood is shown below:

$$C_A = \frac{C_v}{\lambda_{b:air} + \dot{V}_A/\dot{Q}_C} \qquad \textbf{Equation 1.2.1.}$$

$C_A$ is alveolar concentration of specific compounds, $C_v$ is the venous blood concentration, $\lambda_{b:air}$is blood: alveoli partition coefficient, $\dot{V}_A/\dot{Q}_C$ is the alveolar ventilation: cardiac output ratio. This model describes steady state inert gas elimination from lung as a single alveolar compartment with alveolar ventilation: cardiac output ratio close to one (8).

However this model seems to fail for compounds such as acetone and isoprene, so revised models has been published recently (9):

$$C_A = \lambda_{b:air} C_a \qquad \textbf{Equation 1.2.2.}$$

$C_a$is concentration of compounds in arterial blood.

The exact location of where metabolic VOCs enter the alveolar compartments are still under research. The relevant compartments and their gas volume are: alveolar walls 250-300 mL, epithelium 60-80 mL, endothelium 50-70 mL, interstitium 100-185 mL, macrophages 50 mL, supportive tissues 250 mL and capillary system 400 mL. Although some VOCs such as ammonia are generated in oral cavity by bacterial action on oral urea.

The VOCs detected in human exhaled breath are reflective of the entire body's metabolism, many cells produce VOCs as products of their metabolic pathway.

Although the mechanism of transportation of systematic VOCs produced in remote parts of the body to lung is well understood (8) (9), however, the biochemical process that leads to VOCs biomarker production in human body can be difficult to determine. The determination of biogenic origin of VOCs biomarker is important providing understanding to the metabolic pathways which links to certain diseases. The presence and amount of VOCs biomarker provides information to biological statues of the body.

Research into production of VOCs from single cell lines helps establishing the origin of many VOCs biomarkers. By studying a single cell line, the researcher is able to control environment, genotype and growth of cells, as well as to eliminate other factors that comes from other cells to establish.

## 1.3. The origin of exhaled breath VOCs

Exhaled breath VOCs can be separated into two categories based on their origin: endogenous and exogenous. Breath VOCs diffuse readily from and into the red blood cells

and rest of the body via alveolar-capillary epithelium. Endogenous breath VOCs are usually end products of metabolic pathways that are excreted via breath, these are compounds such as isoprene and acetone. Exhaled breath VOCs are reflective of the entire human body's metabolism, as many cells emit VOCs. However, many sources of endogenous breath VOCs and their links to metabolic pathways are not yet understood.

The sources of exogenous breath VOCs can be through inhalation through ambient air, ingestion of food and drinks or skin absorption. These compounds are possibly metabolised in the body and excreted in breath. Philips *et al* investigated the endogenous and exogenous nature of commonly occurring breath VOCs by comparing room air and alveolar breath (10). Out of the 3481 VOCs observed, 1753 VOCs had positive alveolar gradients and 1728 had negative alveolar gradient where the concentration of the VOCs is higher in the room air than in exhaled breath. For exogenous compounds not significantly metabolised or catabolised such as room air contaminants, their alveolar gradient is near zero. The equilibrium between VOCs in the blood and in the alveolar air is assumed be rapid and near complete. Due to large proportion of exhaled breath VOCs being compounds of an exogenous origin, it is important to be able to distinguish these compounds from the truly endogenous compounds.

## 1.4. The variability of exhaled breath VOCs

An average breath sample could contain several hundreds of VOCs, however, apart from the commonly occurring compounds such as acetone or isoprene, the compounds found in human breath can differ significantly between individuals. Philips *et al* studied the breath profile of 50 healthy humans and found that out of the 3481 different VOCs identified, only 27 can be observed in all 50 participants (10), thus indicating the inter-individual variation. Due to the high sensitivity of the increasing breath analysis method, there is wide range of factors that can cause the variability to individual breath profiles; the main factors are age, gender, human body state, food/drink and exposure to air contaminants.

Several studies have found direct link of age and gender of subject to breath VOCs. Isoprene concentration is found to be in significantly lower level in children compared to adults, and it is also found to increase during puberty (11) (12). It is also found to be in higher level in male subjects compared to female. Breath ammonia level was reported to increase directly with age (13).

The other major factor to variability in breath VOCs is food or drink intake. Smith *et al* has investigated the effect of glucose ingestion and alcohol consumption on common breath metabolites such as ammonia and acetone using SIFT-MS. The study has found decrease in ammonia and acetone level following food/drink intake (14) (15).

Food and drinks are known to emit trace level VOCs in the human body over time. Recent study has used monitored the catabolism of isotope-labelled ethanol oral ingestion using real-time breath gas analysis with PTR-MS (16). It has found ethanol was transformed into deuterated acetone and isoprene. The absence of deuterated acetaldehyde indicated more rapid metabolism preventing it from entering blood stream. Other study monitored breath VOCs following the ingestion of raw garlic, it has found that rapid increase in sulphide compounds and slow increase of acetone even after 30 hours of ingestion (17). These studies have demonstrated the duration of release of trace volatiles from food can vary significantly depending on the metabolism process it is involved in, thus also demonstrating the complexity of breath VOCs.

Exposure to air borne contamination can also significantly influence the composition of exhaled breath. Studies have found compounds present in cigarette smoke such as 2,5-dimethylfuran, acetonitrile, benzene, toluene and styrene are present in exhaled breath of both smokers and passive smokers (18). Additionally, acetonitrile, hydrogen cyanide, ethane, pentane and benzene are known to be present in exhaled breath of smokers when compared to non-smokers (19).

Air borne contaminants can also enter the body through inhalation and skin absorption, and can be released at different rates through breath or other excretion. Common air contaminants such as toluene, benzene or limonene emitted from pollution or household products are commonly found in exhaled breath, these compounds can be absorbed by blood during inhalation and released over a long period of time. Toluene and benzene are known to be carcinogenic and therefore can trigger adverse biochemical reactions in body.

## 1.5. Exhaled breath VOCs as diagnostic tool and applications

### 1.5.1. History

Breath analysis has been used to detect diseases since the times of Hippocrates in ancient Greek, physicians used the smell of breath to identify status of health. Even in modern times, the smell of breath is still a valid indicator of diseases. It is known for many years a fruity smell of acetone in breath indicate diabetes, fishy smell of breath indicate liver disease, whereas urine smell of breath may indicate kidney diseases.

However, in order for breath analysis to become an objective diagnostic tool, it has to progress beyond the realm of subjective sniffing of breath. This process was started around 200 years ago by Antoine-Laurent de Lavoisier the “father of modern chemistry”, he analyzed the breath of guinea pigs and discovered animals produced carbon dioxide from consuming oxygen. He also created breath trap where carbon dioxide produced by human is

reacted with a potassium hydroxide solution through the mercury to form precipitate. Since carbon dioxide is very abundant in human breath, it is relative easy to detect, but many other VOCs that are present in trace amounts in breath are much more difficult to trap in detectable quantities.

The modern age of breath analysis began in 1971, when Noble prize winner Linus Pauling discovered these trace amount VOCs in human breath and proved the complexity of breath, he was able to reveal the presence of 250 VOCs in an average breath (20).

In 1999, Michael Philips and his co-workers were able to demonstrate the variation in human breath of 50 participants. He revealed presence of thousands of VOCs among the 50 participants and 27 common VOCs that is found in all subjects. The VOCs that are common in all subjects indicates the essential VOCs from human metabolic pathways (10).

### 1.5.2. Exhaled breath VOCs in Asthma

Asthma is a common chronic inflammatory lung disease. It is also becoming increasingly common in developed country and has become the most common chronic condition in the west. Anybody at any age can develop asthma, although it seems to be more common in children. In the UK, more than 5.2 million people suffer from asthma which is 9% of population. Internationally, 300 million people are affected by asthma, and 250,000 people die from asthma attacks every year. But 90% of asthma death is preventable with proper treatment. With the advances in medical treatments, the asthma death rate is decreasing, but even mild asthma will still pose risks of death. The symptoms of asthma include coughing, wheezing, shortness of breath and chest tightness.

Asthma is a complex disease with many factors that control the level of asthma symptoms. There are two general types of asthma: atopic and non-atopic. Atopic asthma is extrinsic and developed toward hypersensitivity allergic reactions to certain environmental factors; it is also known as allergic asthma. The environmental factors which can cause atopic asthma are variable to individuals and there are many potential causes. Some common causes include air pollution, pollen and house dust mites. The second general type of asthma is non-atopic asthma, although both type of asthma have the same symptoms, but non-atopic asthma is intrinsic and might be caused by factors such as excise, stress, anxiety, cold air, smoke, viruses or other non-allergic factors; this type of asthma is also known as non-allergic asthma. Research suggest asthma is developed early in life during the development of human immune system through a combination of genetic factors and environmental exposure to allergens, pollution or stress etc.

Asthma is clinically classified by four severity categories: intermittent, mild persistent, moderate persistent and severe persistent. The severity of asthma is determined mostly by frequency of symptoms, forced expiratory volume in 1 second ($FEV_1$) and peak expiratory flow rate (PEF). Other factors to be considered when determining asthma severity include frequency of nocturnal symptoms, frequency of exacerbations and $FEV_1$/FVC ratio. Severe asthma sufferers will show daily or continual symptoms, frequent exacerbations, FEV1 lower than 60% predicted, frequent nocturnal symptoms and limited physical activities, whereas mild asthma sufferers will only have occasional symptoms less than once a week, occasional nocturnal symptoms less than once every two months and $FEV_1$ higher than 80%. Severe asthma will need to be treated high dose of corticosteroid plus long-acting inhaled β2-agonist and another controller, but mild asthma wouldn't need any controller at all. A table showing classification of asthma severity is shown below:

**Table 1.5.2. The classification of asthma based on the severity of the symptoms (21).**

| Severity | Symptoms | Night time symptoms | Lung function |
|---|---|---|---|
| Severe persistent | Continual symptoms<br>Frequent exacerbations<br>Limited physical activity<br>SABA used several times a day | Frequent | FEV1<60% predicted<br>FEV1/FVC reduced <5% |
| Moderate persistent | Daily symptoms<br>Daily use of SABA<br>Exacerbations>2/week<br>Exacerbation affects activity | >1/week | FEV1>60% predicted<br><80% predicted<br>FEV1/FVC reduced 5% |
| Mild persistent | Symptoms >2/week but not daily<br>Exacerbations may affect activity | 3-4/week | FEV1>80% predicted<br>FEV1/FVC normal |
| Mild intermittent | Exacerbations brief<br>Symptoms < 2/week<br>No controller needed | <2/week | FEV1>80% predicted<br>FEV1/FVC normal |

As the lung becomes inflamed during an asthma exacerbation, the epithelial cell lining of bronchioles is disrupted and the epithelial cells start to desquamate. The eosinophilic cells increase in the bronchial wall. The cellular changes cause the bronchial wall to swell. The muscle around the airway contracts creating resistance to airways. The goblet cells start to

secrete mucus into the airway; the mucus will also cause additional obstruction in the airways. All the constriction in the airway causes it to narrow which result in coughing, wheezing and shortness of breath. However, this obstruction in the airway unlike irreversible chronic obstructive pulmonary diseases is reversible with early and proper treatment. If it is left untreated, asthma can cause irreversible and long term loss of lung function or even death.

Asthma is normally diagnosed and classified using a spirometer. The spirometer measures the volume of inhaled and exhaled air. It is used to produce measure vital capacity (VC), forced vital capacity (FVC) and forced expiratory volume (FEV). Another way of accessing asthma condition is by is by bronchoscopy. However, it is invasive and may cause discomfort.

In 1991, the field of breath analysis for asthma was started by Gustafsson who suggested who demonstrated the presence of nitric oxide (NO) in animals and human (22). He also indicated NO was endogenously produced in the lung by an NO synthase and suggested its important role in lung function. In 1994, Kharitonov tested the breath of asthma patients, and determined an increased NO level in asthma patients compared to control group (23). NO is currently used as biomarker of asthma, and different types of NO sensor is available commercially.

Hydrocarbons have known to be products of lipid peroxidation in the lung. During asthma symptoms, immune and inflammatory cells release reactive oxygen species (ROS) which will induce lipid peroxidation of membrane fatty acids. This is likely to damage membrane function, hence causing constriction to the airways. In recent years, saturated and unsaturated hydrocarbon compounds have been suggested as markers of asthma.

### 1.5.3. Exhaled breath VOCs for Lung cancer

Lung cancer is a form of cancer that is caused by uncontrollable cell growth in lung tissue. 98% of lung cancer is a result of transformation of healthy epithelial cells into primary carcinomas. The cause of this transformation is damage to genome of cells due to exposure to carcinogens. This uncontrollable cell growth if not treated will quickly spread to rest of the lung by the process of metastasis, and into other parts of body. The cancer tissue is likely to block the normal function of the lung and the resultant symptoms are difficulty breathing, chest pain and weight loss.

Internationally lung cancer is the one of most common and serious types of cancer, people diagnosed with lung cancer has 86% death rate within 5 years. However, if detected and treated early, the survival rate is likely significantly increases from 10~23% (stage 3 lung

cancer) to 40~50% (stage 2 lung cancer) or 60~80% (stage 1 lung cancer). The purpose of breath analysis of lung cancer is to provide early detection at stage 1 or stage 2 of lung cancer before it develops into stage 3.

The breath analysis research into finding marker compounds of lung cancer started in 1985 by Gordon and his colleagues (24). They used computerised GCMS to indicate 22 possible lung cancer markers out of 150 compounds among 12 lung cancer patients and 17 controls. Out of the 22 candidate compounds, 16 were oxygen containing compounds and 4 was sulphur containing compounds. Although they couldn't determine the exact identity of 22 compounds, it had shown the enormous potential of breath analysis for lung cancer detection.

In 1988, O'Neil and Gordon classified 386 human breath compounds into 16 chemical classes using GCMS method with 8 lung cancer participants (25). Out of the 16 chemical classes, they were able to identify 1-oxygen and 2-oxygen containing classes to be potential lung cancer markers. They also suggested 9 potential lung cancer markers: propenal, acetone, 2-butanone, phenol, benzaldehyde, acetophenone, nonanal, ethylpropanoate and methylisobutenoate. However, no sulphur containing compounds were found to be potential markers.

In 1999, Michael Philips and co-workers began large scale lung cancer breath analysis project. They were able to recruit in total 108 participants with abnormal chest radiograph who were scheduled for bronchoscopy and 50 healthy participants as control. Lung cancer was later confirmed to 60 out of 108 participants. The breath samples were analyzed by GCMS method. They were able to identify 22 breath VOCs that are discriminated between lung cancer and healthy participants. Out of the 22 VOCs, 15 were alkane or alkane derivatives, the other 6 were isoprene, benzene or benzene derivatives. It seems to have supported some of O'Neil's potential marker compounds (26).

In 2003, Michael Philips and co-workers was able to determine the presence of 9 lung cancer markers based on 178 bronchoscopy patients and 41 healthy participants (27). Out of 178 bronchoscopy patients, 87 were diagnosed with lung cancer. The type of cancer diagnosed in the 87 participants was also separated into primary lung cancer and metastatic lung cancer. Participants with primary lung cancer were found to have elevated level of alkanes and monomethylated alkanes, but this is less likely in metastatic lung cancer participants. The 9 marker lung cancer compounds are butane, 3-methyl tridecane, 7-methyl tridecane, 4-methyl octane, 3-methyl hexane, heptanes, 2-methyl hexane, pentane and 5-methyl decane. Out of the 9, butane was found to be the best discriminator.

### 1.5.4. Summary of recent lung disease studies using exhaled breath VOCs

Below is a summary of lung disease biomarkers studies using exhaled breath VOCs in the last five years from 2012 to 2016.

**Table 1.5.4. List of biomarkers studies for asthma, COPD and lung cancer using exhaled breath VOCs from 2012 to 2016**

| Year | Authors | Type of lung disease | Participant number | Collection method | Analysis Method | Data analysis method | Identified markers |
|---|---|---|---|---|---|---|---|
| 2012 | Caldeira (28) | Allergic asthma in children | 27 control, 32 with allergic asthma, 10 with allergic rhinitis | Tedlar bag | GC × GC-TOF-MS | PLS-DA | nonane, 2,2,4,6,6-pentamethylheptane, decane, 3,6-dimethyldecane, dodecane, and tetradecane |
| 2012 | Buszewski (18) | Lung cancer | 44 control, 29 with lung cancer | Tedlar bag | GC-TOF-MS | ANOVA | Butanal, 2-butanone, ethyl acetate, ethylbenzene, 2-pentatone, 1-propanol, 2-propanol |
| 2012 | Basanta (41) | COPD | 32 control, 39 with COPD | Thermal desorption tubes | GC-TOF-MS | PCA, MLR and ROC | Undecanal, Hexanal, Dodecanal, Decanal, Nonanal, Pentadecanal, Oxirane-dodecyl, Cyclohexanol, Buanoic acud, pentanoic acid, 2-pentylfuran |
| 2012 | Phillips (42) | COPD | 63 control, 119 with COPD | Bio-VOC | GC-MS | ROC | Isoprene, Acetic acid, Benzaldehyde, Benzene, Butanea, Carbon dioxidea, Hexanal, Nonadecane, Phenol, Phthalic anhydride, Sulphur dioxide Toluene |
| 2013 | Gahleitner (32) | Asthma in children | 12 control and 11 with asthma | Thermal desorption tubes | GC-MS | PCA | (1-(methylsulfanyl)propane, ethylbenzene, 1,4-dichlorobenzene, 4-isopropenyl-1-methylcyclohexene, 2-octenal, octadecyne, 1-isopropyl-3-methylbenzene and 1,7-dimethylnaphtalene) |
| 2013 | Robroeks (33) | Asthma in children | 40 children with asthma | Tedlar bag | GC-TOF-MS | SVM | 2-ethyl-1,3-butadiene, Cyclohexane, 2-octen-1-ol, 1.2-methyl-4H-1,3-benzoxathiine, Benzene |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2014 | Martinez-Lozano Sinues (34) | COPD | 25 with COPD and 25 controls | Direct | TOF-MS | PCA | Acetone and Indole |
| 2014 | Smolinska (43) | Asthma in children | 252 children | Tedlar bag | GC-TOF-MS | PCA | Acetone, 2,4-dimethylpentane, 2,4-dimethylheptane, 2,2,4-trimethylheptane, 1-methyl-4-(1-methylethenyl) Cyclohexen, 2,3,6-trimethyloctane, 2-undecenal, Biphenyl, 2-ethenylnaphtalene, 2,6,10-trimethyldodecane, Octane, 2-methylpentane, 2,4-dimethylheptane, 2-methylhexane |
| 2014 | Fu (36) | Lung cancer | 88 controls, 97 with lung cancer | Tedlar bag | FT-ICR | Wilcoxon test | 2-Butanone, 2-hydroxyacetaldehyde, 3-hydroxy-2-butanone, 4-hydroxyhexanal |
| 2014 | Handa (37) | Lung cancer | 39 controls, 50 with lung cancer | Direct | IMS | Decision tree algorithm | n-Dodecane, 3-methyl1-1 = butanol, 2-methylbutylacetat or 2-hexanol, n-nonal or cyclohexanon, isopropylamin, ethylbenene, hexanal, cyclohexanon, heptanal |
| 2015 | Cazzola (38) | COPD | 7 control, 27 with COPD | Tedlar bag | GC-MS | PLS-DA | Benzene, 1,3,5-tri-tertbutyl-, Decane, Butylated hydroxytoluene, Decane, 6-ethyl-2-methyl-, Hexane, 3, ethyl-4-methyl- Hexyl ethylphosphonofluoridate, Limonene, 1-Pentene, 2,4,4-trimethyl-, 2-Propanol |
| 2015 | Ligor (39) | Lung cancer | 361 controls, 123 with lung cancer | Tedlar bag | SPME-GC/MS | n/a | 1-Propanol, 2-butanone, 3-butyn-2-ol, benzaldehyde, 2-methyl pentane, 3-methyl pentane, n-pentane, n-hexane |
| 2016 | Gaida (44) | COPD | 52 healthy non-smokers, 31 smokers with COPD and 30 ex-smokers with COPD | Tenax DA filter | GC-MS | ANOVA and LDA | m/p-Xylene, 1,6-Dimethyl-1, 3,5-heptatriene, o-xylene, 1-Ethyl-3-methyl benzene, Phenole, m/p-Cresol, linalyl acetate, Tridecane, Indole |

## 1.6. Exhaled breath VOCs collection and analysis methods

### 1.6.1. Current exhaled breath VOCs collection methods

Exhaled breath VOCs are usually pre-concentrated by analysis due to most trace VOCs are in the ppbv to pptv range. Also it is not always possible to have sample analysis system where the test subjects are, so breath samples are usually collected on-site and transported to the laboratory later. The most common breath collections methods used are Tedlar bags, adsorbent tubes and canisters. The method and materials used to for breath collection must be not absorb, adsorb or react with any VOCs to maximise recovery.

Tedlar bags are made from poly(vinyl fluoride) films developed by DuPont Corporation in 1960s. Each bag had Teflon hose/valve where gas can enter. Tedlar bags come in a variety of sizes ranging from 0.6L to 25L, with 1L size the most commonly used for breath collection. Due to its low cost and easiness to use, they have been used widely for breath collection. However, Tedlar bags are permeable to low molecular VOCs such as methanol and 1,3-butadiene which can cause poor recovery at 50% to 80% (45). A storage study monitored a mixture of common VOCs and found rapid decline in VOCs concentration level over 70 hours period (46). Breath samples collected using Tedlar bags are recommended to be analysed within 10 hours. The losses of VOCs were due to a combination of adsorption and diffusion through bag wall. Additionally, Tedlar bags are prone to carry over between samples if the bags are re-used, residual VOCs have shown to be difficult to remove even after flushing and heating cleaning procedures (47). Tedlar bags are more suitable for non-polar and medium polarity compounds.

Canisters are stainless steel containers with inert inner surface to avoid adsorption and permeability. A wide range of VOCs collected in canisters have shown to be stable for 30 days or longer. It can vary in sizes from 1L to 6L. Canisters can be treated after sample and re-used with no sample carry over. Canisters are much more expensive compared to other two collection methods, so it is less widely used in comparison.

Adsorbent tubes are typically made of stainless steel and can contain a variety of solid adsorbent materials. This includes activated carbon, silica gel, organic porous polymer such as Tenax or Amberlite resins. Different types or combination of sorbents materials can be selected for trapping a wide range of compounds with high recovery (>90%). The trapping efficiency is dependent upon the chemical properties of the compounds of interest and adsorbent material. Trapped compounds can be removed efficiency when heated to a high temperature during thermal desorption, this ensures no carry over between samples. However, the range of VOCs that can be collected is limited by the adsorbent materials used. Generally, it is more suitable to less volatile and polar VOCs with carbon number

ranging from 3 to 30. It is relative low cost compared to canister method. Typically, 10 mL to 3 L of breath samples are collected onto adsorbent tubes for analysis.

Solid-phase microextraction (SPME) is another pre-concentration method that can be used for breath collection. It uses a fiber coated with extracting phase which can be liquid or solid sorbents. Due to the physical properties of the fibers, only a relatively small volumes 10 to 20 mL of sample can be collected with limited range of VOCs. However, SPME is not affected by water vapor in breath as much as adsorbent tubes due to its high selectivity for target compounds.

### 1.6.2. Current exhaled breath VOCs sampling devices

In principle, there are two approaches to exhaled breath VOCs collections:

- Mixed expiratory sampling – Total breath collection including dead space air and alveolar air
- Alveolar air sampling – Only pure alveolar gas is collected

Mixed expiratory collection method is easy to perform with no additional sampling equipment required, it has been most frequently used in practical applications. However, pure alveolar collection is recommended due to the significantly increased level of endogenous VOCs collected compared to mixed expiratory collection method. This is because for VOCs exchanges between blood and alveolar air, dead space air is considered a contaminant and dilutes the concentration of VOCs when breath is collected.

The first modern exhaled breath VOCs sampling device was developed by Pauling *et al* (20). Exhaled breath volatiles was collected using a cold trapping method by blowing through a stainless steel tube chilled in isopropyl alcohol dry ice bath. The stainless tube was then heated and analysed by GC-FID. They were able to observe around 250 different VOCs. This sampling collection method collected mixed expiratory air and required considerable respiratory effort from the subject for breath collection. Since then, advances in technology have made breath VOCs sampling more user friendly and is able to selectively collect alveolar air. Schuert *et al* developed a breath sampling device using end-tidal $CO_2$ to separate dead space gas from alveolar gas from patients under assisted mechanical ventilation (48). 1 L exhaled breath was collected onto activated charcoal trap and analysed by GC-FID. The study has reported doubling in expired acetone and pentane concentration using the $CO_2$ controlled alveolar air sampling method compared to mixed expiratory sampling. Philips *et al* developed a breath collecting apparatus (BCA) to collect alveolar air (49). The device utilises microprocessor controller to collect alveolar breath onto adsorbent tube. The participant wears a nose clip and breath in and out through a wide bore tube which

presents very little resistance to expiration. Breath samples were collected for 5 minutes at 2 L $min^{-1}$. A typical chromatogram identified 150 to 200 different VOCs.

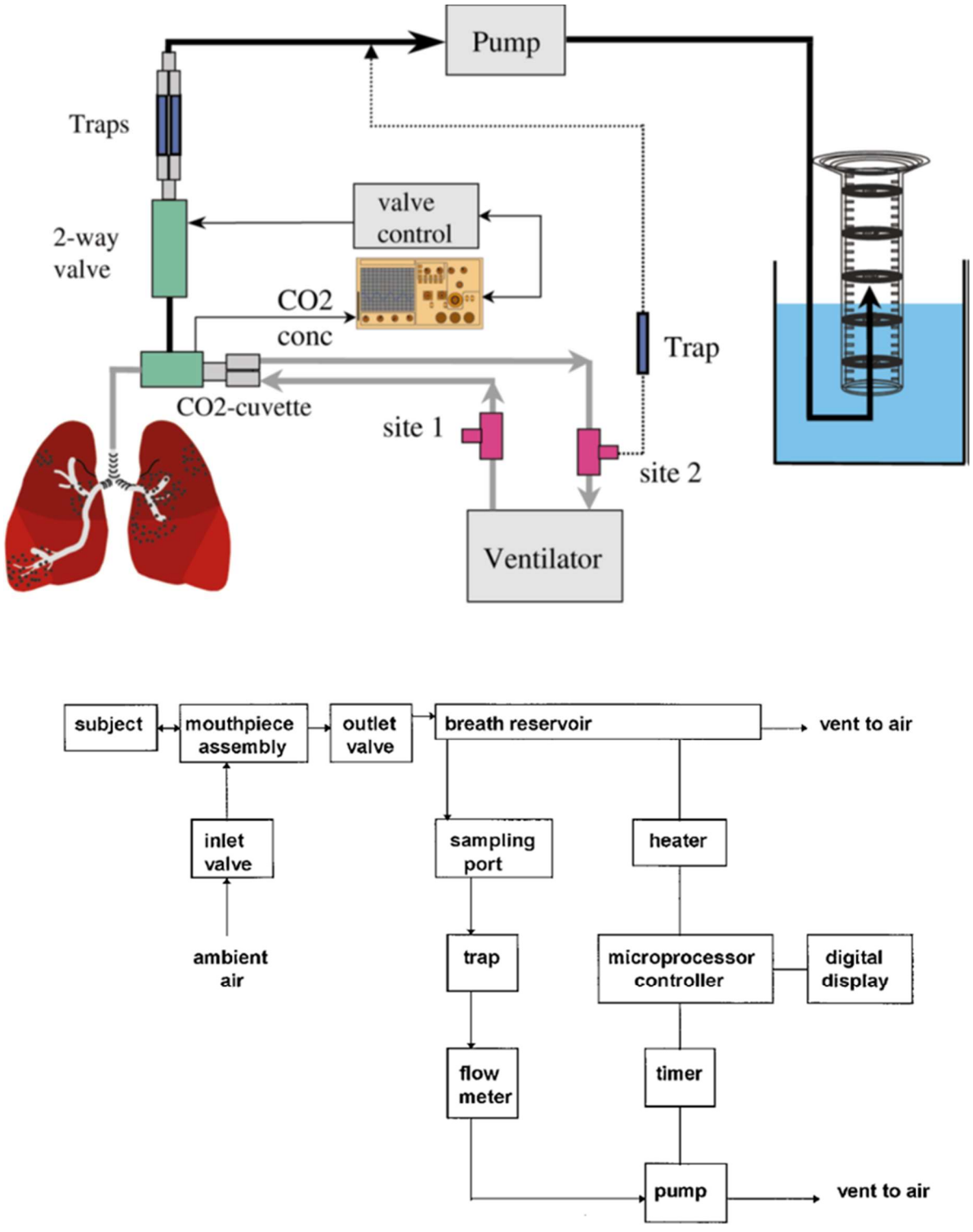


**Figure 1.6.2. Sampling devices which collected alveolar air. (Top) $CO_2$ controlled sampling of alveolar gas in mechanically ventilated patients by Schuert *et al* (48) (Bottom) Breath collecting apparatus (BCA) developed by Philips *et al* (49).**

### 1.6.3. Current exhaled breath VOCs analysis methods

There are two general types of analytical technique for breath analysis: real-time measurements, and off-line analysis. Real-time analytical techniques have the advantages of breath VOCs readily analysed without pre-concentration steps or separation technique, however, this can also lead to loss of sensitivity compared to off-line techniques. Real-time breath analysis techniques include: Proton transfer reaction mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS), laser absorption spectroscopy (LAS) and ion mobility spectrometry (IMS). In these techniques, breath VOCs samples are analysed as the participant breath into the apparatus which allows breath to breath resolution, thus has enables real-time continuous analysis during an intervention. Karl *et al* measured breath isoprene using PTR-MS technique, the study has found breath isoprene concentration increases within a few seconds after exercise is started as result of increase in heart rate (50).

In off-line analysis, samples are usually collected on-site and then transported to laboratory for analysis. These techniques usually involve pre-concentration and separation technique which is more time consuming, but also generates very high sensitivity. Gas chromatography – mass spectrometry (GC-MS) is the most commonly used off-line analytical technique. The MS data sets are easy to handle and suitable for multivariate analysis, also the existence of extensive compounds library such as NIST makes compound identification much easier. However, off-line technique such as GC-MS also have extensive requirements such as expertly trained staff for operation and data analysis, extra time for storage and transportation, also the need for detailed procedures to follow.

Another breath VOCs analysis technique that has seen rapid progress in recent years is the development of chemical sensor technology, also called ‘electronic nose’. This device consists of head space sampling, sensor array and pattern recognition modules in order to mimic human olfaction. The disadvantage of electronic nose include lack of sensitivity and selectivity compared to other methods, but in recent years, studies have reported the potential of electronic nose for breath analysis in the detection of lung diseases. Natale *et al* investigated the potential of using electronic nose to diagnose lung cancer using breath VOCs profiles (51). Geffen *et al* reported electronic nose was able to distinguish between COPD and healthy subjects (52).

### 1.6.4. TD-GC-MS setup for breath analysis

This section will describe the TD-GC-MS setup employed for the current breath analysis used in this thesis.

## 1.6.4.1. Gas chromatography

### 1.6.4.1.1. The GC elution process

Gas chromatography (GC) is a physical separation method which compounds are separated between two phases: one is a liquid or solid stationary phase with large surface area; the second one is a gas unidirectional mobile phase which carries the compounds through the column. The compounds going through the column are then separated based on the distribution of each individual compounds between the mobile phase and stationary phase. Since compounds that are distributed in stationary phase become static, they only move while they are in the mobile phase, so compounds that have higher distribution in the mobile phase will pass through and leave the column faster than compounds with lower distribution.

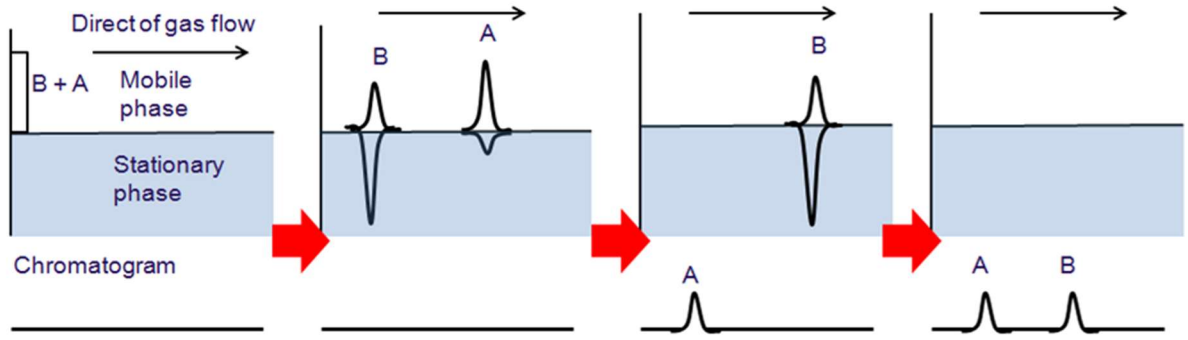


**Figure 1.6.4.1.1.1. The GC separation process**

Compounds with lower distribution coefficient will be eluted from column first. The distribution coefficient K of a compound can be calculated using the following equation:

$$K = \frac{C_S}{C_M}$$ **Equation 1.6.4.1.1.**

$C_s$ is the concentration of solute in the mobile phase; $C_m$ is concentration of solute in the stationary phase.

The process which compounds are transported and separated through chromatographic system is known as chromatographic development. There are different types of chromatographic development, but elution development is the only development that occurs in GC. Elution development is consisting of a series of absorption-extraction processes that occurs as the sample move through the chromatographic system. As the sample travels along the column, equilibrium is established between gas mobile and stationary phase so that the probability of sample compounds colliding with large surface area of stationary phase and entering it is the same as the probability of a compounds obtaining enough kinetic energy to leave the stationary phase and entering the gas mobile phase. The distribution of

a compound between the two phases is always thermodynamically driven towards equilibrium. However the gas mobile phase is constantly moving displacing concentration of solute in the gas mobile phase forward, so concentration of solute at the front of peak in the mobile phase will increase and exceeds the equilibrium concentration. As a result, the solute in the front of the peak will be continuously entering the stationary phase to reach the equilibrium concentration. At rear of the peak, the concentration of solute in gas mobile phase will be lower than the equilibrium concentration since the solute is being displaced forward, the solute now enter the gas mobile phase from stationary phase to attain equilibrium. However, only the solute molecules with kinetic energy greater than the potential energy of their association with molecules of stationary phase will leave and enter the mobile phase. When the number of molecules with enough kinetic energy to enter the mobile phase equals the number of molecules with not enough kinetic energy and retained in the stationary phase, the equilibrium is attained (55) (56).

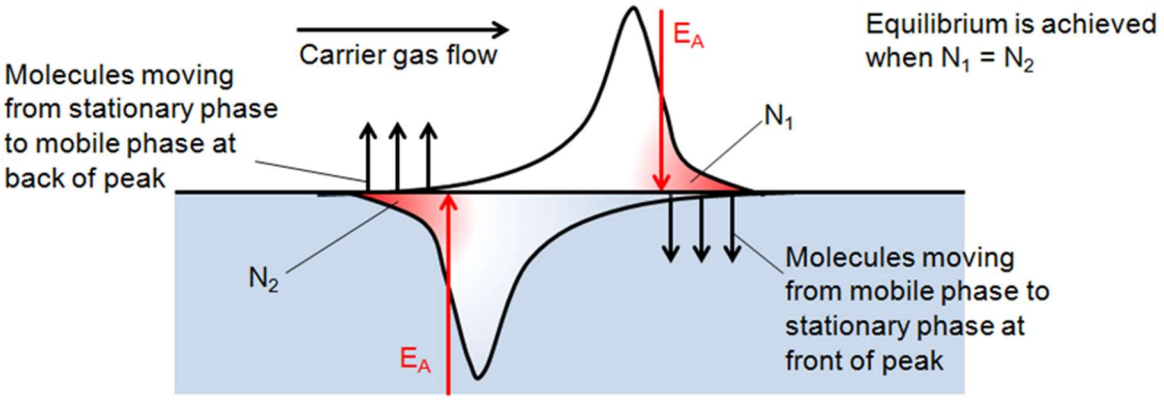


**Figure 1.6.4.1.1.2. The elution process**

## 1.6.4.1.2. The plate theory

In order to understand how separation can be achieved, an equation needs to be derived for retention volume of a compound, hence equation for elution curve of the compound. The plate theory was originally created by Martin to derive an expression for elution curve of the compound. The plate theory is based on the assumption that a compound is always in equilibrium between the two phases. However, due the constant movement of the gas mobile phase, equilibrium is never truly achieved. So the column is considered to be divided into number of plates, each plate has a certain length, solute will spend a finite time in each plate. The size of each plate will provide enough dwell time for the solute so equilibrium is achieved between the two phases, therefore the smaller the plate is, the more efficient the solute exchange between the two phases are, the more plates are present in the column.

The number of theoretical plate N in a column is also known as column efficiency and reflects the separation power of a column. A equation can be derived to determine plate number by measuring retention time $t_R$and peak width $W_h$:

$$N = 5.54\left(\frac{t_R}{W_h}\right)^2$$ **Equation 1.6.4.1.2.1.**

A large plate number and small peak width indicates an efficient column.

Another way of expressing the efficiency of column is plate height H. It is height of column occupied by one plate. H has units of length and is better than N for comparing efficiencies of columns of different length, it is also known as Height Equivalent to One Theoretical Plate (HETP). Plate height $H$ can be calculated by following equation:

$$H = \frac{L}{N}$$ **Equation 1.6.4.1.2.2.**

$L$ is the column length. A small H will indicate an efficient column.

### 1.6.4.1.3. The rate theory

When sample is first injected into a GC column, it is a narrow band, but as the sample travels through the column the solute will start to randomly disperse and spread. The distribution of sample molecules will form a Gaussian shape. The dispersion of sample is very important to consider, since if the dispersion is great, then different compounds will mix together and peaks that emerge will be have low resolution. The dispersion of solute is due to physical processes which take place which can be explained partly by the plate theory, but it didn't address non-equilibrium conditions, so another approach known as the rate theory was published by Van Deemter in 1956 to describe the kinetic factors in peak dispersion. The rate theory defines three important factors that contribute to peak dispersion in packed column: multi-path dispersion, longitudinal diffusion, resistance to mass transfer in the two phases. The Van Deemter equation that describes variance per unit length of a packed column to linear velocity of mobile phase in terms of plate height ($H$) is shown below. As you can see, it is separated into three parts, A, B and C each represent a dispersion factor, and $\mu$ is the linear velocity.

$$H = A + \frac{B}{u} + Cu$$ **Equation 1.6.4.1.3.1.**

Most of columns that are used currently are capillary column, so the Van Deemter equation cannot be applied to them. Since in cappilary columns there are no packing particles, so all the solute molecules will take the same path, hence multi-path dispersion (Factor A) will not occur.

Longitudinal diffusion (factor B) occurs due to the natural random diffusion process of solute contained in gas or liquid, which produces Gaussian form distribution. The solute diffuse from region of high concentration to an area of low concentration. Van Deemter derived the following equation for longitudinal diffusion:

$$\boldsymbol{B = \frac{2D_m}{u}}$$ **Equation 1.6.4.1.3.2.**

$D_m$is diffusivity of the solute in the mobile phase.

Small value of diffusion coefficient will generate small factor B and small H. Since B is divided by u, by using a high linear velocity carrier gas or flow rate it will minimise factor B by decreasing the time solute spends in column and solute diffusion.

Factor C is resistance to mass transfer in both mobile phase and stationary phase. This resistance to mass transfer occurs when the molecules close to boundary of the two phases will travel to the other phase before the molecules that are further away and will have longer distance to travel. As a result, the moving mobile phase will push the molecules that are closer further away from molecules that are further away, thus will result in dispersion. Factor C can be separated into two equation for the two phases. $d_f$ is effective thickness of stationary phase, $d_p$ is effective thickness of mobile phase. $f_1$ and $f_2$are both constants, $D_m$ is diffusivity of solute in mobile phase, $D_s$ is diffusivity of solute in stationary phase and $k$ is capacity ratio of solute.

$$\boldsymbol{C = \frac{f_1 k d_p^2}{D_m} u + \frac{f_1 k d_f^2}{D_s} u}$$ **Equation 1.6.4.1.3.3.**

To minimise Factor C, stationary or mobile phase should be thin and diffusion coefficient needs to be large. Rapid diffusion through mobile or stationary phase will keep the solute molecules together. Thin stationary phase will speed up mass transfer into and out of stationary phase. Creating thin stationary phase can be achieved by applying thinner coating on column, but lowering diffusion coefficient can only achieved by using low viscosity stationary phase.

The modified equation for open tubular column or capillary column is invented by Golay, so it is known as the Golay equation:

$$H = \frac{2D_m}{u} + \frac{f_1 kr^2 u}{K^2 D_s} + \frac{f_2 kr^2 u}{D_m}$$ **Equation 1.6.4.1.3.4.**

$r$ is the column radius and $K$ is the distribution coefficient of the solute between the two phase.

## 1.6.4.2. Thermal desorption

In recent years, thermal desorption (TD) has become the method of choice for most ambient monitoring applications due to the 1000 fold sensitivity enhancement when compared with solvent extraction. A wide range of weak, medium and strong commercial sorbents are available for a wide of applications. Therefore sorbent selection compatible for TD and analyte of interest is essential to consider prior to experimental use. Sorbent packed tubes can typically contain between 1 and 4 sorbents arranged in order of increasing sorbent strength from sampling end. Multi-sorbent tubes are especially useful when monitoring a wide range of compounds at different volatility. A range of factors need to be considered to sorbent selection for multi-sorbent tubes including the volatility range of target compounds, sorbent temperature compatibility, storage stability and water retention (53). There is no such thing as a truly 'universal' adsorbent tube that can cover all applications, however, the most broadly applicable multi-sorbents combination for a single tube is Tenax TA backed up by a medium strength graphitized carbon black (eg. Carbopack B or Carbograph 1 TD) backed up by a strong carbonized molecular sieve sorbent (eg. Unicarb or carbonxen 1003). This multi-sorbents combination can be used for quantitatively a wide volatility range of compounds from $C_3$ to $C_{26}$. However, there is one limitation to this sorbents combination which is the strong carbonized molecular sieve sorbent is prone to water retention, due to the high level of humidity in human breath, carbonized molecular sieve sorbent isn't an ideal sorbent material. Standard sized tubes packed with equal bed length and weight of Tenax TA and Carbograph 1TD is selected for measuring breath VOCs for this study. When using multi-sorbent tubes, it is essential to use backflush desorption where the flow of gas through the tube during desorption is the opposite direction of flow during sampling, thus protecting the stronger sorbents from higher boiling point analytes.

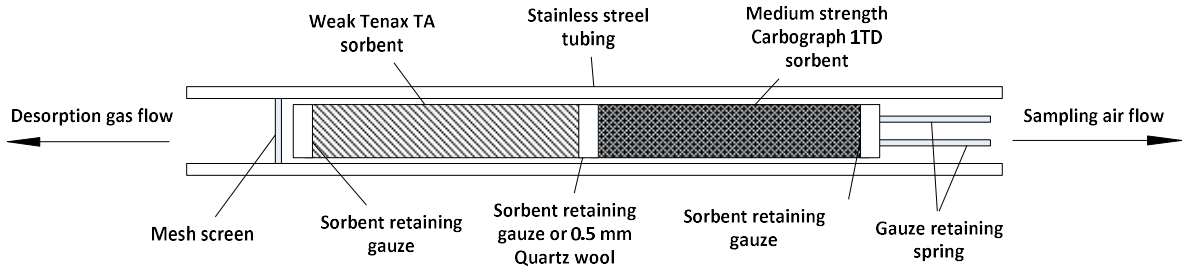

**Figure 1.6.4.2. Schematic diagram of multi-sorbent tube with weaker Tenax TA sorbent and medium strength Carbograph 1TD sorbent.**

The analytes retained by the adsorbent tube can be injected into the GC in either a 1-stage or 2-stages thermal desorption process. In a 1-stage process, the analytes are thermally desorbed directly into the GC. For 2-stage thermal desorption, a focusing device is used to trap and rapidly inject the analytes into the GC. The 2-stage process is the preferred process, the introduction of a focusing device such as capillary cryofocusing or cold trapping significantly improve chromatography. Capillarycryofocusing collection involvespassing carrier gas through capillary tubing at very low temperature (as low as -160°C depending on the application), this method produces very good chromatography by reducing peak distortion and improving peak resolution, but the method can be costly due to the use of cryogen, and the water vapor contained in the sample can be deposited as ice causing blockage to the capillary and dilute the sample., Therefore cold trapping is the preferred focusing device used in thermal desorbers. In the first stage of thermal desorption, the adsorbent tube is heated to high temperature under Helium carrier gas in the reverse flow of sampling. The analytes are transferred and retained onto the electrically cooled cold trap. At the second stage, the cold trap is rapidly heated to high temperature (Up to 100°C $s^{-1}$) causing the analytes to transferred into the GC. This rapid second stage thermal desorption process allows analytes to be transferred in as little as 100 to 300 µl of carrier gas, thus significantly improve analyte sensitivity and chromatography (54).

### 1.6.4.3. Quadrupole Ion trap mass spectrometry

The Varian quadrupole ion trap MS instrument used have two different ionisation methods: electron ionisation (EI) and chemical ionisation (CI). In EI, the ion source exposes the sample to 70 eV of electrons from filaments, as analyte molecule interact with the thermal electrons from the beam, the sample molecule will have one electron knocked off and become positively charged ion. Since the positively charged analyte ion has gained energy through energy transfer, it is likely to fragment to loss energy. It is also likely the initial energy transfer to the molecule will cause fragmentation as well. EI is also known as hard ionisation due to the high level of fragmentation it causes, but the fragmentation pattern it generates is unique to specific compound and can be used for qualitative analysis. The ionisation process of molecule is shown below:

$$\boldsymbol{M + e^- \rightarrow M^{+\cdot} + 2e^-} \qquad \textbf{Equation 1.6.4.3.}$$

The quadrupole ion trap mass spectrometry is three dimensional analogue of the more widely used linear quadrupole mass filter. The ion trap consists of three electrodes with hyperbolic surfaces: the central ring electrode and two adjacent endcap electrodes (See Figure

1.6.4.3.1.). The electrodes are separated by ceramic spacers. A radio frequency (RF) generator generates a voltage that is applied to the central ring electrode which alternatively reinforces and dominates the direct current (DC) voltage that is applied to the endcap electrodes, thus generates a three dimensional hyperbolic electrical field within the ion trap. Ions generated from ionisation source which enter the electrical field are subjected to stabilising and destabilizing forces, and oscillate in both R- and Z-directions. The R- direction is a combination of X and Y axis. Therefore the ions are trapped in a three dimensional stable trajectory in between the ring electrodes and end cap electrodes which allows no degrees of freedom (57).

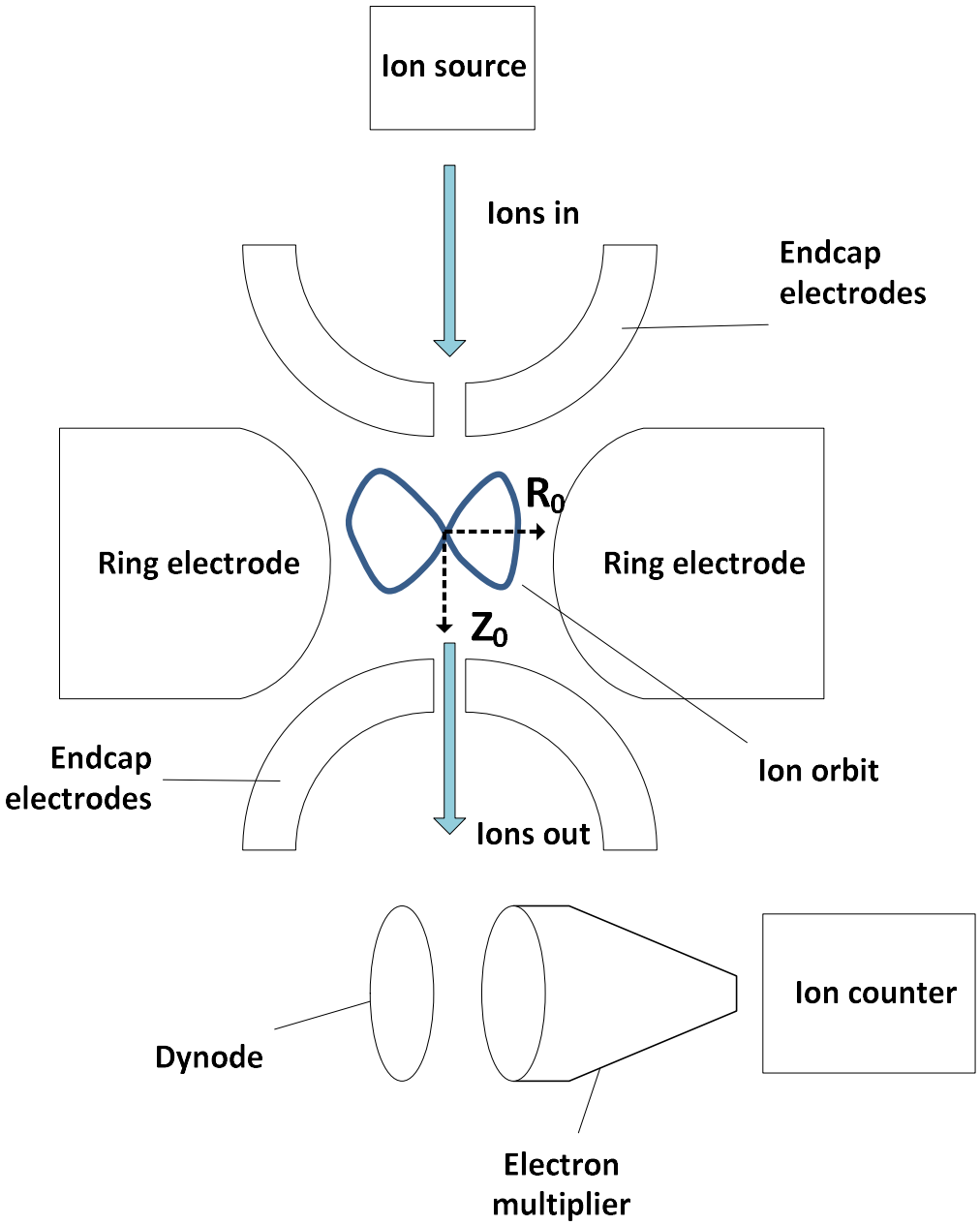


**Figure 1.6.4.3.1. Schematic diagram of quadrupole ion trap consisting of the central ring electrodes and two adjacent endcap electrodes. The ion trap is radially**

**symmetrical with hyperbolic cross section, $R_0$ and $Z_0$ represent its size. R-direction is a combination of X and Y planes.**

Similar to linear quadrupole mass filter, the trapped ions are then ejected by increasing RF voltage, this pushes the ions into unstable orbit and exit through the end electrode. The Mathieu stability diagram describes RF and DC voltages that determines the range of ion mass that can are in stable trajectory inside the ion trap. Radial stability is expressed in terms of $a_r$ and $q_r$ must be simultaneously maintained. As RF voltage increase, ion move along the $q_z$ axis until they become unstable at the boundary where $q_z$ = 0.908. When ions reach the unstable region, their kinetic energy increase causing ions to be ejected out of their stable trajectory in the Z-direction, finally they exit the ion trap through endcap electrode. The ejected ions then enter the conversion dynode and electron multiplier where it will send a signal response to form mass spectrum.

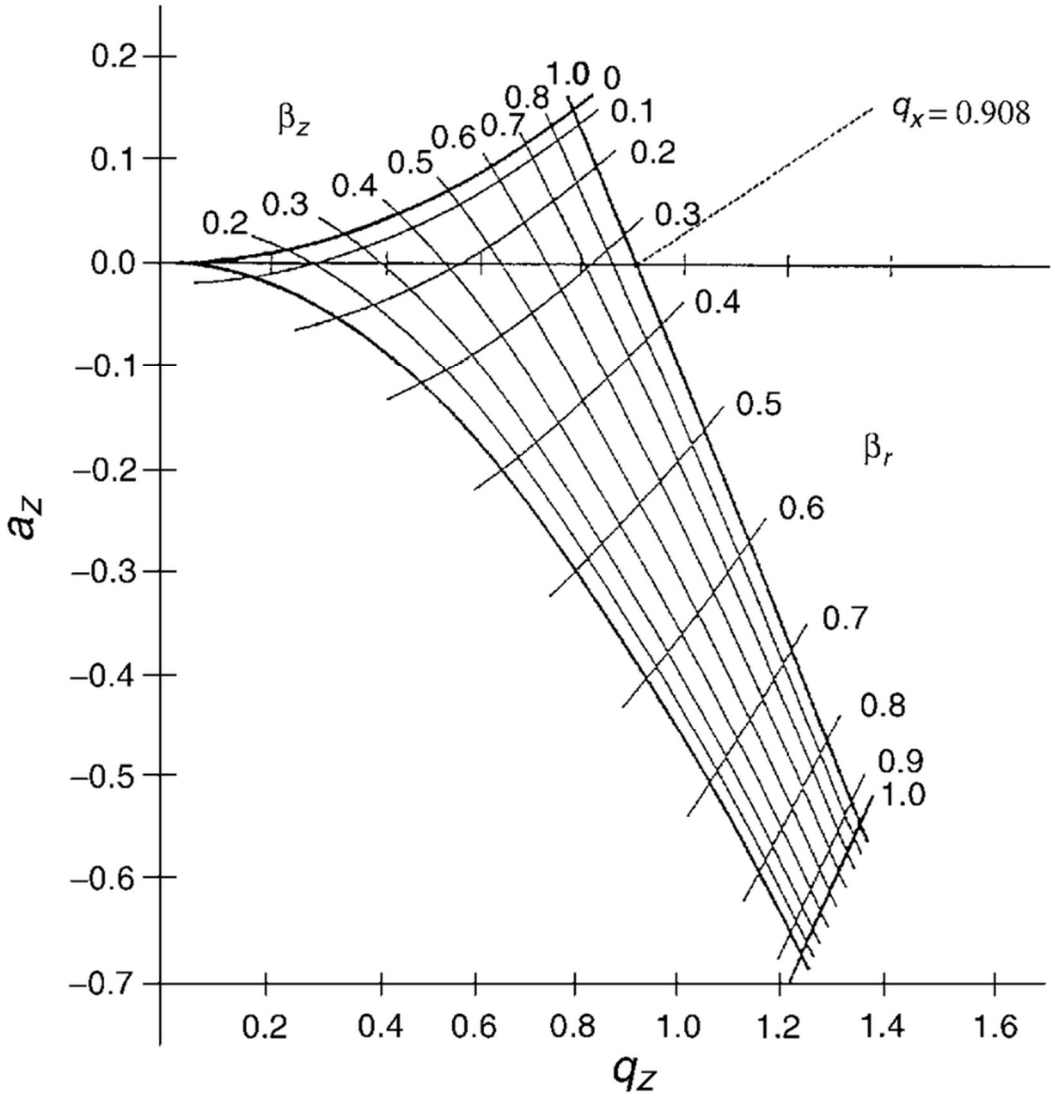


**Figure 1.6.4.3.2. The Mathieu stability diagram for quadruple ion trap (58). Ion are simultaneous stable in both the R- and Z- planes if their $a_z$ and $q_z$ are within the**

**shaded stable region. As RF voltage is increased, $q_z$ value increases to the value of 0.908 while $a_z = 0$, then ions become unstable and are ejected out of ion trap.**

# CHAPTER 2. EXHALED BREATH ANALYSIS WORK-FLOW

## 2.1. Introduction

The current exhaled breath analysis work-flow employed can be separated into 3 main stages: Exhaled breath sampling, breath sample analysis and data processing. This section will describe each stage of the work-flow in the detail.

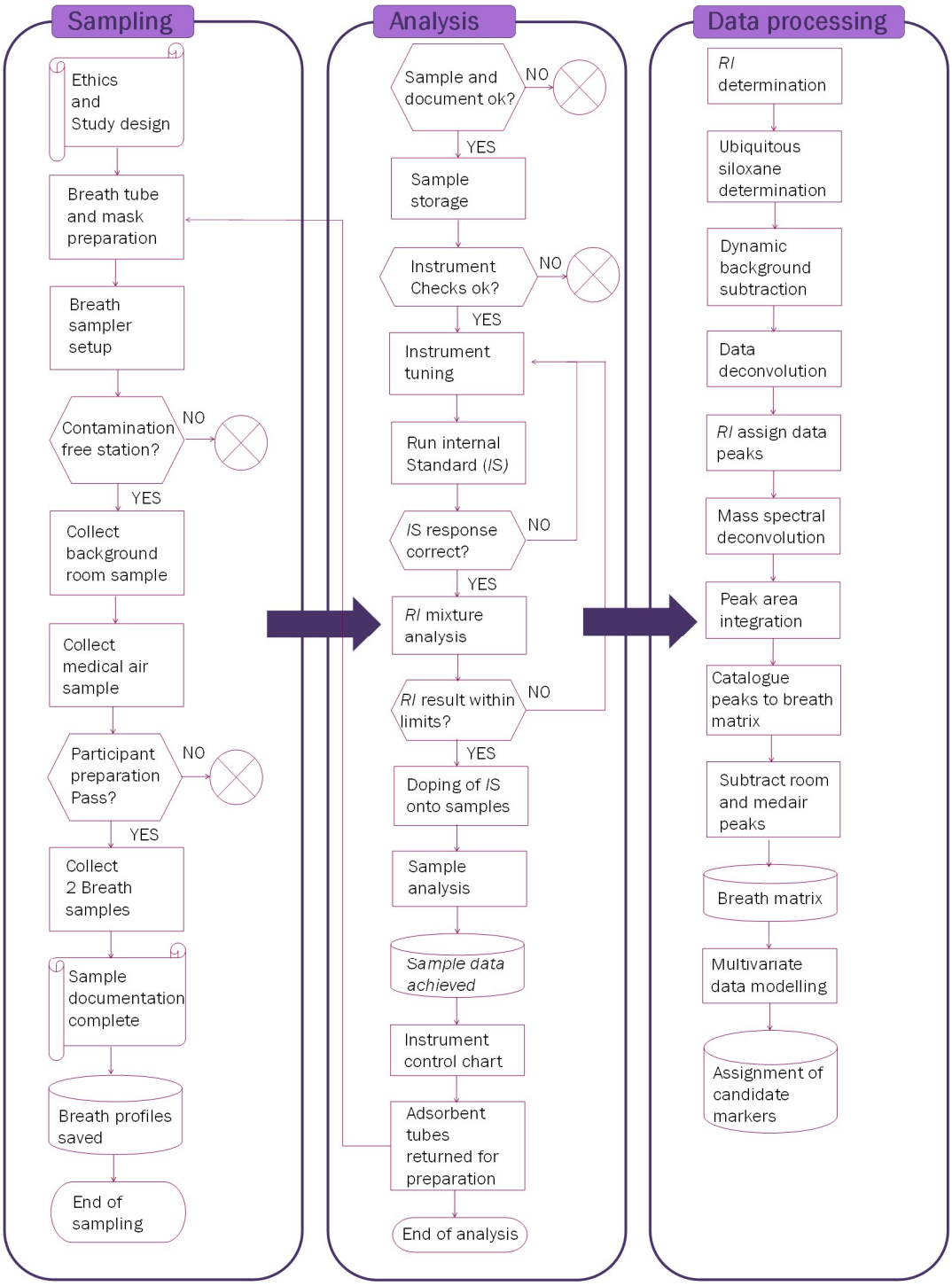


**Figure 2.1. The current exhaled breath VOCs analysis work-flow chart with 3 main stages: Exhaled breath sampling, breath sample analysis and data processing.**

## 2.2. Exhaled breath VOCs sampling

### 2.2.1. Ethics

All studies were conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. Detailed exhaled breath VOCs sampling protocol based

coded G09-05 on the current breath analysis work-flow was submitted and approved by ethics advisory committee at Loughborough University. Research proposals for any subsequent breath VOCs sampling studies involving human subjects were approved separately by ethics advisory committee at Loughborough University.

### 2.2.2. Preparation for sample collection

#### 2.2.2.1. Breath masks preparation

All breathing apparatus that are in direct contact with subjects are thoroughly cleaned and disinfected prior to experimental use. This include Mirage S2 full face masks (ResMed, UK), PTFE interface connections and sampling valves. The cleaning procedure employed follows recommended ResMed face mask cleaning procedure in accordance with the standard ISO 17664: Sterilisation of Medical Devices.

The breathing apparatus were first full disassembled and disinfected with ortho-phthalaldehyde CIDEX OPA 0.55% solution (Johnson and Johnson, UK). The disinfected breathing apparatus were then rinsed with 5 L of warm water to clean off any residue disinfectant solution. Finally, the breathing apparatus were air dried and then vacuum dried at 50°C for 2 hours before being reassembled. The breath masks were then carefully packed in individual container before transported to breath sampling station.

#### 2.2.2.2. Adsorbent tubes preparation

The adsorbent tubes used for exhaled breath collection were conditioned and analysed prior to experimental use. The adsorbent tubes used in our breath collection studies were multi-sorbent tubes consist of Tenax TA 35/60 mesh and Carbograph 1TD 20/40 mesh (Markes International, UK) with a stainless steel tube casing on the outside (L89 mm × 6.4 mm O.D.). The two sorbent trap materials have very different physical properties; The Tenax TA has a recommended conditioning temperature of 325°C and Carbograph 1TD has a higher temperature limit at 350°C to 400°C (59). The adsorbent tubes were conditioned using a modified Hewlett Packard 5890 GC oven (Agilent Technologies, UK) at 310°C for 2 hours under high purity nitrogen gas from a Nitrogen generator (Peak Scientific, UK). The adsorbent tubes were conditioned in the opposite flow direction to sampling at a flow of 100 ml/min. The tubes were cooled downed to 30°C for 1 hour before capped with 1/4” brass caps fitted with PTFE ferrules using spanner.

The conditioned adsorbent tubes were then analysed on TD-GC-MS at isothermal temperature program at 300°C for 10 minutes to check for any VOCs residuals. The tubes were initially cooled to -10°C and desorbed at 300°C for 5 minutes. The inspected

conditioned adsorbent tubes were capped and stored at 4°C before transported to sampling station.

### 2.2.3. Participant preparation

All participants were given information sheet explaining the aim of study and brief breath sampling description. Prior to study, participant have been asked to avoid the use of make-up and fragrance which can cause contamination to the sample collection. Depending on the study, participants might also be asked to fill in health questionnaire, consent form and exhaled breath collection questionnaire, or additional questionnaires. Exhaled breath collection questionnaire examines the participant's food and drink intake prior to the start, any make-up or other products with strong odour that can cause contamination to the breath sampling.

Participants have also been informed to terminate the breath sampling at any time if discomfort is felt by either pulling the safety strap on the full face mask or ask the breath sampling staff to take off the mask. Participant is also encouraged to try breathing in the medical air for a few breaths before the mask is strapped on to check comfort level. There is also a 5 minutes climatization period before breath sample collection.

### 2.2.4. The adaptive breath sampler setup and exhaled breath collection

The adaptive breath sampling apparatus was described in detail previously by Basanta *et al* (41). It consists of 3 main units: medical air supply, breath sampling unit and control interface on laptop PC.

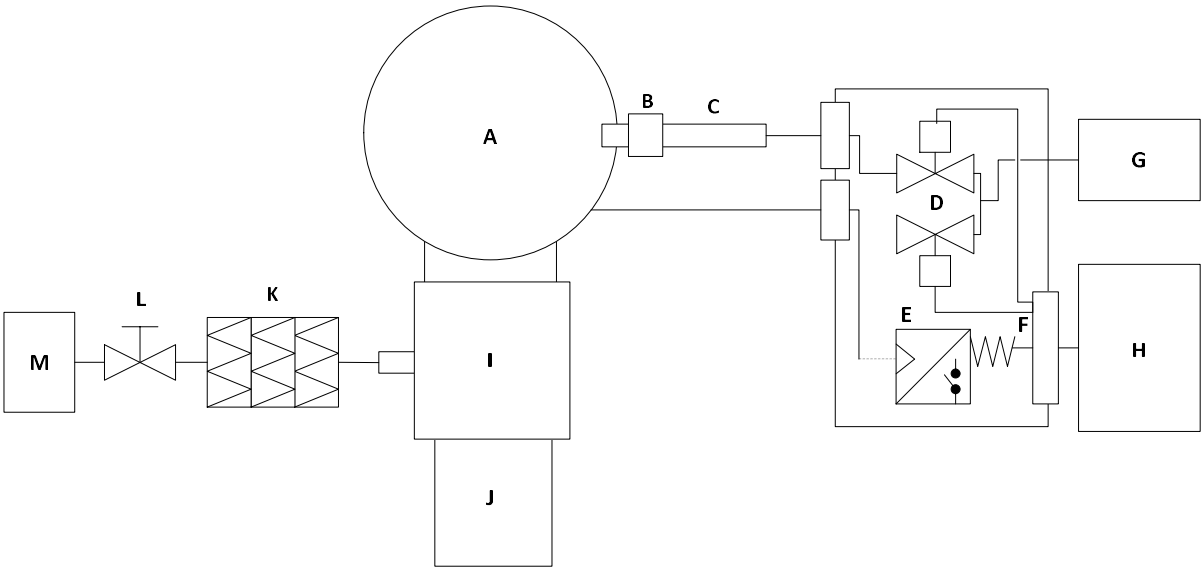


**Figure 2.2.4. Schematic diagram of adaptive breath sampling system. (A) full face breathing mask; (B) PTFE connector with sampling capillary; (C) adsorbent tube; (D) micro-control valves; (E) pressure transducer; (F) electronics interface; (G) sampling pump; (H) Laptop computer running DAC card and Labview software; (I) PTFE T-piece connector; (J) one-way valve; (K) 3-stage filtration assembly; (L) pressure regulator; (M) air supply generator.**

The medical air for breath sampling is supplied direct to the face mask generated by nitrogen generator (Peak Scientific, UK). The air supply from the generators pass through 3-stage filtration assembly: 100-12-DX filter element, 100-12-BX filter element and CI100-12-000 2002-series adsorbent filter element (2000 Balston series, Parker Corporation, USA). 100-12-DX and 100-12-BX filter elements are 2-stage coalescing particle filters that can remove 99.99% of 0.01 Micron particles. The third stage filter is an activated carbon adsorbent that can remove any trace oil vapours from the compressor. Each filter element contains an automatic float drain. When used in combination with air compressor and aftercooler, the purified air supply after 3-stage filtration is able to meet the requirement of ISO8573-1:2010 Class 1.X.1 which specifies maximum particle concentration of 0.08 ppm, maximum pressure dewpoint of -70°C and maximum oil vapour concentration of 0.008 ppm (60).

The purified medical air (> 20 L $min^{-1}$) was then supplied to the face mask through a custom made PTFE T-piece (Tefloturn Ltd, UK) using silicone tubing connection (4.8 mm bore, RS components, UK). A one-way valve (Clement Clarke International Ltd, UK) was attached to the bottom of PTFE T-piece to avoid room air contamination.

The breath sampling unit utilises a custom made sampling control unit with pressure sensor to control alveolar air collection. The sampling control unit consists of two electrofluidic

micro-switch valves (Lee, LFA1201718H, UK) with driver electronics and an integrated circuit pressure sensor which incorporated a Luer fitting connection that allowed it to be connected to face mask via silicone tubing. The sampling control unit is connected to a Laptop PC via a 25-way D-type electrical connector fitted with 6024E PCMCIA data acquisition card (National Instruments, UK). A custom built Labview virtual instrument program (National Instruments, UK) was used to operate the sampling control unit. The pressure sensor output voltage tracks the subject's inhalation and exhalation inside the full face mask, and is plotted in real-time as continuous waveform generating a breath profile on the Labview software interface. In order to sample alveolar air, the user can specify the proportions of the breath profile to be collected. The Labview software is then able to assess the real-time breath profile and enable targeted breath sampling based on user setting. The breath profile can be saved in .csv format at end of breath sampling.

The adsorbent tube is attached to the face mask via custom made PTFE connector (Tefloturn Ltd, UK). An approximately 5 cm MXT guard column sampling capillary (Thames Restek, UK) was fitted inside a Luer fitting into the face mask positioned close to participant nose. The other end of the capillary is positioned inside the adsorbent tube inlet end close to the mesh screen. As subject inhales and exhales, the two micro-switch valves controlled by Labview interface switches between sampling and vent modes to enable targeted alveolar breath sampling. During sampling mode, the sampling pump (Escort ELF pump, MSA, UK) draws subject's exhalation into the adsorbent tube at a flow rate of 0.8 L $min^{-1}$. During vent mode, no breath is collected onto the adsorbent tube. The exhaled breath is collected until the target volume has been reached. Breath collection usually takes around 5 to 10 minutes to achieve 2 L to 2.5 L target volume. If time allows, 2 breath samples are usually collected for each participant. The participant is asked to maintain relaxed normal breath pattern through nose during breath collection (Figure 2.2.4.). Before breath collection, participant is asked to acclimatise to the purified air for 3 to 5 minutes.

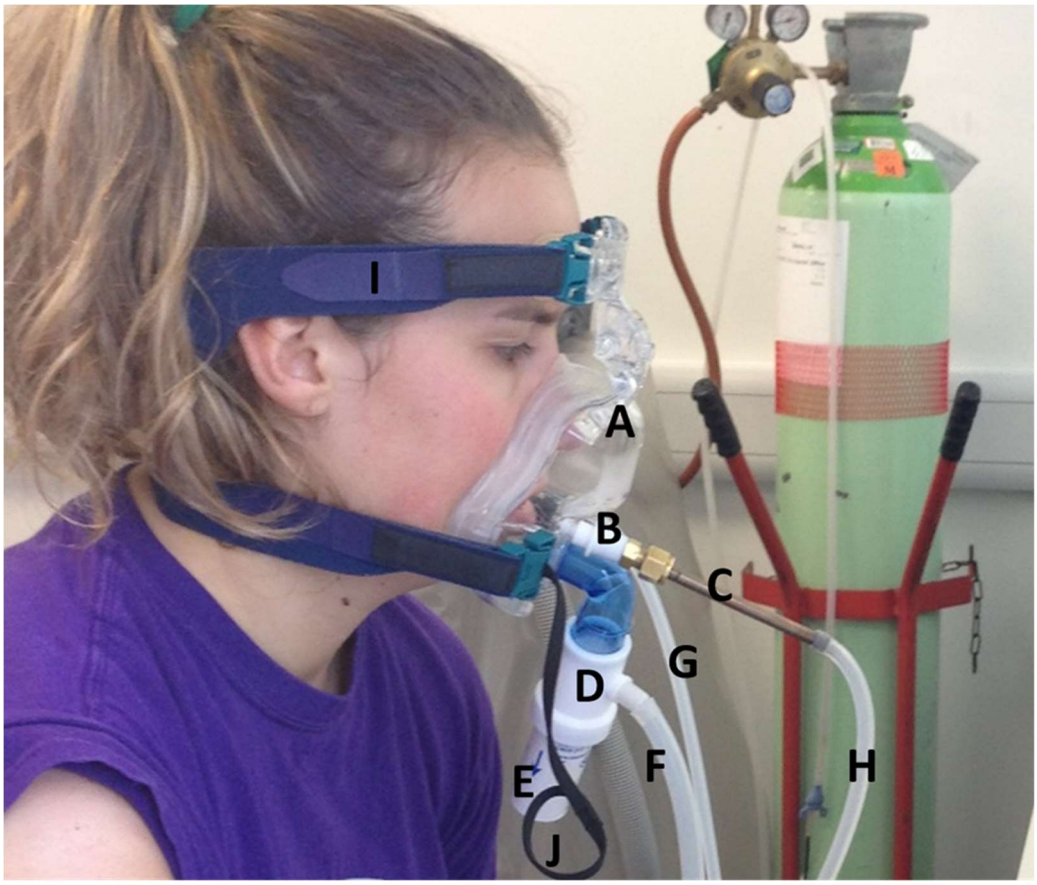


**Figure 2.2.4. the participant is strapped to full face mask (A) using headgear clips (I). An adsorbent tube (C) is attached to the face mask via a custom made PTFE connector fitted with sampling capillary (B). Sampling pump is used to suck exhaled breath into the adsorbent tube via silicone tubing (H). A 3.4 mm bore silicone tubing (G) is connected to other port on the face mask which is connected to the sampling control unit that monitors the pressure output voltage inside face mask and is plotted as continuous waveform. Purified medical air is supplied via silicone tubing (F) through a custom made PTFE connector (D) fitted with one way valve (E) into face mask. The full face mask also contain an emergency release cord (J) that can be pulled by participant to remove mask.**

### 2.2.5. Background air collection

Additional to the breath samples collected, 1 room air sample and medical air supply sample are collected on the same day. A 2.5 L (equivalent volume to breath sample collected) room air sample was collected onto a conditioned adsorbent tube for 2.5 minutes at a flow rate of 1 L $min^{-1}$ using Escort ELF sampling pump. The medical air supply sample can be collected by adjusting the pressure regulator to 1 L $min^{-1}$ and sampling for 2.5 minutes onto a conditioned adsorbent tube.

## 2.3. TD-GC-MS instrumentation and sample analysis

The exhaled breath VOCs samples collected were analysed using thermal desorption – gas chromatography – mass spectrometry (TD-GC-MS) system (Figure 2.3.). The exhaled breath VOCs trapped onto the adsorbent tubes were injected into the analysis system using a 2-stage thermal desorption process. The thermal desorption unit consists of Unity Series 1 thermal desorption unit coupled to an Ultra thermal desorption auto sampler unit with 100 adsorbent tubes capacity (Markes International Ltd, UK). During primary desorption stage, the compounds trapped onto the adsorbent tube were thermal desorbed onto U-T2GPH general purpose hydrophobic cold trap (Markes International Ltd, UK) by heating the tube to 300°C with a desorption flow of 50 ml $min^{-1}$ for 5 minutes. The desorbed compounds were retain onto the cold trap at - 10°C. During the secondary desorption stage, the retained compounds were rapidly desorbed off the cold trap and injected into the GC-MS analysis system by heating the cold trap to 300°C at maximum heating rate with a flow rate of 50 ml $min^{-1}$ for 5 minutes.

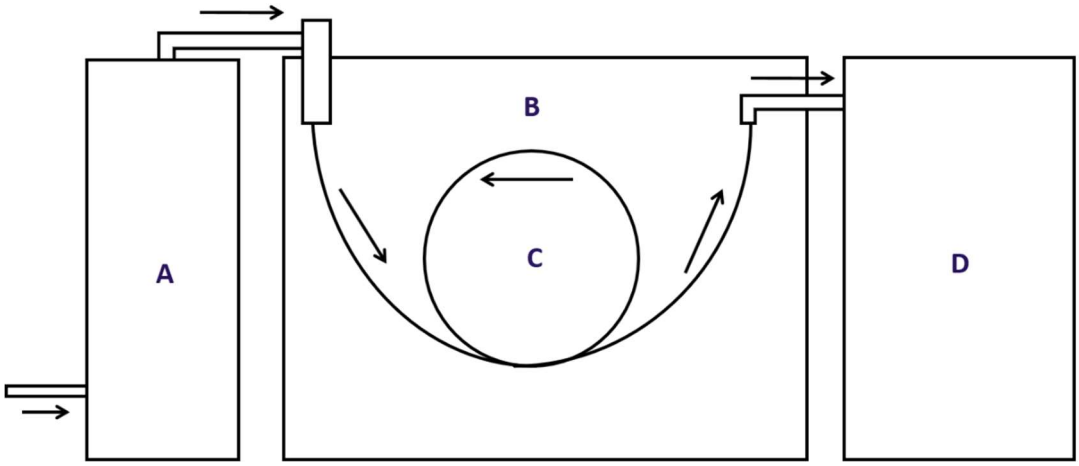


**Figure 2.3. Schematic diagram of TD-GC-MS setup used in breath analysis. High purity helium is pumped into Unity Series 1 thermal desorption unit coupled to an Ultra thermal desorption auto sampler unit (A) where breath sample collected on adsorbent tubes is injected into Varian 3800 gas chromatograph (B) and separated using a DB-5 capillary column (C), the separated breath sample is then analysed using a Varian Saturn 4000 ion trap mass spectrometer (D)**

The breath analytes are injected into the Varian 3800 gas chromatograph at a flow rate of 2 ml $min^{-1}$ via a fused silica column transfer line (0.25 mm I.D, Suepelco, UK) heated to 280°C. The analytes then pass through a DB-5 capillary column ( (5%-Phenyl)-methylpolysiloxane, L30m × 0.25 mm I.D. × 0.25 µm film thickness, Agilent Technologies, UK) at a constant flow rate of 2 ml $min^{-1}$. The GC oven uses temperature ramp program to separate the analytes with an initial temperature 40°C heated to a final temperature 300°C (Hold time 8 minutes) at

a rate of 5°C/minute. The separated analytes are then transferred into the ionisation source of Varian Saturn 4000 ion trap mass spectrometer and ionised through electron ionisation. The resultant ions responses are measured by the detector as mass to charge ratios information.

$$K = \frac{C_S}{C_M}$$

$$\boldsymbol{M + e^- \rightarrow M^{+\cdot} + 2e^-}$$

## 2.4. Exhaled breath VOCs data analysis

### 2.4.1. Data processing work-flow

The data processing methodology work-flow employed is illustrated in Figure 2.4.1. The workflow consists of mainly 3 stages: initial data deconvolution, creation of breath matrix and multivariate analysis of the created breath matrix.

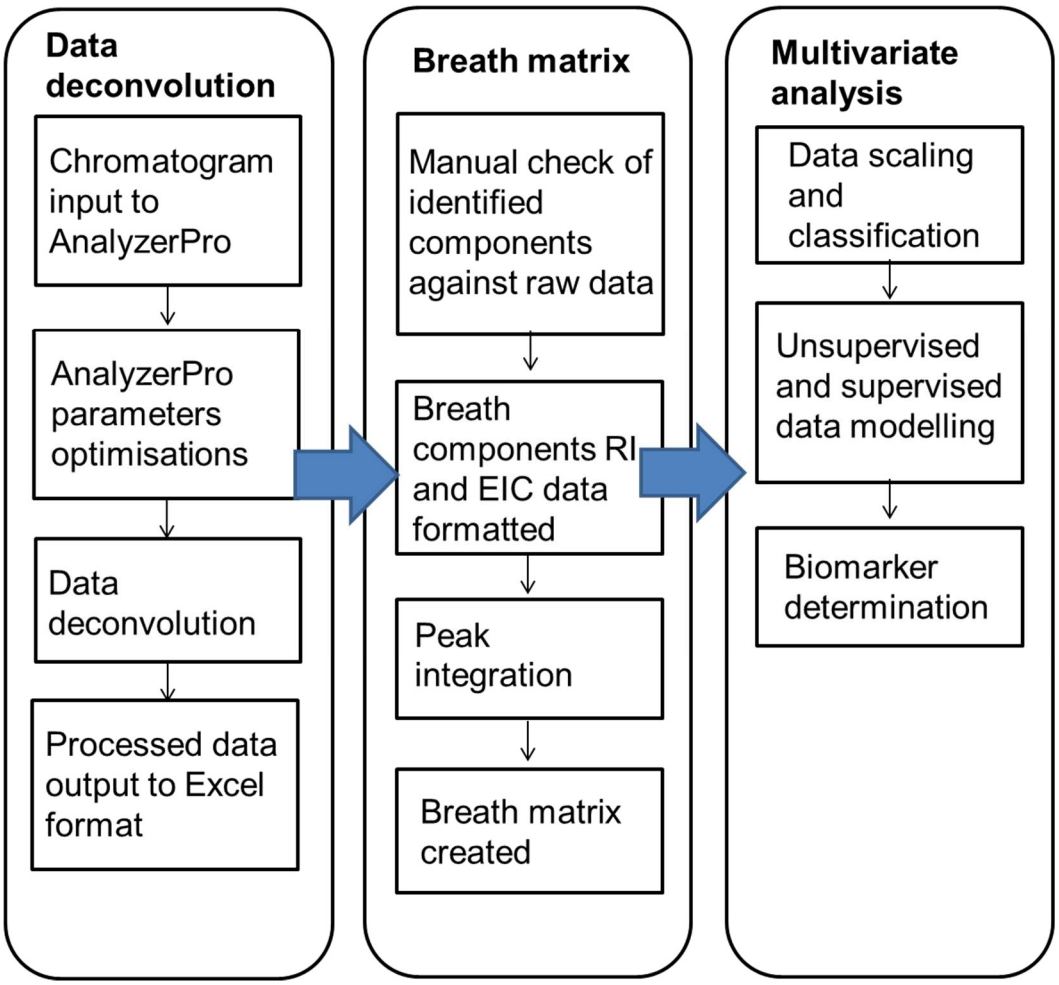


**Figure 2.4.1. The work-flow diagram of exhaled breath data processing method consisting of data deconvolution, creation of breath matrix and multivariate analysis.**

## 2.4.2. GCMS data deconvolution

The first stage in the data processing work-flow is deconvolution of raw data generated by GCMS. The data is first checked through for any major contamination, high level of volatile contaminants from participant can cause high intensity peaks at the start of chromatogram, the over loading peaks can cause ion suppression to low intensity peaks and makes chromatogram unusable.

The usable raw data was then deconvolved using AnalyzerPro software version 2.5.1.7. (Specktralworks, UK). The data deconvolution parameters were optimised using test data with known co-eluting compounds. The data deconvolution parameters used is summarised in Table 2.4.2. Retention index ladder was also added to each chromatogram prior to deconvolution in AnalyzerPro software. The creation of retention index ladder will be discussed in the next section. The deconvolved data was exported into .csv format which can be latter assessed in Excel spreadsheet (Microsoft, USA). Each exhaled breath sample typically generates around 400 to 600 compounds. Each identified component was defined by retention time, retention index, scan number, intergrated peak area, peak height, peak width, signal to noise ratio, peak purity, base peak and number of ions. The output data is then manually checked against raw GCMS data to check for false positives or any components missed by the software.

**Table 2.4.2. Data deconvolution parameters used in AnalyzerPro software.**

| Deconvolution parameters | Settings |
|---|---|
| Area threshold | 500 counts |
| Width threshold | 0.05 Min |
| Signal to noise ratio | 3 |
| Resolution | very low |
| Scan window | 1 |
| Gaussian smooth | 3 |
| Minimum ions in a component | 1 |

## 2.4.3. Retention index

Retention index (RI) ladder is incorporated into breath sample analysis to allow for data alignment. The use of retention index is also essential for inter-laboratory data comparison since retention index is independent of instrument type, column length, flow rate or condition. The Kovats retention index is well established method that was first outlined in 1950s when performing research into the composition of essential oils:

$$\boldsymbol{RI = 100 \times \left[ n + \frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right]}$$ **Equation 2.4.3.**

$RI$ is the retention index value, $n$ is the number of carbon atoms of straight chain alkane that elutes before the unknown analyte with a retention time of $t_{r(n)}$. $t_{r(N)}$is the retention time of the straight chain alkane that elutes after the unknown analyte, and finally $t_{r(unknown)}$is the retention time of the unknown analyte of interest.

The retention index standard used for breath analysis consists of a 18 compounds mixture made from straight chain alkane, chlorinated alkanes and alcohol functional classes (See Table 2.4.3.1.). Pure chemical standard for each compounds was purchased from Sigma Aldrich (USA) or Fisher Scientific (USA) and diluted using dichloromethane to a final concentration of 50 ppm. The RI solution was then injected into a blank thermal desorption tube using a 1 µl syringe at a injection volume of 0.1 µl, the spiked thermal desorption tube was then analysed using TD-GC-MS.

Using the known retention times of a series of straight chain alkane mix from carbon number $C_8$ to $C_{20}$, retention index values are assigned to each alkane based on its carbon number (See Figure 2.4.3.). The assigned retention index values are then plotted against alkanes' retention time to form the primary retention index ladder.

**Table 2.4.3.1. List of compounds that consist the retention index standard used for breath analysis**

| Functional group class | Compound name | Carbon number | Assigned RI number |
|---|---|---|---|
| Alkane | Octane | 8 | 800 |
| | Nonane | 9 | 900 |
| | Decane | 10 | 1000 |
| | Undecane | 11 | 1100 |
| | Dodecane | 12 | 1200 |
| | Tetradecane | 14 | 1400 |
| | Heptadecane | 17 | 1700 |
| | Eicosane | 20 | 2000 |
| Chlorinated alkane | 1- Chlorohexane | 6 | |
| | 1- Chloroheptane | 7 | |
| | 1- Chlorooctane | 8 | |
| | 1-Chlorononane | 9 | |
| | 1- Chlorodecane | 10 | |
| Alcohol | 1-Hexanol | 6 | |
| | 2- Heptanol | 7 | |
| | 2- Octanol | 8 | |
| | 5- Nonanol | 9 | |
| | 1- Decanol | 10 | |

Due to the high humidity in breath samples, the stationary phase of the capillary column is continuously degraded. The water content of the breath samples cause hydrolysis to the

siloxanes of the stationary phase, multiple reactions can occur and results in formation of cyclosiloxanes which are observed in the chromatogram (See Figure 2.4.3.). These cyclosiloxanes are found in every breath sample chromatogram and are used as secondary retention index ladder. The 5 commonly found siloxanes which cover a wide duration of chromatogram were assigned a retention index value based on the primary alkane retention index ladder (See Table 2.4.3.). The assigned retention index values of these cyclosiloxanes are then plotted against their retention times for each breath sample, thus this is then used to align all breath sample data.

**Table 2.4.3.2. The 5 commonly found cyclosiloxanes used in the secondary retention index ladder for aligning breath sample components.**

| | Common breath siloxanes | RI value | Expected retention time | Extracted ion chromatogram |
|---|---|---|---|---|
| S1 | Cyclotetrasiloxane, octamethyl | 998 | 10.768 | 281 |
| S2 | Cyclopentasiloxane, decamethyl | 1142 | 15.357 | 355, 267, 73 |
| S3 | Cyclohexasiloxane, dodecamethyl | 1303 | 20.128 | 73, 341, 429 |
| S4 | Cycloheptasiloxane, tetradecamethyl | 1516 | 24.448 | 73, 281, 327, 415 |
| S5 | Cyclooctasiloxane, hexadecamethyl | 1664 | 28.091 | 355, 73, 221, 147, 401, 281 |

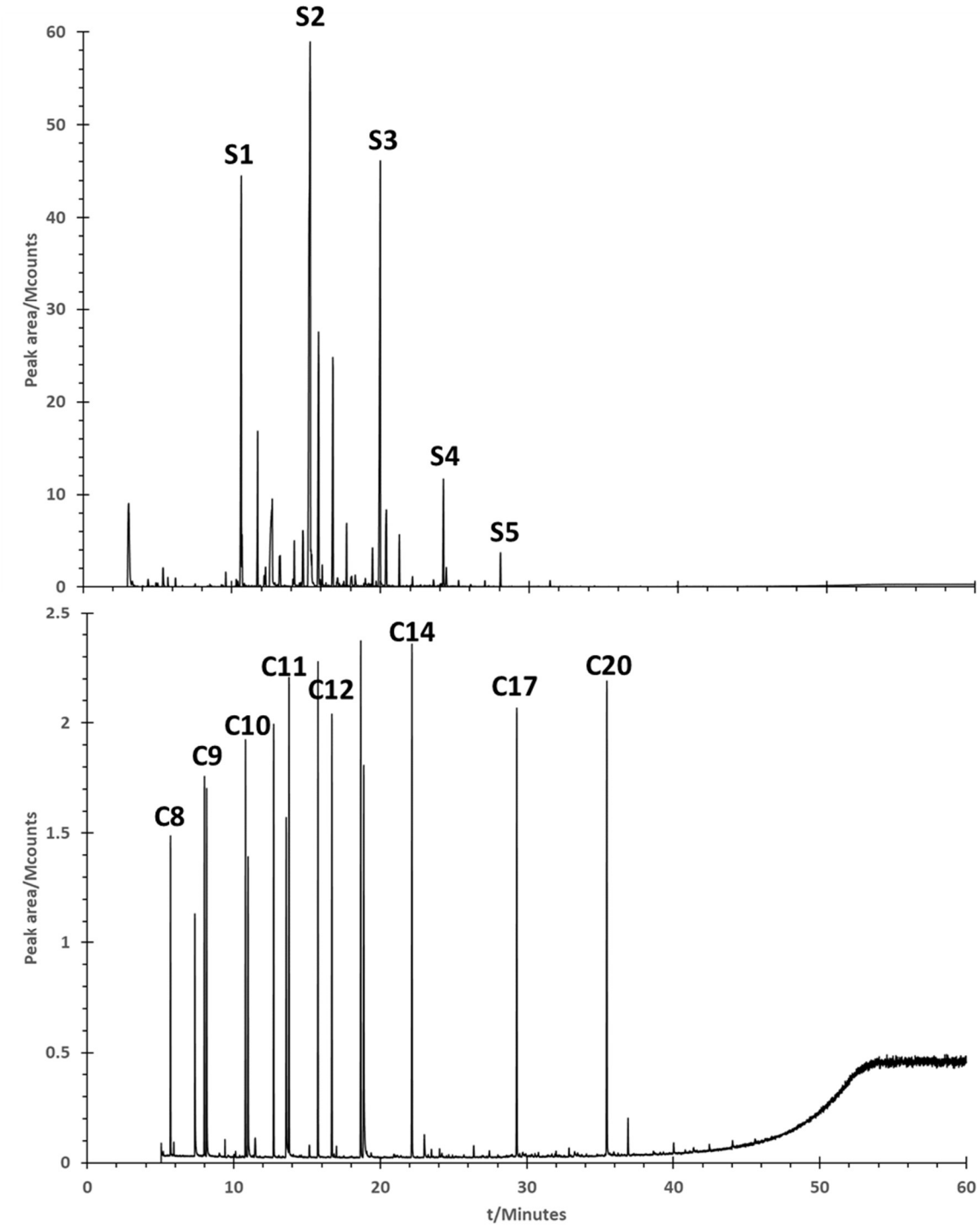


**Figure 2.4.3. TIC chromatograms of primary retention index ladder using alkane compounds from C8 to C20 (bottom) and secondary retention ladder using breath cyclosilane compounds from S1 to S5 (see Table 2.4.3.2.) (top)**

## 2.4.4. Creation of breath matrix

The breath matrix is a spreadsheet of integrated peak areas for each identified breath component plotted against sample name. Each identified breath component is assigned

breath library reference consisting of the calculated RI value and its de-convolved mass spectrum, the breath library reference is written in the format of BRI-XXX-YY-YY-YY-YY-YY. XXX stands the RI value and YY are the 5 most abundant ions in its extracted ion chromatogram. The list of identified breath components was input into Varian MS workstation software to generate a spectrum library where it is then processed against raw data files for peak area integration. The peak area integrated by Varian software is manually checked against raw data files. The final peak areas were used to create breath matrix.

### 2.4.5. Multivariate analysis

Due to complexity of the breath matrix generated, it is not usually not possible to summarise and virtualise the data without using statistical tools. Multivariate analysis (MVA) is based on the statistical principle of multivariate statistics, where multiple variables are analysed at the same time with observations. The aim of MVA is to reduce the amount of data if there is correlation present between variables and observations, thus determining the variable that correlates with the factors of interest (eg. Disease state).

The breath matrix created was imported into MVA software SIMCA-P Version 12 (Umetrics, Sweden). The dataset was first pre-treated by applying the same block weight and Pareto-scaling for each variable. In scaling methods, the data are treated by dividing each variable by the scaling factor which adjust the fold difference between metabolites by converting the data into difference in concentration relative to scaling factor. Pareto scaling divide each variable by the square root of the standard deviation, this ensures large fold changes are decreased more than small fold changes, hence small changes in dataset becomes more dominant, i.e. compounds at trace ppbv or pptv level becomes more apparent in the model. Pareto scaling also stays closer to the original measurements than other scaling methods by retaining the original unit (61).

The pre-treated breath matrix then undergo unsupervised principal component analysis (PCA) and supervised orthogonal partial least square discriminant analysis (OPLS-DA) or partial least square (PLS) data modelling. The unsupervised PCA modelling is usually applied first to the initial pre-treated breath matrix as overview of the dataset for determining trends and outliners. After determining a group of variables with correlation to the observation, the supervised OPLS-DA is then applied to discriminating between groups (eg asthmatic and healthy) and identifying biomarker candidates.

PCA uses orthogonal transformation to combine the original variables (ie. $X_1$, $X_2$…) into a set of linear uncorrelated variables called principal components (ie.$Z_1$,$Z_2$…). The new principal components are chosen so that the first principal component accounts for the most of

variation of the dataset, and the second principal component accounts the next largest variation in the dataset and so on (62).

$$Z_1 = a_{11}X_1 + a_{12}X_2 + a_{13}X_3 + \cdots a_{1n}X_n$$ **Equation 2.4.5.1.**

$$Z_2 = a_{21}X_1 + a_{22}X_2 + a_{23}X_3 + \cdots a_{2n}X_n$$ **Equation 2.4.5.2.**

$a_n$ are coefficients values are chosen so the new principal components are uncorrelated.

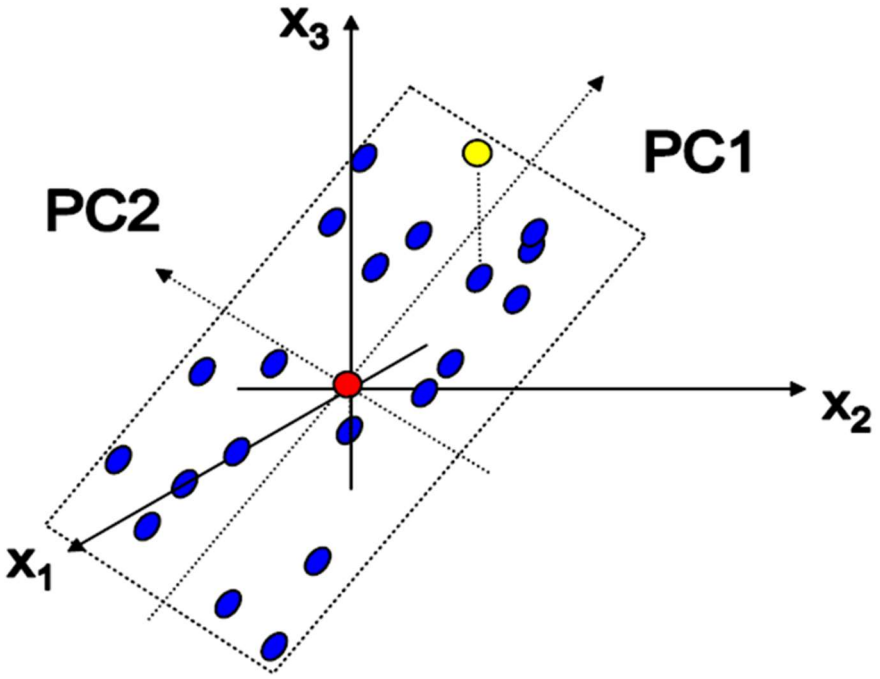


**Figure 2.4.5. The projection of principal components PC1 and PC2 along axis, PC1 and PC2 are orthogonal to each other.**

The loading plot is used to determine the relationship between principal components to the original variables by summarising the variables and explaining the position of observations in the scores plot. Each original variable is assigned $p$ value which is weigh or importance of the variable to the model. If axis of the original variable lines up with the principal component, then $p$ is close to 1 therefore it has a strong influence on the principal component. If the component is orthogonal to the variable, then $p$ value is close to 0 and hence no influence on the principal component. If the component is opposite to the variable axis, the $p$ value is close to -1 and this shows a strong negative influence on the principal component. The loading plot is useful reducing the breath matrix to the smaller dataset that can be used for supervised modelling.

After identifying initial trends and reducing down the variables, a new dataset which have shown correlation to the observations is created and a supervised OPLS-DA or PLS model is applied. The aim of supervised pattern recognition methods is determine the relationship between the X and Y matrices. It uses a learning or training dataset to allocating unknown variables to the correct group. The starting point of PLS is linear discriminant analysis which is a linear combination of the original variables $X_1$, $X_2$ etc (62):

$$\boldsymbol{Y = a_1X_1 + a_2X_2 + \cdots a_nX_n}$$ **Equation 2.4.5.3.**

The original $n$ measurements for each object have been combined into a single value of $Y$, so the data have been reduced from $n$ dimensions to one dimension. The coefficients $a$ are chosen so $Y$ value reflects the maximum difference between groups. OPLS model is extension to the PLS regression method which use information in the $Y$ matrix to decompose the $X$ matrix into blocks of structured variation correlated to and orthogonal to $Y$. The main advantage of OPLS-DA is its ability to separate predictive from the non-predictive orthogonal variation making the loading vector $p$ easy to interpret (63). OPLS model is built on class membership 0 or 1, therefore it is used mainly for datasets with 2 classes (eg asthmatic or healthy).

The SIMCA-P software uses a cross validation approach which divides the 70% of the data into a training set used to calculate the $Y$ values, and 30% of the data into a test set that is used to identify the correct group. The process is repeated until the model finds an optimum result.

S-plot is score plot of $p$ values which describes the magnitude of each variable against $p(corr)$ describes the reliability of each variable. It combines the covariance and correlation from the supervised OPLS-DA model to determine the variables that discriminate between groups. Peaks with low importance are grouped in the middle, variables with high covariance and reliability are located at the far corners, thus enables good visualisation of the data and useful to determining potential biomarkers (64).

## 2.5. Research objectives

This chapter has explored the aspects of exhaled breath VOCs, and its applications as a diagnostic tool for respiratory diseases. It has also described in detail the methodology and its theoretical principles used for exhaled breath analysis including breath sampling, analysis and data processing.

There are many confounding factors to the current methodology that need to be considered. As discussed in the first chapter, inter- breath sample variability can be due to a huge variety of factors. The condition of participant such as exposure to airborne contaminations, diet

intake, menstrual cycle and diurnal variation all adds to the complexity of breathomics, the introduction of external artefacts can significantly increase the difficulty in detecting small changes in participant breath VOCs profile. Therefore it is essential to achieve optimum standardised and reproducible approach to all aspects of breath sample collection.

Many of the participant factors are difficult to manage, since everyone has a distinctive diet, metabolism, lifestyles and body conditions. However, a standardised and reproducible approach to the exhaled breath sampling methodology can still significantly reduce variability to the breathomics data. This thesis aims to explore a number of approaches to standardise the breath sampling method:

- **The storage stability of exhaled breath VOCs retained on the multi-sorbent tubes** – Clinical studies can take months or even years to finish due to participant shortage. The analysis of these breath samples collected from participants can be spread out over several month if not years. This can introduce inter-instrumental variability to the data that can cause significant difficulties during data processing. A long term storage experiment of exhaled breath VOCs was carried out to establish a validated storage protocol for exhaled breath sampling.
- **The development of standardised portable breath sampling system** – it is not always feasible for collect breath sample from participants in designated clinical locations, remote breath sampling can enable a much wider range of breath studies to be carried out. However, the level of VOCs at different locations can vary significantly. Therefore it is important for participants to be provided with standardised inspired air. The design and development of the portable breath sampling system utilising standardised air supply is described in Chapter 3.
- **The application of the standardised approach to clinical study of exercised induced bronchoconstriction (EIB)** – Exercise-induced bronchoconstriction (EIB) is a common pulmonary disorder characterized by airflow obstruction occurring after several minutes of physical activity. This study sought to determine if there is detectable changes in participant exhaled breath VOCs profile after high intensity cardiopulmonary exercise testing (CPET). The portable breath sampling system developed will be used to collect breath samples from participants before and after their CPET inside sports facility. Sports facility can introduce extra air borne contaminants from human sweats and odour. This study will test the application of the developed protocol and device in a clinical study.

# CHAPTER 3: THE DEVELOPMENT OF A BREATH ANALYSIS METHODOLOGY: TOWARDS A REPRODUCIBLE APPROACH AND SAMPLE STORAGE

## 3.1. Introduction

### 3.1.1. Evaluation of the current workflow

Exhaled breath VOC analysis has experienced growing attention recent years as a method for observing biochemical processes, and discovering biomarkers. The non-invasive and safe nature of breath VOC analysis has excited significant interest in its potential as a clinical diagnostic test. In particular has been the focus of research on diseases such as lung cancer, COPD, tuberculosis, asthma and other types of cancer (65) (66) (42) (67) (68).

The current work-flow for exhaled breath analysis employed was reviewed by Guallar-Hoyas *et al*, which can be separated into 3 stages: sampling, analysis and data processing. 2.5 L breath samples were collected using an adaptive breath sampler at optimised flow rate of 1 L $min^{-1}$. The breath sampler was connected to a full face mask. The samples were collected in Tenax/Carbotrap adsorbent tubes and stored at 4°C until they were analysed within 72 hours of sampling.

Exhaled breath samples were analysed using two-stage thermal desorption (Unity series 1, Markes International, USA) interfaced to gas chromatography (Varian CP-3800, Agilent, USA) and ion trap mass spectrometer (Varian 4000, Agilent, USA). A mixture of straight chain hydrocarbons, chlorinated hydrocarbons and alcohol standards were used for retention indexing. This retention index ladder created was then mapped onto the retention times of ubiquitous siloxanes to produce a secondary retention index ladder. Resultant data from exhaled breath samples were processed to create a breath matrix based on retention index and mass spectra. The breath matrix consists of the peak area of isolated VOCs compounds coded by RI number and ion fragments against participant codes. This breath matrix can be input straight into multi-variate analysis (MVA) to identify candidate biomarkers.

Exhaled breath samples are saturated with water vapour, a 2.5 L sample contains approximately 125mg of water. Such levels of water degrade gradually the stationary phase in the GC column affecting the retention time of chromatographic peaks and causing variation in the data. The profiling of VOCs compounds using retention index and mass spectra has proven to be effective for reducing data variation, especially against the effect of water vapour. This provides data suitable for inter-laboratory and inter-study comparison.

This method uses retention index to manage variation, but the manual syringe injection involved in retention index standards, introduces other aspects of analytical variability that prove difficult to manage. This approach could be enhanced by the incorporation of stable isotope-labelled internal standards into breath samples.

This current method requires a breathable air supply and as such is restricted to locations where this is available. Certain studies require samples to be collected in the field or clinic or community and transporting participants to a location with an air supply is rarely practical, or welcomed by participants. Additionally, the level of exogenous compounds within breathable air supplies originating from background room contamination or the manufacture of components may often be present at higher concentrations than exhaled VOC biomarkers. This present challenges for the development of clinical breath sampling protocol and so the development of a portable breath sampler presented an opportunity to reduce such background contaminants.

### 3.1.2. Increasing storage times

The storage stability of breath sample adsorbent tubes for thermal desorption is an important aspect of breath analysis methodology. Currently, the samples are stored at 4°C and should be analysed within 72 hours due to the risk of low-volatility compounds migrating onto stronger adsorbents inside the sampling tube. The stronger carbon black adsorbent is not completely inert, and may cause degradation of labile low-volatility compounds leading to irreversible adsorption and incomplete recovery during desorption (53). Often the collected breath VOC adsorbent tubes are analysed immediately after sampling to minimise any variability, however it might not be possible to always achieve this. In such cases, the adsorbent tubes should be be put into adequate storage conditions until sample analysis. The storage conditions adopted varies depending on the type of adsorbent tubes used. Single Tenax TA sorbent tube have been observed to be stable under room temperature conditions for up to 1 year for benzene, xylene and toluene, and up to 2 years for stable chlorinated hydrocarbons (69) (70). Although trapped VOC are more stable in single adsorbent tubes, multi-bed adsorbent tubes are used more often in breath research because a wider volatility range of compounds may be captured. Multi-bed adsorbent tubes need to be stored under refrigerated conditions for no longer than the maximum recommended period of 30 days for targeted compounds (53) (71) to supress the migration of VOCs from weaker to stronger adsorbents within multi-bed adsorbent tubes. Migration can lead to irreversible adsorption and hence incomplete recovery during the analysis. This type of migration can be minimised by increasing the length of weaker adsorbent or adding a medium strength adsorbent. Additionally storing the samples at a lower temperature, reduces the amount of kinetic energy the VOCs have to migrate between adsorbents and

hence minimises the rate of migration. The effects of storage have been revealed to be compound dependent (71). A recent short-term (2 weeks) storage and transportation study of multi-adsorbent breath VOCs samples reported no significant difference on 15 breath compounds related to asthma (72). However no study to date has investigated the long-term effect of storage on multi-adsorbent exhaled breath samples on a metabolomics scale. Additionally, past studies have often investigated exogenous breath compounds that are not related to biochemical pathways in the human body and may behave differently to endogenous breath components during storage. As the area of breath VOCs research grows it is essential to understand the impact of storage on the whole breath metabolomics profile for method validation.

### 3.1.3. Experimental objectives

The experimental objectives were identified for the method development activity in this project:

- enhanced quality assurance through the incorporation of internal standards
- fully mobile breath sampling
- higher purity air supplies
- longer storage times

## 3.2. Varian TD/GC/MS system long term breath sample reproducibility and quality control

### 3.2.1. Introduction

The elements of the method development activity were embodied in a study to determine the effect of long-term (12 month) storage at - 80°C on the whole breath profile. The hypothesis was that storage at - 80°C would minimise the VOC migration between adsorbents enabling multi-bed adsorbent samplse to be stored for a long time prior to analysis. 25 breath samples, obtained rapidly from a single participant were analysed in 5 batches by TD-GC-MS at 5 time points throughout 12 months storage. The storage stability of the trapped and recovered compounds were evaluated

Instrumentation and/or storage conditions can introduce variations to breath sample analysis which may introduce difficulties to processing and modelling the data. By applying internal standards to breath samples it should be possible to account for such variations and this study investigated the feasibility of using deuterated standards to manage variability in breath analysis. The main aims are to determine:

- Intraday breath samples variability

- Long term breath samples variability
- Use the deuterated standards to establish instrument quality control and minimise its variability
- Application of this method into studies

### 3.2.2. Internal standards breath sample doping system setup

The deuterated compounds were chosen to span the retention times of the chromatogram with an RI range from 755 to 1605 (5 minutes to 30 minutes); the range where most VOCs in breath samples occur.

**Table 3.2.2.1. Internal standards used for breath analysis**

| Compound name | Formula | Molecular weight | Calculated RI |
|---|---|---|---|
| Toluene-$d_8$ | $C_7D_8$ | 100.1877 | 755 |
| Decane-$d_{22}$ | $C_{10}D_{22}$ | 164.4172 | 969 |
| Hexadecane-$d_{34}$ | $C_{16}D_{34}$ | 260.448 | 1605 |

The internal standards were made into permeation sources that were placed in a test atmosphere generator that diluted the compounds released by diffusion with high purity nitrogen at low pressure. Low pressure was essential to minimise error since high pressure nitrogen flow will reduce the permeation rate. Gas flows were monitored and adjusted to constant values and the permeation source temperatures were maintained at 40°C throughout experiment (See Figure 3.2.2.).

The permeation source doping system conditions used are shown in Table 3.2.2.2.

**Table 3.2.2.2. Permeation source doping system conditions**

| Parameter | Flow-rate | Pressure | Doping time | Temperature | Split |
|---|---|---|---|---|---|
| Setting | 50 ml.min$^{-1}$ | 10 psi | 30 s | 40°C | 0 |

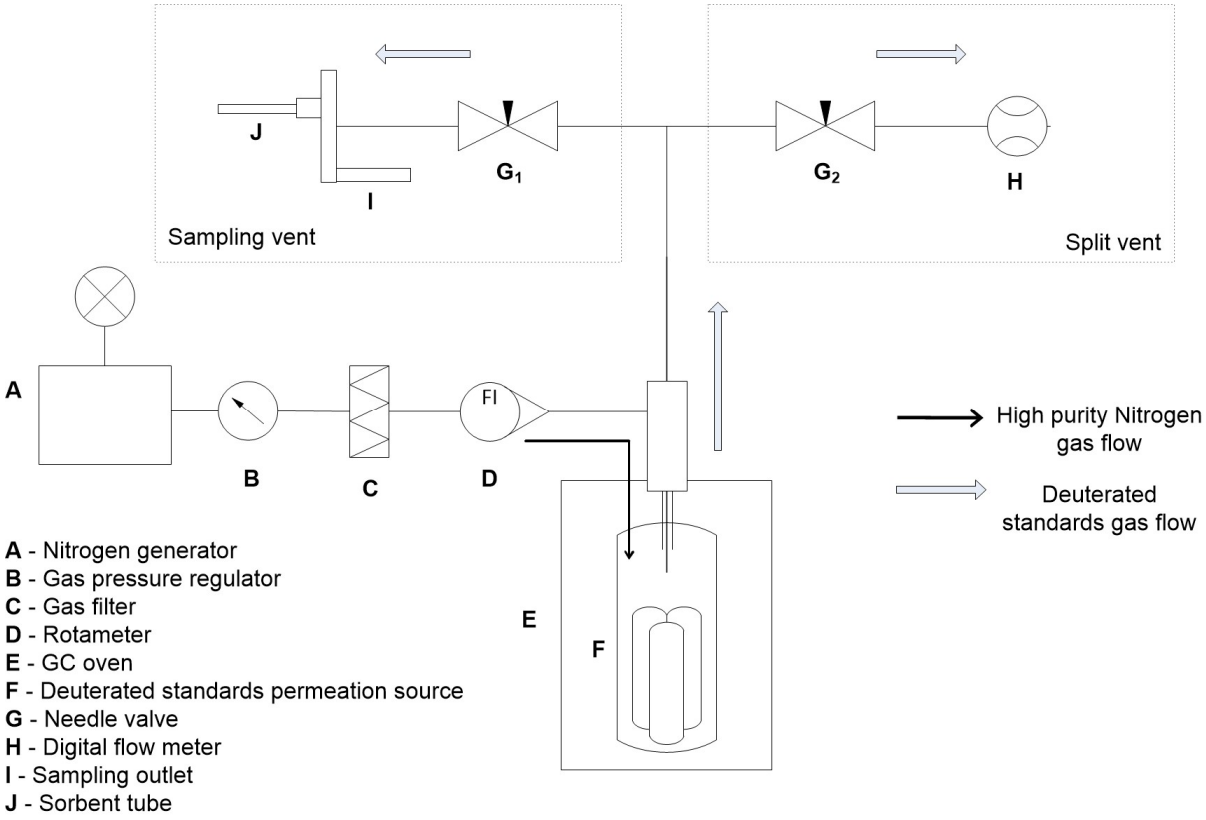


**Figure 3.2.2. Schematic diagram of internal standards permeation system**

### 3.2.3. Breath sample analysis reproducibility quality control study

#### 3.2.3.1. Method

Breath sample variability was investigated by taking 6 breath samples doped with same amount of internal standards were analysed on Varian GCMS within the same day since January 2012 on a weekly basis until September 2012. The peak area (MCounts) for internal standards were calculated and recorded. The RSD% of the internal standards' peak areas from these 6 samples was used to estimate the intraday variation. The long term variability can be calculated as RSD% of peak area over time.

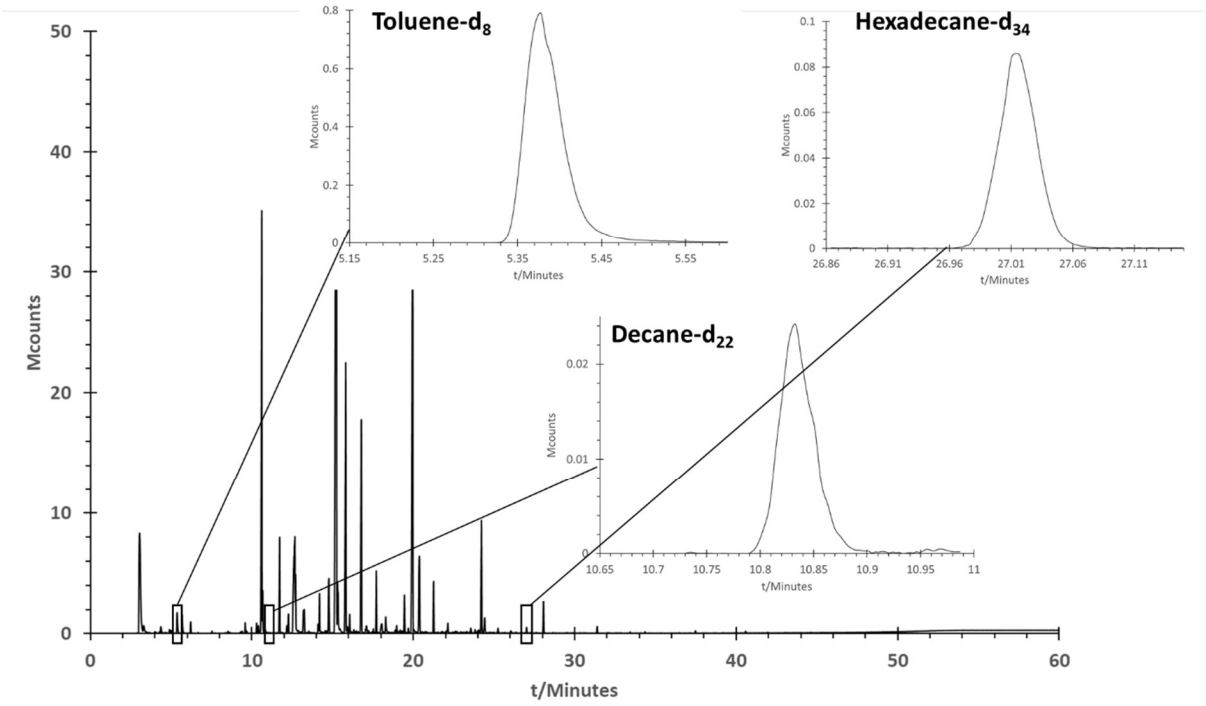


**Figure 3.2.3.1. TIC chromatogram of 2.5 L breath sample doped with internal standards toluene-$d_8$, decane-$d_{22}$ and hexadecane-$d_{34}$. The EIC chromatogram of the three internal standards are shown on the right hand.**

The peak area data from the internal standards were also used for introduce instrumental quality control. The responses to the internal standards were established by calculating the mean EIC peak areas from 20 doped breath samples. The upper control limit (UCL) and lower control limit (LCL) for each standard were also established as ± 2 standard deviations from the mean EIC peak areas of the standards recovered from the 20 doped breath samples.

The peak areas of the internal standards responses were evaluated continuously and if they did not fall within between the LCL and UCL the instrument parameters were re-tuned to re-establish the optimised responses.

Breath samples introduce contamination into the ion trap mass analyser which reduces the response. Observation of the internal standards' responses alongside the instrument parameters enabled the contamination level of the ion trap to be determined. If retuning the instrument failed to bring the instrument into compliance within the control limits the ion trap and ionisation sources were disassembled and serviced.

The Varian 4000 mass spectrometer default parameters for achieve optimised responses are shown in Table 3.2.3.1.

**Table 3.2.3.1. Summary of Varian 4000 mass spectrometer parameters**

| Parameters | Settings |
|---|---|
| Mass range | 40 to 450 m/z |
| Scan type | full |
| Run time | 60 minutes |
| Maximum ion time | 25000 µs |
| Ionization type | Internal EI |
| Emission currents | 10 µAmps |
| Electron multiplier range | 0 to 3000 Volts |
| Scan time | 0.38 seconds/scans |
| Scan frequency | 2.63 Hz |
| Transfer line temperature | 300℃ |
| Manifold temperature | 50℃ |
| Ion trap temperature | 150℃ |

### 3.2.3.2. Results

Average intraday variation was calculated to be 10.3% ± 4.6% from a total of 156 doped breath sample data throughout 9 months (See Figure 3.2.3.2.1.). The sources of this variation may be attributed to variations in: auto sampler injection, manual doping; and degradation of the ion trap from breath contaminants; causing reduction in response. In some breath samples, degradation in the responses to the internal standards response can be observed as the number of samples analysed increase. This was thought to be due to the water vapour in breath samples. interacting with active phases in the thermal desorption cryogenic trap, transfer components and GC column to form cyclic siloxanes. This phenomenon also caused reductions in retention time and peak. The effect of siloxane emphasise the importance of RI and internal standards for breath analysis. The effect of siloxane can be eliminated by either system bake out or increase electron multiplier voltage. The relationship between ion time and internal standards response will be discussed in the next section.

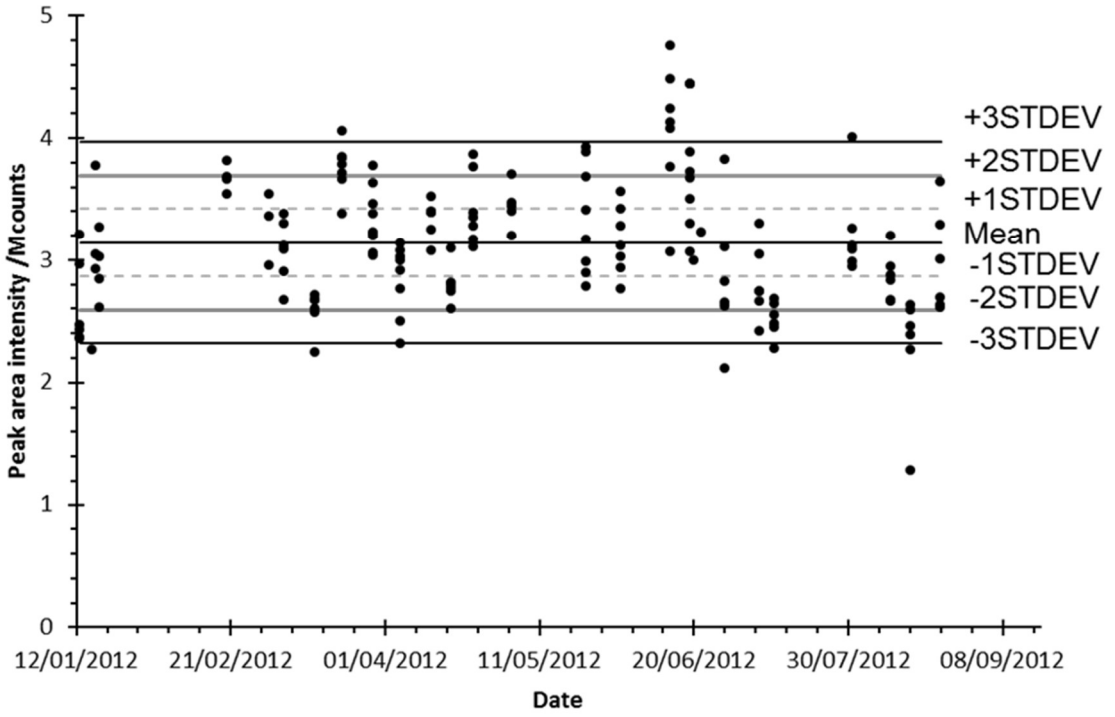


**Figure 3.2.3.2.1. Variation of Toluene-$d_8$ response for duration of 9 months on Varian GC/MS**

Long term sample variability was calculated as the total variation of the internal standards' responses (RSD %) of 156 samples as 17.4%. 8.8% of samples were outside 3 standard deviation range, 28.2% of samples were outside 2 standard deviation range (See Figure 3.2.3.2.1.). The samples response outside 3 standard deviation indicates system retuning or service was required.

The ion time (μs) is the ionization time of the last scan in a Varian 4000 mass spectrometer. It indicates the amount of time ions were accumulated in the ion trap. The maximum ion time is set at 25000 μs for full scan. This is an essential instrument parameter for monitoring the state of the instrument. A reduction in ion time from its maximum on instrument parameter usually indicate possible contamination of the ion trap (perhaps from siloxanes) which reduces the ion accumulation time of analyte ions in the ion trap, and hence reduces the responses to analytes.

22 doped breath samples were analysed throughout 1 month where the instrument parameters were kept constant except for ion time. The build-up in contamination with increasing breath samples was evident with the ion time decreasing accompanied by a reduction in the Toluene-$d_8$ response, see Figure 3.2.3.2.2. The reduction in ion time was rectified by either a "bake out" or a system service.

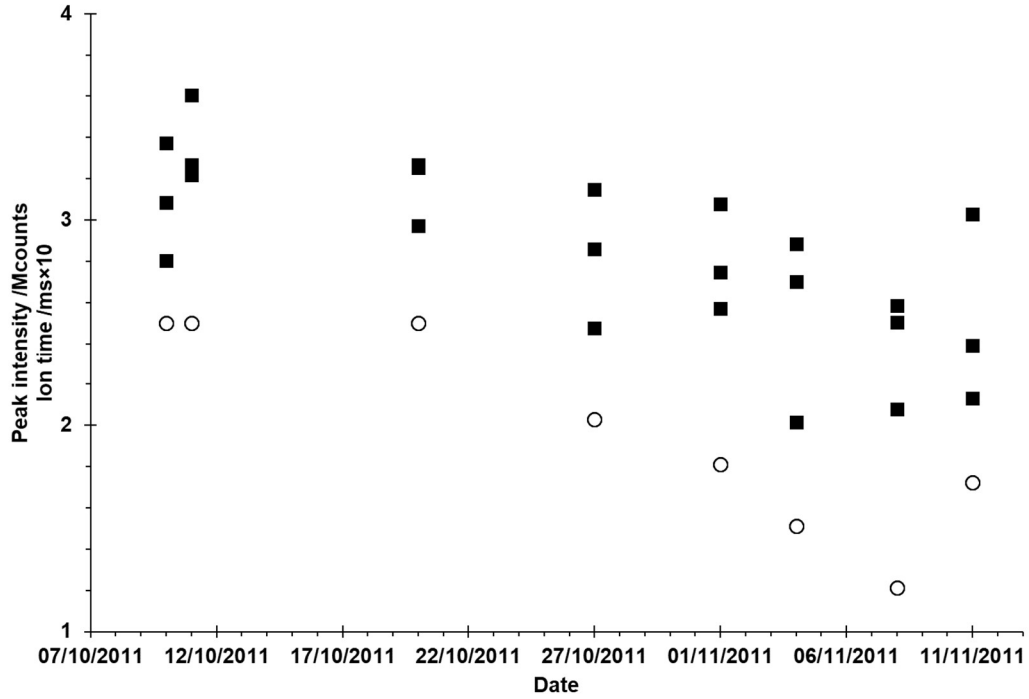


**Figure 3.2.3.2.2. Peak areas of toluene-$d_8$ internal standard measured from 22 breath samples (black squares) and ion time measured before sample analysis (white circles) plotted against analysis date for one month duration.**

### 3.2.3.3. Summary

Long term and intraday variability in breath samples were evaluated and the effect of various instrumental parameters on responses to internal standards was observed. The combination of internal standards and instrumental parameters tuning was successfully used to improve instrument stability, reduce variability and enhance the established method. Overall, the variability of the method was established to be 17% under this method. The rigorous use of formal quality control approaches and reports was intended to improve the reliability of data processing, and the application of these approaches to the sample storage will be shown in later section.

## 3.3. The stability of volatile organic compounds trapped onto a dual-bed Tenax TA: Carbograph 1TD adsorbent trap from exhaled breath

### 3.3.1. Breath sample collection

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. The research method followed non-invasive exhaled breath sampling protocol coded G09-P5 that was approved by Ethics Advisory Committee at Loughborough

University. The participant gave written consent and was screened by a health questionnaire.

One healthy male participant was recruited for this study to provide 25 exhaled breath samples. To minimise variation between samples, all breath samples were collected within a 3 hours' time frame. The participant was encourage to remain hydrated and drink water every 30 min. In addition to the breath samples, 5 room air samples and 5 medical air supply samples were collected after breath sample collection.

Exhaled breath samples were collected from the participant fitted with a non-vented medical full face mask (ResMed, Mirage Full Mask Series 2) supplied with purified medical air at approximately 35 L $min^{-1}$. The participant was first acclimatised to the purified air supply for 10 min while breathing in a relaxed manner; through their nose. A pressure sensor fitted through one of the mask inlets measured and recorded pressure output voltage change inside the sealed mask tracking participant's breath profile. Labview virtual instrument software controlled micro-valves in the sampling control unit that switched between sampling and vent modes based on participant's breath profile. These software controlled valves enabled reproducible and targeted sampling of small airway exhaled breath. An inert capillary was connected to a second inlet on the facial mask to collect breath sample when the micro-valve switched to sampling mode; causing breath to be drawn through the mixed adsorbent trap (Tenax® TA/ Carbograph 1 TD, Markes International, UK). Each 2.5 L breath sample takes around 10 to 15 minutes to collect. A detailed adaptive breath sampler setup and workflow used here has previously been described (41).

### 3.3.2. Heart rate and blood pressure measurements

The participant's blood pressure and pulse rate were measured every 15 minutes throughout the experiment using an automatic blood pressure monitor HPL-300. Metabolic rate is reflected in blood pressure and pulse rate and changes in these physiological parameters affects the level of many endogenous VOCs in breath, hence efforts were taken to keep the participant in a constant physiological state. Room temperature was also monitored every 15 minutes.

Participant's systolic blood pressure was measured with average of 148 mmHg with ± 4% of variation, diastolic blood pressure was measured with average of 103 mmHg with ± 6% of variation, pulse rate was measured with average of 69 with ± 8% of variation, temperature was measured with average of 17.4°C with ± 1% of variation.

### 3.3.3. Long term breath sample storage at - 80°C

All exhaled breath samples collected were randomised and then sealed with air tight storage packaging (73). After sample adsorbent tubes have reached - 80°C, their caps were retightened to avoid cap loosening. The samples were packed into air tight box and stored in - 80°C freezer. In order to assess the long term storage stability of breath samples, the collected samples were stored for up to 1 year.

### 3.3.4. Sample analysis on TD-GC-MS

During the storage experiment, batches of 5 randomised and replicate breath samples, along with 1 room air sample and 1 air supply sample were analysed at 5 time points respectively. The time intervals for sample storage and then analysis were based on a central composite design and set at 0 month no storage, 1.5 month, 6 month, 10.5 month and 12 month. The last time point 12 month was delayed to 12.5 month due to instrument service.

The breath VOCs collected were analysed using a Varian 3800 GC interfaced to a Varian Saturn 4000 ion-trap mass spectrometer. The sampled VOCs were recovered and injected onto the GC-MS by a 2 stage Markes Unity Series 1 thermal desorption unit using a general purpose hydrophobic cold trap. The instrumentation parameters are summarised in Table 3.3.4. The stored samples at - 80°C were allowed to warmup to room temperature for 3 hours before analysis with all frozen condensate thawed and evaporated. Instrument parameter checks and quality control evaluation were performed prior to analysis to ensure that the system was operating within control limits. One internal standard was run before sample analysis as part of quality control. Two blanks were run between each sample to avoid carry over contaminations.

**Table 3.3.4. Instrumental parameters**

| Thermal desorption | | Gas chromatography | | Ion trap mass spectrometer | |
|---|---|---|---|---|---|
| Parameters | Setting | Parameters | Setting | Parameters | Setting |
| Pre-desorption purge | 1 min | Initial temperature | 40°C | Mass range | 40 m/z to 445 m/z |
| Tube desorption | 5 min at 300°C | Final temperature | 300°C | Scan mode | EI auto |
| Pre-trap purge | 1 min | Hold temperature/time | 300°C for 8 min | Emission current | 10 μAmps |
| Trap desorption | 5 min at 300°C | Rate | 5°C/min | Target TIC | 20000 counts |
| Heating rate | Maximum °C/min | Initial pressure | 27.3 psi | Maximum ion time | 25000 μseconds |
| Flow path temperature | 180°C | Final pressure | 56 psi | Scan time | 0.38 seconds/scan |
| Trap low temperature | -10 °C | Hold pressure/time | 56 psi for 7.83 min | Data rate | 2.63 Hz |
| HV temperature | 180°C | Rate | 0.55 psi/min | Trap temperature | 150°C |
| Cold trap flow rate | 50 $cm^3$/min | GC column | DB-5 60m length × 0.25mm i.d. × 0.25 μm film thickness | Manifold | 50°C |
| Cold trap packing | General purpose hydrophobic | Total analysis time | 60 min | Transferline temperature | 300°C |
| Split ratio | Splitless | Carrier gas | Helium | | |
| | | Carrier gas flow rate | 2 $cm^3$/min | | |

### 3.3.5. Quality control

The relationship between retention index and retention time was determined by the analysis of retention index standard mixture consisting of 16 hydrocarbon compounds at a concentration of 75 ppm (w/v) dissolved in dichloromethane was injected manually before sample analysis to act as quality control.

The retention index analysis enabled instrument performance checks before the sample analysis and provided the retention index scale for the subsequent breath components. However it could not be used to account for within-day and between-days reproducibility, hence internal standard dopants were infused onto breath samples. The internal standard dopants were the deuterated compounds toluene-$d_8$, decane-$d_{22}$ and hexadecane-$d_{34}$. The permeation sources are stored under a constant temperature of 40°C, high purity nitrogen at flow rate of 50 $cm^3$ $min^{-1}$ was used to dope headspace VOCs from the permeation sources onto breath sample tubes at outlet for set duration of 30 seconds. Permeation rates of internal standards were measured by changes in weigh and their on-column masses were calculated to be 19.5 ng, 2.7 ng and 1.3 ng for 2.5 L sample volume. The peak area, retention time and peak parameters of internal standards were plotted into control chart to assess the sample stability across the year.

### 3.3.6. Data deconvolution

GC-MS data were deconvoluted using AnalyzerPro (Spectral Works, UK), one breath sample typically yielded approximately 500 components. All isolated components were assigned a retention index value based on a primary retention ladder generated from the retention index standards and a secondary retention ladder based on five ubiquitous siloxane components present in all breath samples. The retention indexed breath components were integrated using Varian MS Workstation software (Varian, UK) generating an extracted ion chromatogram for each component.

Because of retention time shifts across the 12 month storage period the peak integration was performed separately for the 5 sample batches with updated retention index/retention time windows. Each breath component was given a unique breath library reference code that started with prefix BRI followed by retention index value and five most abundant ions in de-convolved mass spectrum (BRI-XXX-XX-XX-XX-XX-XX). A breath matrix was created from the extracted ion chromatography peak areas for all the breath components against the sample name. The data processing workflow adopted here has been discussed in detail in a previous publication[13]

## 3.4. Results and discussion

### 3.4.1. Determination of system variability using internal standards quality control chart

Peak areas of internal standards from every breath sample and background samples were deconvoluted and recorded throughout the 12 month storage period. The variation in peak areas of internal standards serves as a method to assess system stability across longitudinal study and enables comparison of breath sample data at different time points. Additionally the determination of system variation in sample set and batch was used to separate system variation from longer term breath sample storage stability variation. The %RSD system variability across the sample set for 12 month duration was 22.1% for Toluene-$d_8$, 26.9% for Decane-$d_{22}$ and 24.2% Hexadecane-$d_{34}$. The mean variation between the three internal standards was calculated to be 24.4% and indicates system variability within the year. The sample set includes 25 breath samples, 5 background room air samples, 5 medical air supply samples and 5 internal standards blanks. Example chromatograms of the sample sets are shown in Figure 3.4.1.1. The sample set was analysed in batches at 5 time points across 12 month period. The %RSD system variation at each sample batch was 11.4% ± 3% for Toluene-$d_8$, 17.8% ± 7% for Decane-$d_{22}$ and 15% ± 3% Hexadecane-$d_{34}$. The mean variation between the three internal standards was 14.9% and indicates the amount of variability of breath samples that exists even if it is taken from the same participant within short amount of time.

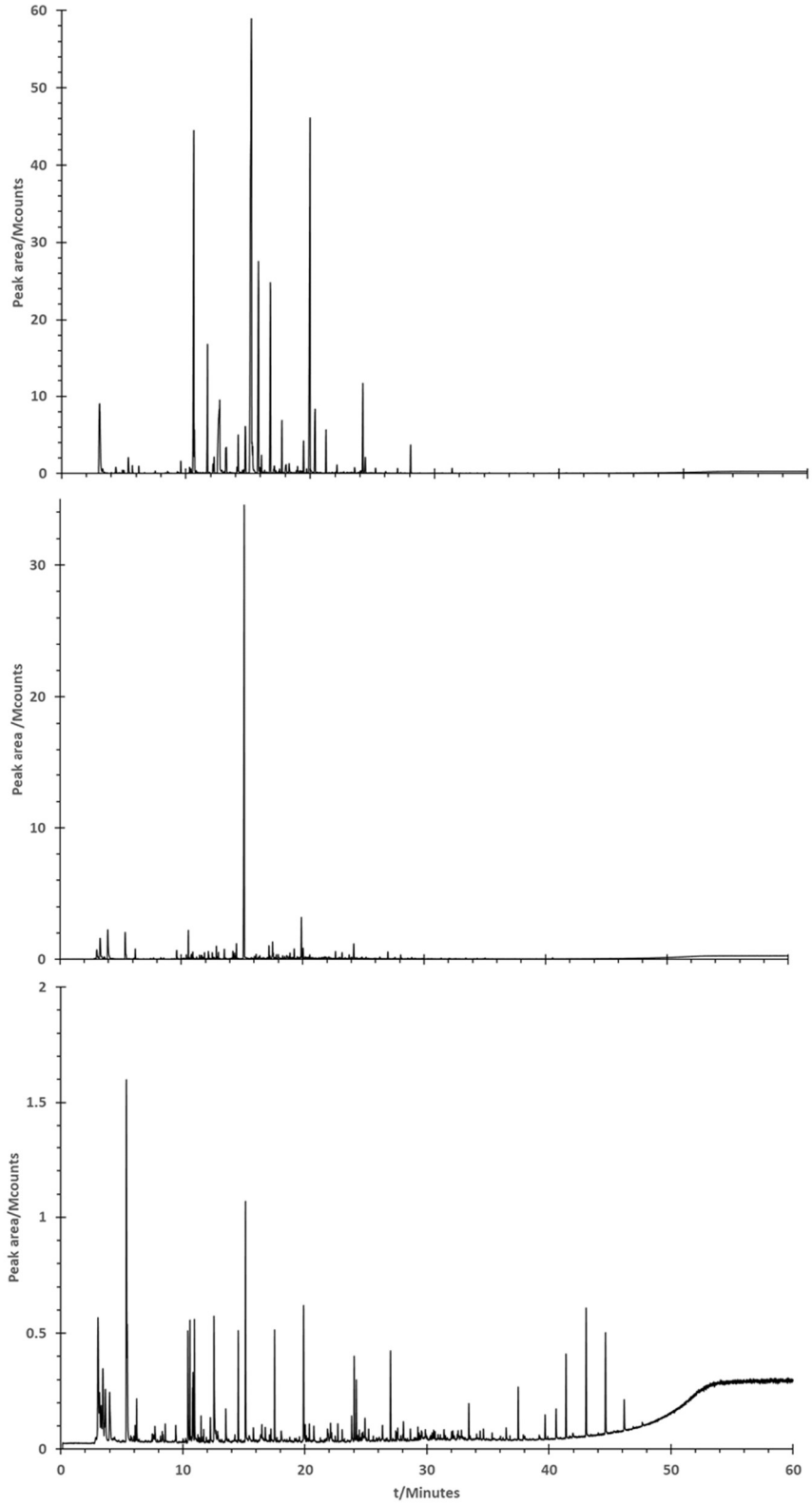


**Figure 3.4.1.1. TIC chromatogram of storage sample set including participant breath sample (top), medical air supply sample (middle) and background room air sample (bottom).**

One contributing factor to the variation in sample set and sample batches was error in manual operation of infusion of internal standards onto breath samples. This error can be observed in the decrease of variation in sample batches analysed at later time points due to better handling of the operation. Figure 3.4.1.2. is a box-whisker plot of the combined marker scores (CMS) of the three internal standards peak intensities across the 12 month storage period. CMS was calculated from collective scores from the three internal standards peak intensities based on their Euclidean distance shown in Equation 3.4.1. Additionally, unsupervised principal component analysis (PCA) of three deuterated standards peak intensities across 12 month was shown in top right corner of Figure 3.4.1.2. In the PCA plot, no distinction between peak intensities obtained different sample storage durations was observed indicating low systematic effect across the sample set.

$$\boldsymbol{CMS} = \sqrt{\sum_{\propto=1}^{3} \boldsymbol{I_R^2}} \qquad \textbf{Equation 3.4.1.}$$

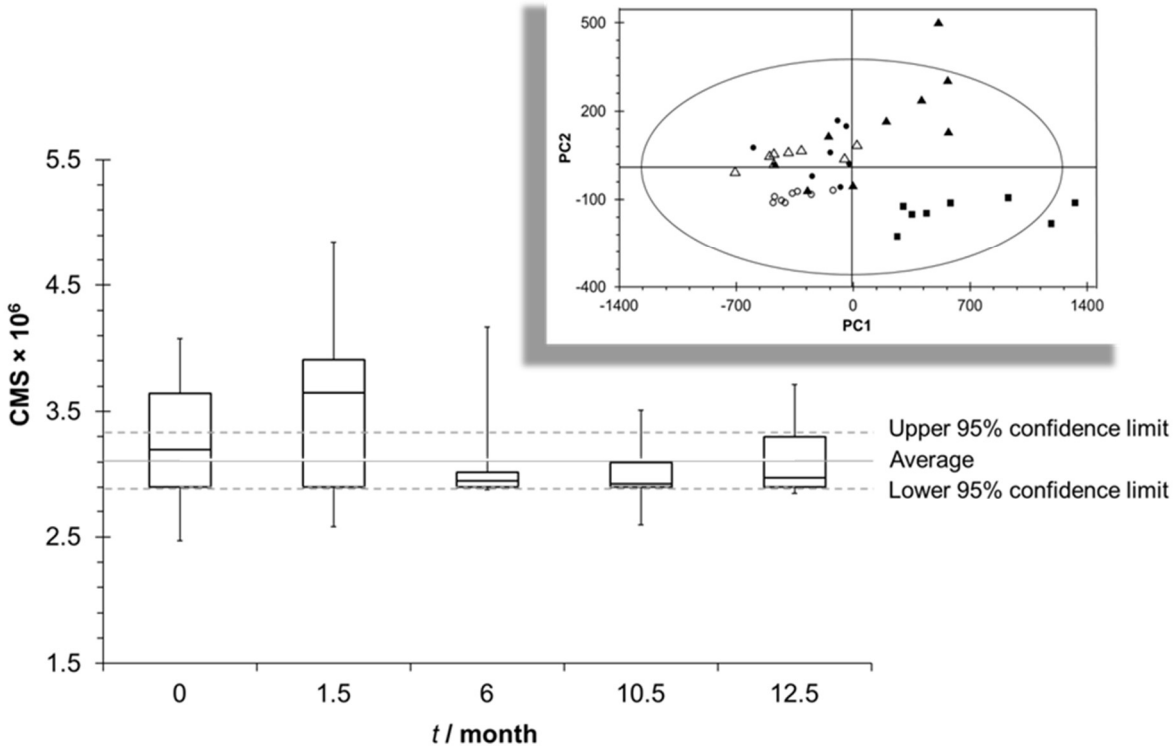


**Figure 3.4.1.2. Box-whisker chart illustrates combined marker scores (CMS) classified by breath sample storage durations. (On the top right corner) Unsupervised principal component analysis (PCA) of all 3 deuterated standards peak areas throughout the year. 5 observations respectively from each time points 0 (black dots), 1.5 month (triangle), 6 month (square), 10.5 month (triangle) and 12.5 month (open circle) are shown.**

### 3.4.2. Relationship between breath components and storage duration

The breath matrix contained 592 breath components separated into 161 endogenous and 423 exogenous breath components. Multivariate analysis was performed on all three data sets using SIMCA-P+ software (Version 12.0.1.0, Umetrics, UK) to determine the effect of long term storage on exhaled breath VOCs of various origins. All three data sets contained a data matrix of library referenced breath components as variables and time points referenced sample names as observations. All variables were assigned to single block weight of 1(1/SQRT) and Pareto base scaling (Par). Pareto base scaling was selected due to its ability to magnify low to medium range signals in analysis without inflating the background noise, and hence stay closer to original data set than UV scaling. This scaling subtracts each variable from the mean of the data set and divides the difference by the square root of the standard deviation. Unsupervised principal components analysis (PCA) was then performed for all three data to determine the correlation between storage time and breath components.

The levels of cyclic siloxane compounds generated from breath samples reflects degradation of the active phases within the instrument and may complicate data modelling. For this study, the same GC column was used for the entire duration. The quality control dataset indicated that the retention time had shifted roughly 1 minute after 12 month and the levels of cyclic siloxanes seem to increase after longer storage periods, and this may due to column degradation as opposed to storage effects. In order to eliminate the effect of GC column degradation from data modelling, all cyclic siloxane compounds were removed from the data set. Compounds that were present in less than 30% of the breath samples were also excluded from data modelling.

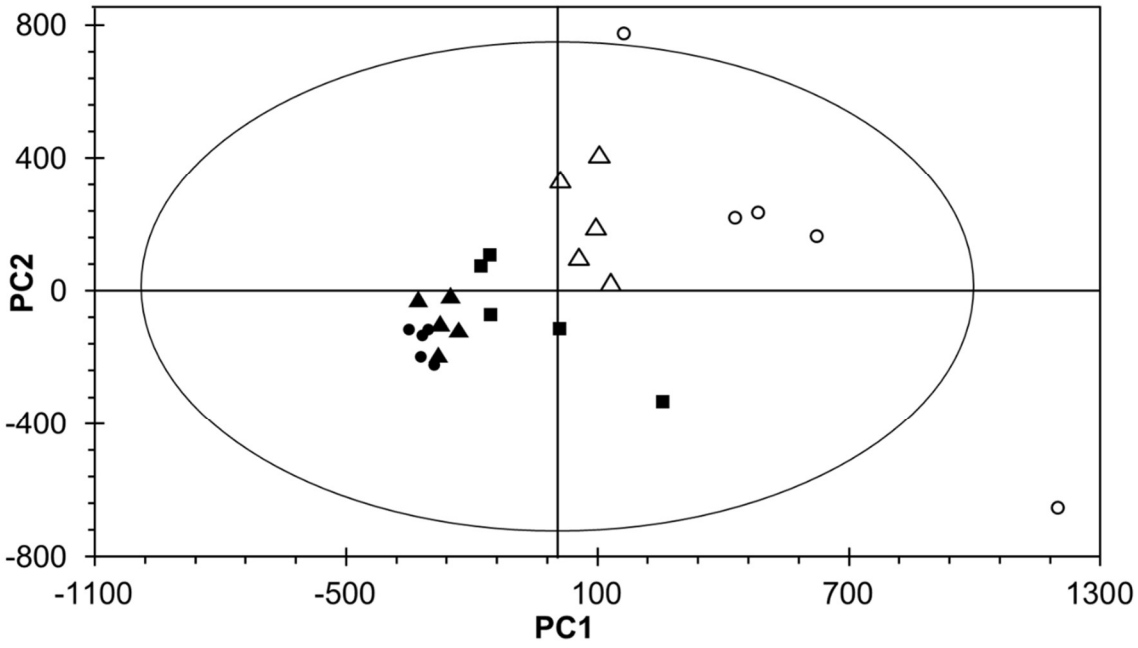


**Figure 3.4.2.1. (A) Unsupervised principal component analysis (PCA) of 592 storage sample components both endogenous and exogenous originals. The model used 25 observations, 5 observations from each time points respectively: 0 (black dots), 1.5 month (triangle), 6 month (square), 10.5 month (open triangle) and 12.5 month (open circle) are shown.**

Figure 3.4.2.1. shows the unsupervised principal component analysis (PCA) score plot of 592 variables with each time point represented by a distinctive symbol. The score plot contains two principal components with total variance explained by the model with the value of 55.0% for PC1 and 29.3% for PC2. This value is the fraction of sum of squares explained in that component. A distinct separation can be observed between breath samples stored for duration within 6 month and beyond the 6 month period. The data response from storage time points within 6 months (0, 1.5 and 6 month) seems to cluster and this clustering decreases significantly after 6 month, indicating increased variation within breath samples components as storage duration becomes longer. A trend in opposition to the decrease in variability observed with the internal standards. The score plot also has calculated sensitivity and specificity of 100% and 94% based on hierarchical clustering. The difference between endogenous and exogenous breath components can be observed from individual score plots in Figure 3.4.2.2. Although as shown in both score plots, a distinction can be made between breath components analysed before and after 6 month storage period, endogenous breath components show additional distinction within the 3 time points under the 6 month storage period. As observed from Figure 3.4.2.2.A, a distinction between endogenous breath

components stored for under and above 1.5 month can be seen, however this separation is a lot less apparent in the score plot generated from exogenous compounds in Figure 3.4.2.2.B. This seems to indicate endogenous compounds are stable for shorter amount of time than exogenous compounds when stored under - 80°C. Score plot generated from endogenous breath components had total variance value of 43.6% for PC1 and 25.8% for PC2 with a sensitivity of 91% and specificity of 100%. As for exogenous components score plot, total variance value was calculated to be 29.0% for PC1 and 13.4% for PC2, it also has sensitivity and specificity of 100% and 83%.

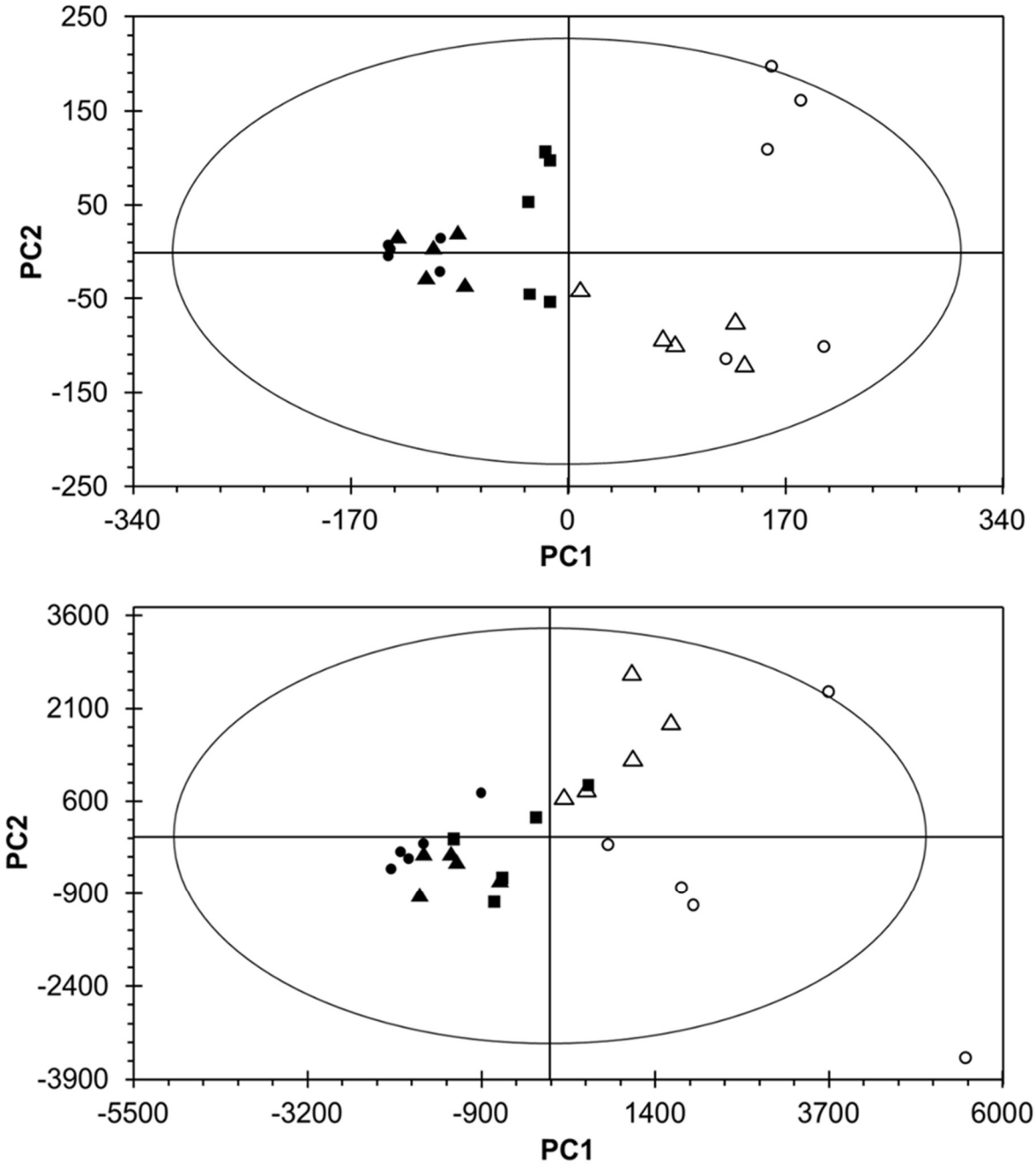


**Figure 3.4.2.2. (A) Unsupervised principal component analysis (PCA) generated from 151 endogenous breath components. (B) PCA of 423 exogenous components identified from storage samples. Both models used 25 observations, 5 observations from each time points respectively: 0 (black dots), 1.5 month (triangle), 6 month (square), 10.5 month (open triangle) and 12.5 month (open circle) are shown.**

The difference was further investigated using hierarchical cluster analysis (Figure 3.4.2.3.). The distance between two clusters is measured by difference in variance. Two sub-clusters can be observed in endogenous components dendrogram between response generated

from time point at 6 month and up to 1.5 month, these sub-clusters cannot be observed from exogenous dataset.

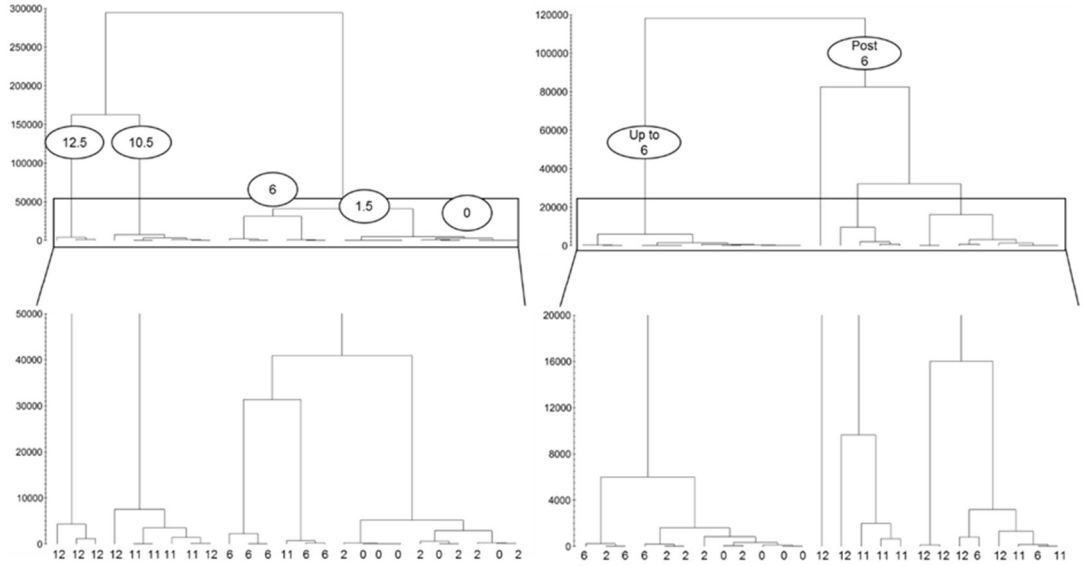


**Figure 3.4.2.3. Dendrogram of endogenous (left) and exogenous (right) breath components dataset generated from hierarchical cluster PCA analysis. This illustrates the difference in clustering between endogenous and exogenous components, although both datasets show distinct separation at 6 month, a sub-clustering of 6 month of endogenous compounds can be observed which is not present in exogenous dataset.**

The trend in the data was explored further with partial least squared regression (PLS) to determine the relationship between peak intensities (predicted variable X) and storage duration (response variable Y) in the endogenous data. The model generated three principal components with a goodness of fit value of 90.8% and a predictive value of 91.2% for principal component 1 (PC1) indicating a strong relationship between X and Y variables. The relationship between X and Y variables can be visualised by plotting PC1 scores against storage duration. PC1 contain 57% of total variance. (See Figure 3.4.2.3. and Equation 3.4.2.)

What is apparent is that endogenous VOC appear stable at 1.5 and by 6 month instability can be detected with variability increasing non-linearly at 10.5 month and 12.5 month. Comparison between the endogenous and exogenous model indicates endogenous breath compounds are more prone to variability during storage. The stability of endogenous breath compounds are explored further in the next section.

$$y = 0.8821x^2 + 12.564x - 130.41$$ **Equation 3.4.2.**

### 3.4.3. Stability of breath components under long term storage

A paired two-tail t-test was carried out on all endogenous compounds peak intensities to assess the difference between each time point against the reference time point (0 month). Systematic variation calculated from internal standards was deducted from the calculation. The *p*-values calculated from differences in peak intensities between the reference time point (0 month) and 1.5 month indicated significant difference for 9 compounds at P = 0.05 (Figure 3.4.3.1.). In terms of the whole endogenous breath components, this suggests that 94% of compounds were stable at this point in time. At 6 month time point, *p*-values from 44 compounds were observed to be significantly different. Out of the 44 compounds, 24 were observed to have shown an increase in intensity at the 6 month time point and the remaining 20 compounds showed a decrease. The stability of whole breath sample had reduced to 73% from 94%. At 10.5 month time point, another 53 compounds were observed to show significant difference compared to their peak intensities at 0 month. The stability had reduced to 41%; the majority of breath components were now unstable. At 12.5 month, another 10 compounds has shown significant variation in their p-value, the reduction in number of compounds that have shown significant variation from 95 to 78 indicates the breath matrix may be stabilising.

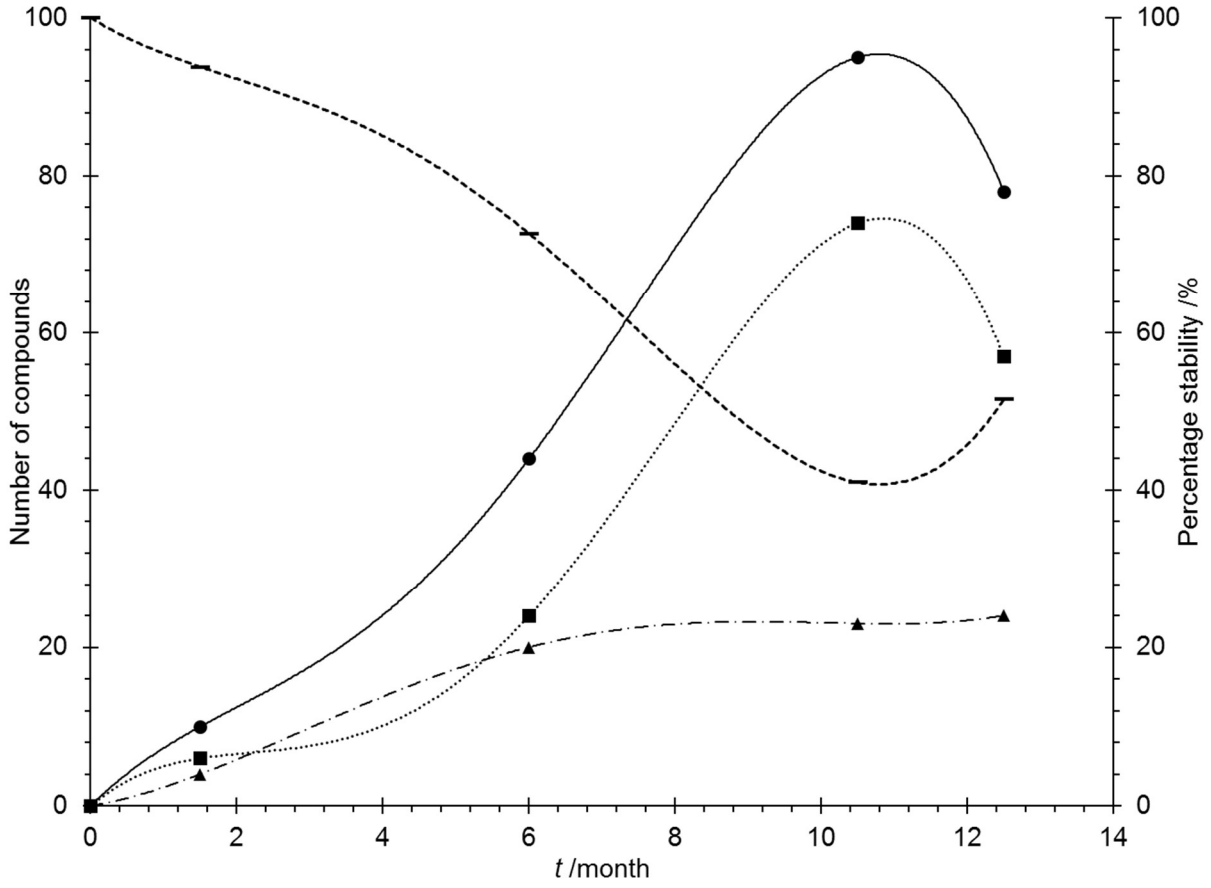


**Figure 3.4.3.1. The number of compounds changing significantly (black dot with solid line) at various time points over the 12.5 month storage period.**

**The other two lines illustrates number of compounds shown significant increase (square symbol with dotted line) and number of compounds shown significant reduction (triangle symbol with dashed line) at various time points.**

**Paired two-tailed t-test was used to determine the significance level for each endogenous breath components at P = 0.05. Additionally, the percentage of compounds stabile at each time points is shown (dash symbol with dotted line).**

PCA and PLS analysis enabled determination of a first order quadratic relationship between changes in breath components and storage time. First order curve line relationship reveal the underlying multi step reactions occurring in breath components during storage. Evaluation of the percentage change in the endogenous breath components revealed how breath components behaved differently compared to each other.

Within a total of 159 endogenous compounds 60% of the compounds were observed to increase in response, and 19% showed a reduced response over 12.5 months storage.

9% of the compounds observed to increase in response were new, appearing after 6 month. The majority, 86%, of compounds exhibiting reduced responses were completely missing after 6 month of storage. This indicates possible decomposition, loss or formation from decomposition occurring during storage.

7% of compounds observed were within the systematic variation of the systems and therefore were identified to be stable throughout storage.

4% of compounds shown increased response after 6 month then reduced response at 12 month time point, and adversely 9% of compounds shown reduced response after 6 month but response increased at 12 month.

These observations suggest complex and as yet undescribed reactions and interactions that combined to form the time dependent relationship shown in Figure 3.4.3.2., thus suggests that there are markers of sample aging within the breath matrix.

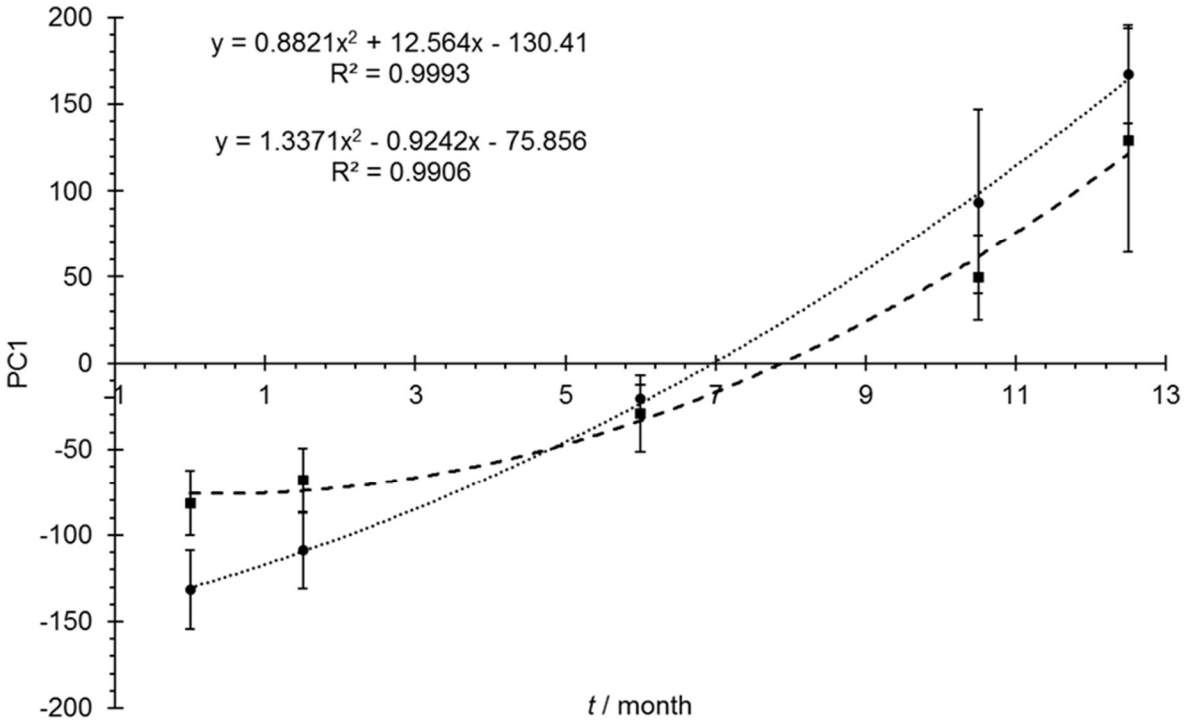


**Figure 3.4.3.2. Scatter plots of the mean of PC1 scores generated from partial least squares regression (PLS) of endogenous breath components (black dot with dotted line) and exogenous breath components (square with dashed line) against storage duration.**

### 3.4.4. Identification of storage variable breath components

The most sensitive endogenous compounds to long term storage are compounds which show either complete loss, or are created during storage. There were 33 compounds in this category.

Out of 25 compounds which show complete loss after 6 month, the majority 56% had mass spectra indicative of hydrocarbon, ester, aldehyde, alcohol or fatty acid functional groups. 36% exhibited m/z 57, 43, 71, 85, 41 fragmentation pattern evident of possible hydrocarbons, ester or aldehyde compounds. It is also possible they can be methylated alcohol or fatty acid compounds. 20% exhibited m/z 69, 67, 81, 83 fragmentation pattern representing possible fatty acids or alcohol compounds. 1 compound belonging to newly formed compounds after 6 month was also elucidated to be a hydrocarbon. 3 out of 33 compounds in both groups observed m/z 69, 111, 110 fragmentation pattern belong to cycloalkane group. Another 2 compounds from both groups contains m/z 91, 92 fragmentation pattern were likely to be Benzeneamide or benzenealdehyde derivatives.

These compounds were present at low peak intensities, for example the mean intensity of the 33 compounds that showed complete loss after 6 month storage was 8734 counts. This may be estimated to be equivalent to an on-column mass of 55 pg of internal standard. With a breath sample volume collected at 2.5 L, the mean exhaled concentration may be estimated to be no lower than 22 ng $m^{-3}$. Such low concentrations might be a potential factor contributing to their increased sensitivity to long term storage.

12 VOCs with the highest correlation to storage duration were matched against the NIST MS library within the Varian MS software, see Table 3.4.4.1. and Figure 3.4.4. The assignments were based on forward and reverse matching of de-convoluted mass spectrum of target compounds to library reference spectrums, a satisfactory match score above 650 must be achieved for identification. If multiple matches with similar forward and reverse scores were found, a comparison of observed retention index value (IU) and reference retention index value from NIST library were performed to achieve the most satisfactory match possible. In cases when no satisfactory matches can be obtained or reference retention index comparison is not successful, an estimation of the functional group of the compounds based on their fragmentation pattern was given. Due to the low intensity of the compounds' peaks, forward and reverse matching of specific compounds could not always be achieved, and so an estimate of compounds identity was generated through a list of library matches with the same fragmentation pattern and within ± 50 IU of retention index value. Some of the low intensity compounds are close to limit of detection, it is possible the compounds haven't

decomposed completely as the peak intensity suggested, but there abundance was below the capability of the instrument.

The functional groups of the compounds that show increased or reduced response are more varied and complex to elucidate, from observing their fragmentation pattern, 18% are possible hydrocarbon, ester or aldehyde functional group compounds, 7% are fatty acids and alcohol functional groups compounds. The discriminators generated from multivariate analysis that exhibited most correlation to long term storage are all from within this category. The 8 VOC variable that generated the highest correlation score of PCA S-plot were elucidated and listed in Table 3.4.4.2.

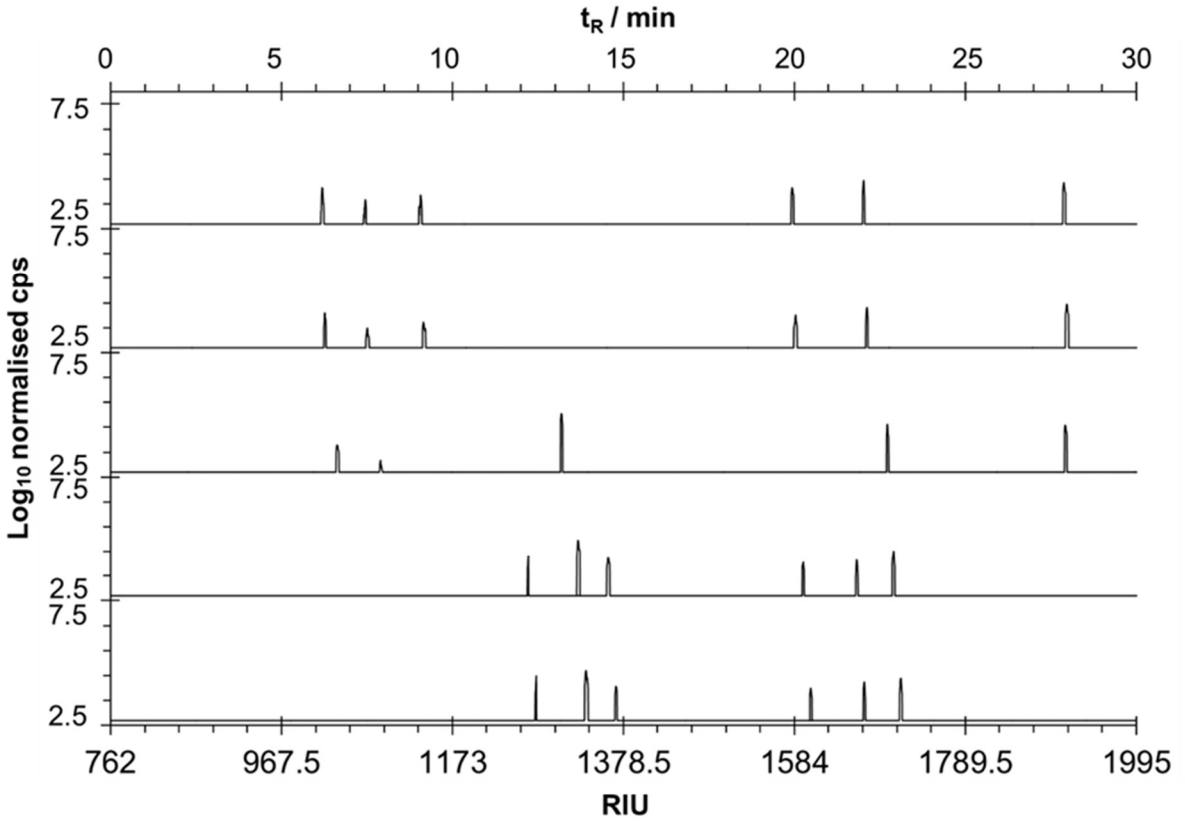


**Figure 3.4.4. Extracted ion chromatograms of 12 endogenous compounds that shown the most significant changes normalised using log10 transformation. Lower x-axis shows retention index units (RIU) and upper x-axis shows their equivalent retention time (tR).**

**Table 3.4.4.1. List of 12 endogenous breath components that were shown most significant difference comparing to starting time point.**

| | Median /month | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Name | 0 | 1.5 | 6 | 10.5 | 12.5 | Libray entry | MS F/R | RI O/E | CAS No. |
| BRI-1525-69-83-41-55-97 | 13892 | 16508 | 14215 | 0 | 0 | Unsaturated fatty acid | | | |
| BRI-1471-71-83-84-70-45 | 7074 | 10024 | 0 | 0 | 0 | Unknown alkane | | | |
| BRI-1124-57-41-70-56-43 | 25599 | 37400 | 51619 | 0 | 0 | 1-Octanol, 3,7-dimethyl- | 804/642 | 1124/1130 | 106-21-8 |
| BRI-1154-85-41-57-43-71 | 2511 | 4173 | 0 | 0 | 0 | 4-Methylpentyl pentanoate | | 1154/1218 | 35852-47-2 |
| BRI-1387-41-56-70-55-68 | 5919 | 3405 | 0 | 0 | 0 | 1-Nonanethiol | | 1387/1219 | 1455-21-6 |
| BRI-1077-57-41-56-43-59 | 4689 | 11354 | 0 | 0 | 0 | Decane, 3-methyl- | | 1077/1051 | 13151-34-3 |
| BRI-913-151-161-133-152-153 | 0 | 0 | 7186 | 10049 | 5314 | | | | |
| BRI-1504-91-92-0-0-0 | 0 | 0 | 0 | 31877 | 17374 | Benzeneamide or benzenealdehyde derivative | | | |
| BRI-1728-57-43-71-41-85 | 0 | 0 | 3171 | 3559 | 5986 | 1-Decanol, 2-hexyl | 566/823 | 1728/1790 | 2425-77-6 |
| BRI-1401-69-111-110-0-0 | 0 | 0 | 0 | 4477 | 8780 | Methylated cycloalkane or alcohol | | | |
| BRI-1007-131-133-0-0-0 | 0 | 0 | 0 | 2256 | 2048 | | | | |
| BRI-954-120-45-64-0-0 | 0 | 0 | 548 | 856 | 1328 | Benzoic acid or benzamide derivative | | | |

**Table 3.4.4.2. List of 8 endogenous breath components that were the highest discriminators in MVA.**

| | Median /month | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Name | 0 | 1.5 | 6 | 10.5 | 12.5 | Libray entry | MS F/R | RI O/E | CAS No. |
| BRI-783-59-60-43-42-44 | $1.5x10^7$ | $1.6×10^7$ | $2.4×10^7$ | $3.6×10^7$ | $9.2×10^7$ | Acetone | | 783/455 | 67-64-1 |
| BRI-789-81-95-94-82-93 | 7975082 | 8522656 | $2.7×10^7$ | $3.4×10^7$ | $5×10^7$ | 3-Methyl-1-penten-4-yn-3-ol | 756/672 | 789/697 | 3230-69-1 |
| BRI-1066-91-108-89-92-90 | $3.6×10^7$ | $4.3×10^7$ | $5.1×10^7$ | $9.4×10^7$ | $1×10^8$ | Unknown fatty acid | | | |
| BRI-1332-91-92-90-0-0 | 14750 | 21325 | 34215 | 49438 | 60238 | Benzeneamide or benzenealdehyde derivative | | | |
| BRI-835-88-73-89-47-90 | 255502 | 296132 | 455604 | 746667 | 802253 | Allyl methyl sulfide | 723/723 | 835/660 | 10152-76-8 |
| BRI-825-61-90-45-41-49 | 50009 | 50789 | 113125 | 180638 | 172868 | Propane, 1-(methylthio)- | 887/863 | 825/670 | 3877-15-4 |
| BRI-820-88-73-89-61-91 | 530068 | 626937 | 961182 | 1622886 | 1663602 | Allyl methyl sulfide | 699/677 | 820/660 | 10152-76-8 |

## 3.5. Summary

A 12.5 month storage study assessed the stability of exhaled breath compounds at - 80°C. Previous studies have focused on the stability of a mixture of synthetic breath compounds or a set of target compounds in human breath. The reported results have varied depending on the compounds and/or adsorbents studied. Chlorinated compounds on a single adsorbent bed have been reported to be stable for 2 years while 2 to 4 weeks stability has been reported for common breath compounds on multi-bed adsorbent tubes (70) (71) (72) (74).

This study is the first that has explored and sought to characterise the changes in the whole breath sample (592 compounds) when stored for 1 year on dual-bed adsorbent tubes. The initial hypothesis was that no change in the VOC profile occurs at - 80°C.

The hypothesis held for samples stored for 1.5 month. Results from paired two-tailed t-tests indicates that 94% of endogenous compounds were stable at the 1.5 month time point and this reduced to 73% compounds stable at 6 month. Longer than this and the percentage of stable compounds drops to 41% at 10.5 month. Although at 12.5 month time point, the stability appears to increase back to 52%.

It seems reasonable to suggest a storage period of 1.5 month for breath VOC compounds as indicated by multivariate analysis and t-test. Even at 1.5 month interval, 6% of endogenous compounds have shown significant difference compared to starting time point. This is likely to be due to complex nature of breath metabolome where stability of compounds vary significantly due to its chemical properties. Therefore the storage method of choice for breath VOC study is highly dependent on the target compounds species. Determination of type of compounds that are more prone to deterioration during storage can be difficult due to breath matrix's complexity.

However some observable patterns have emerged; over half 56% of endogenous compounds that shown complete loss after 6 month have the m/z 57, 43, 71, 85, 41 or m/z 69, 67, 81, 83 fragmentation pattern and it is likely compounds with these fragmentation pattern belong to hydrocarbon, ester, aldehyde, alcohol or fatty acid functional groups (Table 3.4.4.1.). If these functional groups are sensitive to storage conditions, and with many breath VOC biomarkers discovered over the years related to various diseases belonging to these functional groups, a more vigorous storage and transportation methodology needs to be adopted to avoid de-stabilising these sensitive compounds in large scale biomarker elucidation studies. It also need to be made clear that although the storage sensitive compounds may belong to hydrocarbon functional group, it is only a small portion of the overall hydrocarbons that exist in breath; majority hydrocarbon groups are observed to be

stable in breath. With many storage sensitive compounds having low peak intensities, it is possible that sensitivity comes from both chemical properties and concentration.

One of the causes of instability of VOCs during storage may be attributed to the dual-bed adsorbent tubes (Tenax® TA/ Carbograph 1 TD) used. Migration of VOCs trapped between the two materials would result in irreversible binding of some species indicating a reduced stability of the breath sample. Although low temperature would reduce this effect, it is seems that migration of VOCs between the adsorbents still occurs, even at - 80°C. The rate of migration is slower than previously reported at higher temperature taking months to become apparent.

It is also possible that high water levels inside the adsorbent beds had significant effect on the stability of breath components, with increases in carbonyl compounds such as acetone after 6 month that may be created through process of hydrolysis. Also the increase in cyclosiloxanes and higher abundance compounds (e.g. acetone) may be obscuring the statistical detection of other much smaller signal responses. This highlights the utility of studying the stability of the whole breath VOC profile as the changes in breath compounds may impact each other.

An important element to this study was the successfully implementation of gas phase internal standards to characterise systemic variation (24.4%) across 12.5 month year and importantly the variation within breath sample replicates (14.9%) taken from the same individual over 3 hours sampling period. The variability of replicate breath VOC profiles is likely to be higher than 14.9% due to changes in a participant's physiology such as changes in pulse rate, water intake and restroom breaks. Psychological stress has also been reported as a factor for specific chemicals in breath (75). This variability is expected to be significantly different from compound to compound and this would have contribute to the hugely varying stability of breath components. Therefore it is possible that a portion of the breath compounds that have shown significantly difference at later time points to be due to variability of the participant rather than storage.

Limitations in this study was that quantification of breath VOCs was not feasible. Although a rough estimation based on the Toluene-$d_8$ internal standard concentration, its true quantity can only be realised with calibration. For example, the concentration of 3, 7-dimethyl 1-octanol (Table 3.4.4.1.) is estimated to be 64.4 ng $m^{-3}$ at starting time point.

This study only investigated the stability of breath samples at - 80°C. The result may not be applied to other storage temperatures. Further study should be focused on long term stability of breath samples at multiple temperatures such as freezer temperature - 20°C, refrigerator

temperature 4°C and room temperature 22°C. This is particularly important for transportation of breath samples between labs, since often refrigerator temperature 4°C is used.

This study collected large amount of replicate breath samples within 3 hr from the same participant. This was to avoid introducing variations from multiple participants. However a set of breath samples collected from a different participant may show deviation to the stability model calculated. A follow-up study on multiple participants would be advised to screen for more storage sensitive compounds and reduce the chance of false discoveries.

Lastly, another limitation is amount of time points chosen for this study; 5 time points were used to ensure sufficient replicates samples for each time point. On the other hand, renewed experiments with more time points would establish accuracy of the current prediction model.

This study indicates a maximum storage period for breath samples at - 80°C should be no longer than 6 month for specific targeted studies, and care will be needed to demonstrate the absence of storage artefacts. The maximum “safe storage time” at - 80°C storage for non-targeted studies is indicated to be 1.5 month. This study also provides method validation for large scale biomarker elucidation studies that require extended storage and transportation up to this time. It also supports with the previous breath VOCs stable period of 2 to 4 weeks for multi-adsorbents tube stored samples.

The complex changes of in the recovered breath VOC profile during storage has revealed the presence of complicated chemical and physical reactions and interactions that cause a loss of sample stability between various chemical species. This further highlights that the sensitivities of target compounds in biomarker studies to other contributing factors other than the diseases also needs to be considered and tested.

# CHAPTER 4. DEVELOPMENT OF PORTABLE ADAPTIVE BREATH SAMPLER FOR REMOTE EXHALED BREATH VOCS COLLECTION

## 4.1. Introduction

The concept of a portable adaptive breath sampler originated from the adaptive breath sampler created in 2006 (41). The original adaptive breath sampler was developed and employed in breath biomarker elucidation studies. These led to the creation of a breath sampling and analysis work-flow that fully utilised the sampler's specifications. A breath sampling control unit enables selected alveolar portions of an exhaled breath profile to be passed through an adsorbent tube while a human participant breathe normally. The adaptive breath sampler was designed for studies with participants with an impaired respiratory function such as people with COPD. The sampler was configured to adapt to variability in participants' breathing during breath collection. The sampler was controlled from a virtual instrument (Labview) that enabled different parts of a breath profile to be selected and sample volumes to be adjusted at will. The use of continuous measurement of the pressure in the upper airways enabled the sampling controls to adapt to different breathing patterns.

The portable adaptive breath sampler developed in this study incorporated the specifications of the original breath sampler, and enhanced it by adding elements of portability and improved background contamination control.

The primary enhancement made in the portable breath sampling was the in-field sampling capability. A significant limitation of the original design was the requirement for a purified air supply. This meant that it had a fixed location which limited participant recruitment. The introduction of this new specification enabled breath sampling to be carried out in the field with no restriction on location. It was designed to be setup quickly in clinical settings, sports venues and even in domestic and community care settings. This feature combined with the easy to use and non-invasive original sampling unit maintained the capability of working with participants with an impaired lung function minimise participant stress level and discomfort.

The original adaptive breath sampler used inert material and air purification filters to minimise the levels of exogenous VOCs level present in breath samples. This restricted the location of the sampler to locations where a medical air supply was available. Further it was not possible to control the purification process that was run with commercial filters. A

custom-built portable purified air supply was developed for the adaptive breath sampler. The air supply had to meet the following criteria:

- efficient drying of the air supply
- purification
- removal of dust and particulates
- based on re-usable materials such as molecular sieve and activated carbon

This enabled better user control of the nature of the samples collected and created standardised background with a low exogenous VOCs level regardless of where a breath sample was taken.

## 4.2. Portable breath sampler design

### 4.2.1. Health and safety

All desiccants and purification media were obtained from by Acros Organics or Fisher Scientific, and were assessed for possible hazards with information taken from Fisher Scientific Material Safety Data Sheets, see Table 4.2.1. All the materials were classified with a health rating of at least 1; direct contact with humans poses a health risk. In particular Ascarite and calcium oxide cause burns and pose a higher health risk (NFPA rating 3), and therefore were excluded. Importantly, desiccants generate dust which causes irritation to eye or skin and upon inhalation result in irritation to the respiratory tract. Chronic inhalation will lead to lung damage. An additional dust filter was specified to eliminate this possibility and prevent damage to a participant's respiratory system.

**Table 4.2.1. Health and safety information on desiccants.**

| Name | Component | NFPA rating | | | Toxicity | Target organ | Stability | MSDS revision date |
|---|---|---|---|---|---|---|---|---|
| | | F | H | I | | | | |
| Molecular sieve | Zeolites (100%) | 0 | 1 | 0 | Contains trace amount of quartz which might lead to fibrotic lung disease, silicosis or cancer.<br>Dust may cause eye and skin irritation.<br>Dust inhalation may cause respiratory tract irritation.<br>Ingestion may cause digestive tract irritation | E, GIT, S Res | Hy | 20/07/09 |
| Silica gel | Silica, amorphours, precipitated and gel (100%) | 0 | 1 | 0 | Limited evidence of a carcinogenic effect based on animal data.<br>Dust may cause eye or skin irritation<br>Dust is irritating to respiratory tract<br>Ingestion may cause gastrointestinal irritation with nausea, vomiting and diarrhoea. | E, GIT, S Res | Hy | 08/01/10 |
| Activated carbon | Carbon (100%) | 1 | 1 | 0 | Dust may cause eye and skin irritation.<br>Dust inhalation may cause lung damage, chronic inhalation may lead to decreased pulmonary function. | Res | S | 09/05/11 |
| Activated alumina | Aluminum oxide (100%) | 0 | 1 | 0 | Dust may cause mechanical eye and skin irritation.<br>May cause repiratory tract irritation and lung damage.<br>Chronic inhalation of fine dusts may cause lung damage<br>Ingestion of large amounts may cause gastrointestinal irritation. | Res | S | 20/07/09 |
| Drierite | Calcium sulfate (95%) | 1 | 1 | 1 | May cause eye and skin irritation., may produce an allergic reaction.<br>Ingestion may cause gastrointestinal irritation.<br>Inhalation may cause irritation and allergic respiratory reaction. | Res, S | Hy | 12/10/09 |
| Ascarite | Sodium hydroxide (90-95%) | 0 | 3 | 1 | Causes burns if in contact with eyes or skins.<br>Cause burns to respiratory tract if inhaled.<br>Cause burns if ingested. | E, GIT, S, Res, | Hy | 06/05/10 |
| Silica amorphous (5-10%) | | | | | Cause burns if inhaled.<br>Cause burns if ingested. | E, GIT, R | | |
| Calcium oxide | | 1 | 3 | 2W | Causes burns if in contact with eyes or skins, and might be harmful if absorbed.<br>Cause burns to respiratory tract if inhaled.<br>Cause burns if ingested.<br>Prolonged skin contact may cause dermatitis; chronic inhalation may cause nasal septum ulceration and perforation. | E, GIT, S, Res, | !-1. | 13/02/08 |
| Bentonite (Montmorillonite Clay) | Aluminium phyllosilicates | 0 | 2 | 0 | Causes eye, skin, and respiratory tract irritation.<br>Animal studies indicate that this may cause cancer<br>The toxicological properties of this material have not been fully investigated. | Res, E, Sk | Hy | 13/06/08 |

Key: E: Eyes; F: flammability; GIT: Gastrointestinal tract; H: Health; Hy: Hydroscopic; I: Instability; Res: Respiratory system; S: stable; Sk: skin

!-1: Air sensitive, reacts violently with water. Moisture sensitive

## 4.2.2. Desiccants and adsorbents for gas purification

A range of desiccant and adsorbent material and their adsorption capacity are discussed in this section.

Silica gel ($SiO_2.nH_2O$) is amorphous silica produced from the reaction of sodium silicate and sulfuric acid. The sodium sulphate is removed by dissolution and the resultant mixture is then coagulated to hydrogel to produce the commercial silica gel. Silica gel's primary use is in drying gas, desulfurization and gas purification.

Molecular sieve belongs to the zeolite class of minerals (hydrated alkali metal or alkaline earth aluminosilicates). The structural units have cavities at their centre creating a porous structure of molecular dimensions and thus causing the sieving action. Some molecular sieves have pores of uniform dimensions and compounds smaller than the pores diffuse into the structure and are retained. The fundamental building block is made of oxygen anions surrounding a smaller silicon or aluminium cation. The zeolite molecular structure can be A, X or Y form. Zeolite A pores are restricted by 8 membered oxygen rings which forms a cubic structure. The sodium ions or other cations make up the positive charge deficit in the alumina terahedra. Different types of cations form different diameter pores: $K^+$ cations form 3A, $Na^+$ cations form 4A, $Ca^{2+}$ cations form 5A. Zeolite X or Y has pores which are restricted by 12 member oxygen rings which form a tetrahedron structure (76).

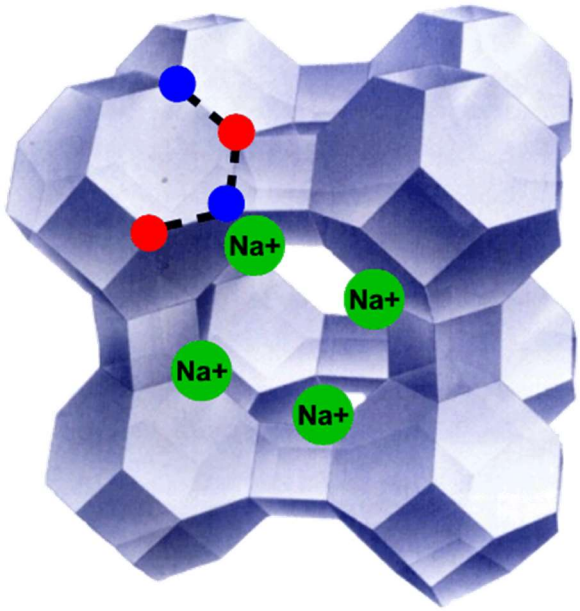


**Figure 4.2.2. Zeolite structure of molecular sieve 4A (76)**

Molecular sieves are generally available as cylindrical pellets, beads or powder. Pellets and beads are commonly used for gas dehydration and purification. The bead is characterised by

a screen cut through which all the beads pass and a screen cut that retains all the beads, in that order, with two sizes separated by an “x”. The two most commonly used screen cuts are 4×8 or 8×12. All molecular sieves are excellent desiccants for dehydrating gas.

Calcium sulfate ($CaSO_4$), better known commercially as Drierite, Calcium sulfate is created by the controlled dehydration of gypsum, acting as a general-purpose desiccant geared mainly toward laboratory use. It is chemically stable, non-disintegrating, nontoxic, non-corrosive, and does not release its adsorbed water when exposed to higher ambient temperatures. The low cost of calcium sulfate must be weighed against its equally low adsorptive capacity: it adsorbs only up to 10% of its weight in water vapor (Figure 4.2.2.). Calcium sulfate also has regeneration characteristics that tend to limit its useful life.

Activated alumina is manufactured from aluminium hydroxide by dehydroxylating it in a way that produces a highly porous material. The compound is used as a desiccant and as a filter of fluoride, arsenic and selenium in drinking water. It is made of aluminium oxide (alumina; $Al_2O_3$). It has a very high surface-area-to-weight ratio.

Silica gel and molecular sieves have the lowest health risks compared to other desiccants. The advantage of molecular sieve compared to silica gel is its better capacity with low relative humidity and elevated temperature gases. They provide extremely low dew points.

## 4.2.3. Gas dehydration design

### 4.2.3.1. Overview of adsorption dynamics

When a gas mixture containing water vapour pass through a bed of fresh adsorbent, the water is adsorbed immediately at the to the bed, dehydrated gas passes through rest of the bed with only a small amount of additional drying taking place. When the adsorbent at the inlet becomes equilibrated with the water in the challenge gas the zone of water adsorption progress through the rest of the adsorbent bed. When this adsorptive wave reaches the outlet, the water content of exhaust gas will increase signifying the breakthrough point.

During the adsorption period, the adsorbent can be characterised into three zones. The equilibration zone, the adsorbent bed is equilibrium with wet air supply. The middle zone is called mass transfer zone (MTZ) where water is being rapidly absorbed from the gas supply. The last zone is the unused adsorbent known as the active zone.

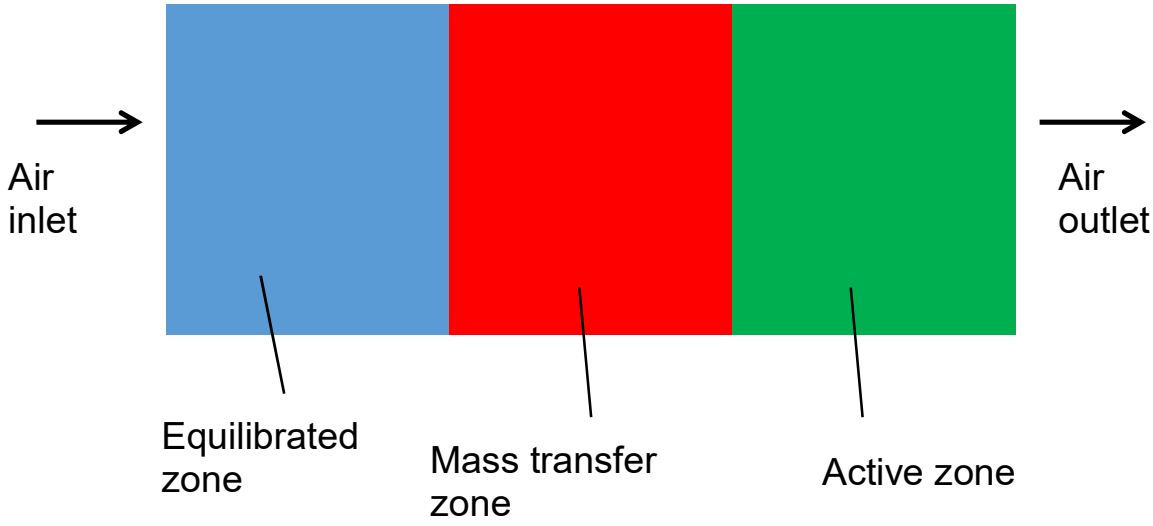


**Figure 4.2.3.1. Principle of desiccant water adsorption**

In order for desiccant not to reach the breakthrough point while sampling, it is important to estimate the mass of desiccant required, bed diameter and bed length to build a cylindrical desiccant bed.

## 4.2.3.2. Gas dehydration desiccant selection

### 4.2.3.2.1. Dew point comparison

Dew point is the temperature which a gas must be cooled at a constant pressure in order for water vapour to condense. Although relative humidity also measures moisture in air, it is dependent on temperature, so “dew point” is a more absolute indicator of moisture in air.

Different desiccants result in different dew points after dehydration. The lower the dew point the desiccant achieve, the drier the air it filtered will be. The chart below shows the dew points the desiccants can achieve.

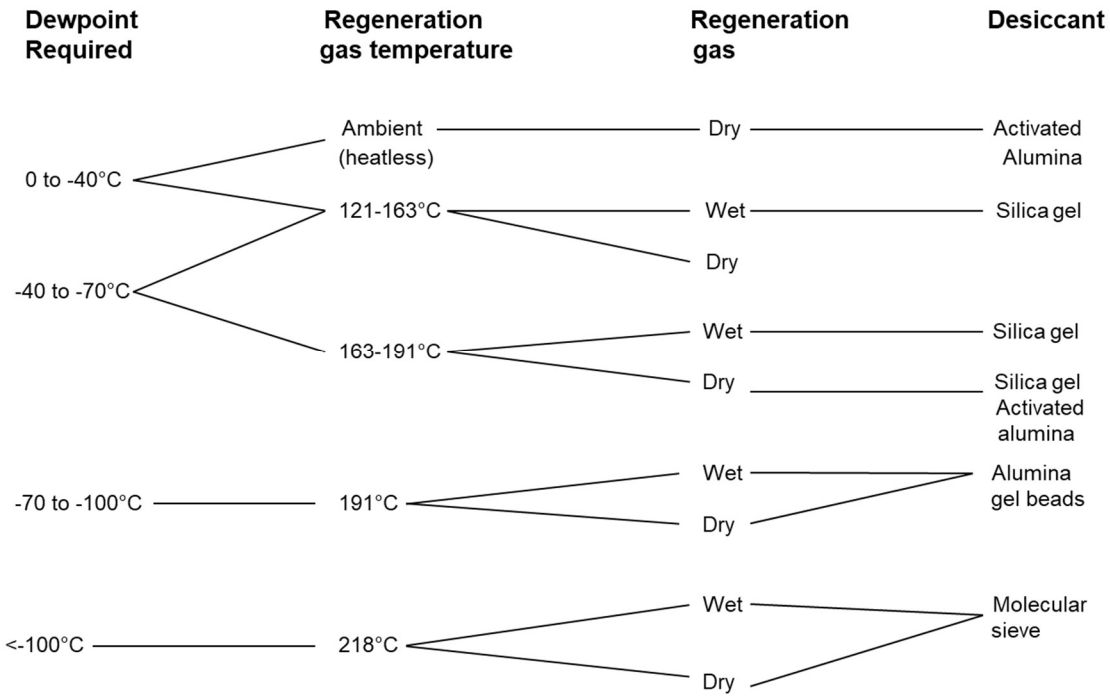


**Figure 4.2.3.2.1. Desiccant dehydration selection chart**

Molecular sieve generates the lowest dew point (< -73°C) and so it was considered the best candidate for gas dehydration from dew point comparison. However water capacity of desiccants also needs to be compared. Note as well that although molecular sieve can achieve very low dew points, it requires higher regeneration temperatures: 218°C.

### 4.2.3.2.2. Water capacity comparison

Water capacity is the ratio of mass of water retained to mass of desiccant. The more water the desiccant can hold the more efficient it is. The aim of this comparison is to determine the desiccant with the highest water capacity for the breath sampler. However the water capacity varies significantly dependent on temperature and humidity, so it is essential the desiccant has stable water capacity across a range of temperature and relative humidity. Indoor temperature is usually kept around 20°C to 25°C, and 30 to 50% relative humidity. But in some locations, this could vary even more, so the desiccant needs to be stable from 10°C to 35°C, and 20% to 60% relative humidity.

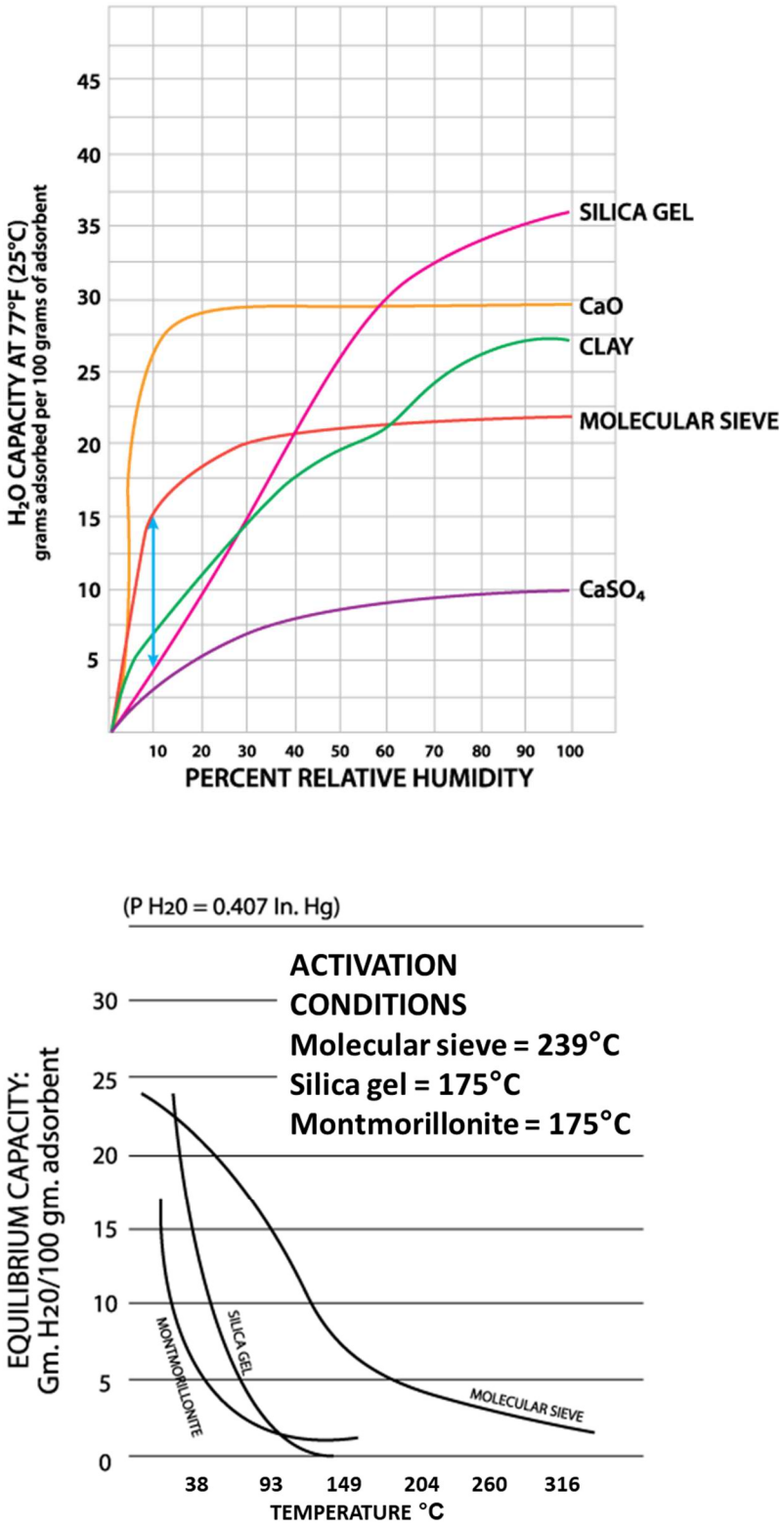


**Figure 4.2.3.2.2. (top) Desiccant water capacity versus relative humidity based on air at atmospheric pressure and 25°C. (bottom) Desiccant water capacity versus temperature** (77)**.**

Figure 4.2.3.2.2 shows silica gel has a higher water capacity than molecular sieve above 40% relative humidity, however at lower humidity, its water capacity is much lower. Although calcium oxide also known as quick lime has a high and stable water capacity at around 30%, it causes burns with any form of contact, so it is not a suitable candidate. Molecular sieve has a very stable water capacity at just above 20% from 0 to 100% relative humidity, so it is the best candidate regarding relative humidity.

By observing Figure 4.2.3.2.2., silica has highest water capacity at lower temperature around 15°C, but its water capacity seems to reduce significantly as temperature increases above 20°C. Molecular sieve has a very stable water capacity at above 20% up to 50°C, so it is the best candidate regarding temperature.

From the comparisons done above, it seems molecular sieve has the most stable water capacity at above 20% from 10°C to 35°C, and 20% to 60% relative humidity (atmospheric pressure).

#### 4.2.3.2.3. Molecular sieve comparison

There are several different types of molecular sieves dependent on their zeolite structures and they are all capable of drying gas, however each has specific properties based on their pore size which gives them specific applications. Molecular sieve with a specific pore size will only absorb compounds that are smaller than the pore. For the breath sampler, it is beneficial to use a molecular sieve which also absorb other impurities in air and $CO_2$.

Molecular sieve beads are characterized by a screen cut, which identifies the Tyler screen size through which all of the beads pass and the size that retains all of the beads. The two common screen cuts are 4×8 or 8×12. Mesh size of molecular sieve indicates the size of sieve beads and has significant impact on the performance of filter. By decreasing the size of sieve, the path length contaminants compounds have to travel is reduced, and the adsorption rate therefore increases, and hence the molecular sieve will be more efficient. 4×8 mesh size indicates the particle will fall through an opening of 4.75 mm and remain on a screen opening of 2.36 mm. 8×12 mesh size indicates the particle will fall through an opening of 2.36 mm and remain on a screen opening of 1.4 mm.

Table 4.2.3.2.3 is a comparison of molecular sieves, and molecular sieve 13X seems to be the most suitable candidate, it absorbs a large range of small hydrocarbon compounds (e.g. di-n-propyl-amine), as well as its main use for the adsorption of $H_2O$, $H_2S$ and $CO_2$. Apart from the wide range of molecules 13X absorbs, it also has the highest water capacity of 28.5%.

**Table 4.2.3.2.3. Common types of commercial molecular sieves**

| Basic type | ⦰ /nm | $\rho$ /g.cm$^{-3}$ | Water capacity (% m/m) | Molecules absorbed | Molecules excluded | Typical applicatiions |
|---|---|---|---|---|---|---|
| 3A | 0.3 | 47 | 20 | $H_2O$, $NH_3$ $H_2S$, $CO_2$, $SO_2$, $C_2H_4$, $C_2H_6$, | Ethane and larger | Dehydration of unsaturated hydrocarbons |
| 4A | 0.4 | 45 | 22 | $C_3H_6$ | Propane and larger Iso compounds, 4 carbon rings and | Static desiccant in refrigeration systems, etc. Drying saturated hydrocarbons |
| 5A | 0.5 | 43 | 21.5 | n-$C_4H_9OH$ Di-n-propyl- | larger | Separates n-paraffins from branched and cyclic hydrocarbons |
| 13X | 1.0 | 38 | 28.5 | amine | $(C_4F_9)_3N$ and larger | Coadsorption of $H_2O$, $H_2S$ and $CO_2$ |

## 4.2.3.3. System design

### 4.2.3.3.1. Bed diameter

Determining bed diameter is important for desiccant bed, since too large a diameter will require a high regeneration gas rate to prevent channelling. Too small a diameter will cause too high pressure drop and damage the sieve. The design pressure drop through the bed should be about 34 kPa . A design pressure drop higher than 55 kPa is not recommended as desiccant is fragile and can be crushed by total bed weight and pressure drop force.

First calculate $V_{max}$ which the maximum superficial velocity is based on pressure drop ($\Delta P$, Pa), length of the packed bed stands ($L$, m), constant 1 ($B$), constant 2 ($C$), viscosity ($\mu$), density ($\rho$, kg.m$^{-3}$):

$$V_{max} = \{(\Delta P/L)_{max}/C\} + [(B/C)(\mu/\rho)]$$ **Equation 4.2.3.3.1.1.**

$(\Delta P/L)_{max}$= maximum allowable is 7.46 kPa.m$^{-1}$

$$V_{max} = \{(\Delta P/L)_{max}/C\} + [(B/C)(\mu/\rho)]$$ **Equation 4.2.3.3.1.2.**

$C$=0.000136, $B$=0.152, $\mu$=viscosity and $\rho$=gas density

The equation below calculates minimum bed diameter ($D_{minimum}$ , m) based on maximum superficial velocity ($V_{max}$, m s$^{-1}$):

$$D_{minimum} = \frac{4q}{\pi V_{max}}$$ **Equation 4.2.3.3.1.3.**

$q$ is the actual gas flow rate, m$^3$ s$^{-1}$, which can be calculated from mass flow rate ($\dot{m}$, kg s$^{-1}$ ):

$$q = \frac{\dot{m}}{60}$$ **Equation 4.2.3.3.1.4.**

Superficial velocity and bed diameter can be adjusted after $D_{minimum}$ is calculated:

$$V_{adjust} = V_{max} \frac{D_{minimum}}{D_{selected}}$$ **Equation 4.2.3.3.1.5.**

### 4.2.3.3.2. Desiccant mass

First estimate the water content to be removed ($W_r$) by the desiccant, then calculate desiccant mass $S_s$ (g) required:

Equation 2

$$S_s = \frac{W_r}{0.09 \times C_{ss} \times C_T}$$ **Equation 4.2.3.3.2.**

$C_{ss}$ and $C_T$ are correction factors for molecular sieve when gas is not water saturated or temperature is above 24°C .

This method replaces the saturation capacity of 13% with an effective desiccant capacity which includes MTZ (mass transfer zone) effect, temperature and relative humidity corrections. An effective capacity of 8-10% is typically assumed.

### 4.2.3.3.3. Bed length

The bed length of equilibration zone ($L_s$) can be calculated:

$$L_s = \frac{S_s \times 4}{\pi \times D^2 \times bulk\ density}$$ **Equation 4.2.3.3.3.1.**

The bed length of mass transfer zone can be estimated:

$$\boldsymbol{L_{MTZ} = (\frac{V_{adjusted}}{35})^{0.3} (Z)}$$ **Equation 3.2.3.3.3.2.**

$Z$ is 0.26 m for 1/16” inch sieve.

The final bed length is the summation of saturation zone and MTZ zone bed length, no less than diameter.

### 4.2.3.3.4. Calculation result

Assuming the flow rate required is 20 L min$^{-1}$, pressure is 101.3 kPa, compressibility factor is 1, air molecular weight is 28.751, relative humidity is 40% and temperature is 22.5°C.

Desiccant type = molecular sieve 13A 1/16” inch bead (8 × 12 mesh) using Equation

Bed diameter minimum = 4.48 cm (using Equation 4.2.3.3.1.3.)

Dehydration period = 24 hr (Estimated)

Superficial velocity = 143 cm $min^{-1}$ (using Equation 4.2.3.3.1.1.)

Water removed for 24 duration = 23 g

Weight of molecular sieve desiccant = 2724 g (using Equation 4.2.3.3.2.)

Bed diameter = 15 cm (using Equation 4.2.3.3.1.3.)

Bed length = 30.28 cm (using Equation 4.2.3.3.3.)

Cylinder bed volume = 5350 $cm^3$

## 4.2.4. Gas purification design

### 4.2.4.1. VOCs removal by active carbon

Active carbon is a term for a range of carbon-based materials that possess adsorptive properties. This includes coal, peat, wood or nutshells. The carbon based materials are activated by high-pressure steam at high temperature, so VOCs present are distilled off, leaving a highly porous structure. The amount of adsorptive activity developed depends upon the raw material. Chemical additives introduced to the raw materials before heating may result in enhanced adsorbent properties.

There are a few guidelines for carbon adsorption:

Larger molecules adsorb more strongly than smaller molecules (compounds with higher boiling point adsorb better than lower boiling point VOCs). Generally, VOCs with molecular weight between roughly 50 and 200 m/z corresponding to boiling points between 67°C and 350°C.

Non-polar molecular adsorb better than polar molecules.

Non-soluble or slightly soluble molecules adsorb better than highly soluble molecules.

Based on the polarity or solubility of molecules, pH may have an influence on level of adsorption.

Other conditions also might affect adsorption capacity:

- The lower the temperature, the better the adsorption capacity
- The lower the relative humidity, the better the adsorption capacity.
- The higher the pressure, the better the adsorption capacity.

The chemicals below are suitable for activated carbon adsorption:

All aliphatic and aromatic hydrocarbons that fulfil above rules such i.e. carbon numbers between C4 and C14+.

Most common halogenated solvents including carbon tetrachloride, ethylene dichlororide, methylene chloride, perchloethylketone and trichloroethylene.

Most common ketones such as acetone and esters such as butyl and ethyl acetate.

Common alcohols such as ethanol, propanol and butanol.

Although all chemicals above are adsorbed by activated carbon, the capacity for different VOCs can vary significantly with: molecular weight, polarity or solubility. Table 4.2.4.1. below shows activated carbon capacity for some common VOCs:

**Table 4.2.4.1. Activated carbon capacity for VOCs** (76)

| Compound name | Molecular weight | Boiling point ℃ | Carbon capacity % |
|---|---|---|---|
| nitrobenzene | 123 | 211 | 51 |
| tetrachloroethane | 166 | 147 | 40 |
| tetrachloroethylene | 165 | 121 | 35 |
| styrene | 104 | 145 | 25 |
| xylene | 106 | 138 | 21 |
| napathylene | 128 | 217 | 20 |
| toluene | 92 | 111 | 20 |
| benzene | 78 | 80 | 12 |
| MTBE | 88 | 55 | 12 |
| hexane | 86 | 68 | 7 |
| ethyl acrylate | 100 | 57 | 5 |
| dichloroethane | 99 | 99 | 7 |
| methyl ethyl ketone | 72 | 80 | 4 |
| methylene chloride | 84 | 40 | 2 |
| acrylonitrile | 53 | 74 | 2 |
| acetone | 58 | 56 | 0.8 |
| vinyl chloride | 62 | -14 | 0.7 |
| chloroethane | 64 | 12 | 0.5 |
| bromotrifluoromethane | 149 | -58 | 0.13 |
| methane | 16 | -161 | 0.0003 |

However there are some VOCs that need to be avoided for activated carbon adsorption:

- Compounds that react with carbon or with steam for regeneration i.e. organic acids such as acetic acid, formaldehyde, cyclohexane and some easily hydrolysed esters
- Compounds that polymerize on the carbon
- High molecular weight compounds that are difficult to remove such as plasticizers, resins, heavy hydrocarbons, phenols, glycols and amines

## 4.2.4.2. System design

### 4.2.4.2.1. Amount of activated carbon required

The Fruendlich isotherm equation is an adsorption isotherm which represents adsorption capacity to equilibrium partial pressure of VOC (78).

$$\frac{x}{m} = kP^n$$ **Equation 4.2.4.2.1.1.**

$x$ is mass of adsorbate, $m$ is mass of carbon, $P$ is partial pressure of VOC in gas (psia), $k$ and $n$ are empirical parameters.

$k$ and $n$ are specific for each VOCs, so each VOCs adsorption capacity needs to be calculated individually.

After adsorption capacities are calculated, calculate the number of moles of air molecules using ideal gas law.

$$PV = nRT$$ **Equation 4.2.4.2.1.2.**

$P$ is the absolute pressure of gas, $V$ is gas volume, $n$ is amount of gas present, $R$ is gas constant, $T$ is temperature.

The amount of carbon required can now be calculated using adsorption capacity.

### 4.2.4.2.2. Bed diameter and length

The equation below calculates minimum bed diameter ($D_{minimum}$ , m) based on maximum superficial velocity ($V_{max}$, m $s^{-1}$):

$$D_{minimum} = \frac{4q}{\pi V_{max}}$$ **Equation 4.2.4.2.2.1.**

The bed length of saturation zone ($L_s$) can be calculated:

$$L_s = \frac{S_s \times 4}{\pi \times D^2 \times bulk\ density}$$ **Equation 4.2.4.2.2.2.**

### 4.2.4.2.3. Calculation results

Assuming the flow rate required is 20 L $min^{-1}$, pressure is 101.3 kPa, compressibility factor is 1, air molecular weight is 28.751, relative humidity is 40% and temperature is 22.5°C.

Based on research on indoor air contaminants, contaminant VOCs have of average 1-10 ppmv, assuming VOCs are in 1 ppmv level, the list of contaminants used to estimate amount of carbon required are shown below.

**Table 4.2.4.2.3. Isotherm data for common indoor VOCs** (76)

| Compounds | molecular weight | Conc in air ppmv | k | m | isotherm pressure Pa | Equilibrium capacity |
|---|---|---|---|---|---|---|
| Benzene | 78 | 1 | 0.597 | 0.176 | 0.69 | 0.118025088 |
| Toluene | 92 | 1 | 0.551 | 0.11 | 0.69 | 0.200056008 |
| acetone | 58 | 1 | 0.412 | 0.389 | 0.69 | 0.011452419 |
| Trichloroethane | 133 | 1 | 1.06 | 0.161 | 0.69 | 0.240605674 |
| Xylene | 106 | 1 | 0.708 | 0.113 | 0.69 | 0.250053684 |
| Phenol | 94 | 1 | 0.855 | 0.153 | 0.69 | 0.208913312 |
| Dichloroehtane | 99 | 1 | 0.976 | 0.281 | 0.69 | 0.073358394 |
| chlorobezene | 113 | 1 | 1.05 | 0.188 | 0.69 | 0.185861441 |
| cyclohexane | 84 | 1 | 0.508 | 0.21 | 0.69 | 0.07342834 |

These compounds are representative of the most prevalent VOCs contaminants in air, however there are many more VOCs present in air, so a calculation such as this is only an estimate for amount of carbon required. Assume there are around 200 types of VOCs present at 1 ppmv in indoor air, and a safety factor of 100% added for a conservative design.

Weight of activated carbon = 695 g

Gas purification period = 3 months

Equilibrium pressure of VOC = 0.69 Pa

### 4.2.5. Gas hydration and purification combination design

#### 4.2.5.1. Mixed bed-designs

Molecular sieve and activated carbon combined together increase the range of compounds that may be removed by adsorption. Molecular sieve adsorbs relatively small polar compounds such as water, carbon dioxide and C1 to C4 hydrocarbons. Activated carbon is suitable for relatively larger compounds such as benzene, and toluene.

Since activated carbon is a stronger adsorbent than molecular sieve, the air inlet goes through the weaker molecular sieve adsorbent first to remove water and relatively small polar compounds, before the relatively larger compounds are absorbed by activated carbon.


**Figure 4.2.5.1. Combining molecular sieve and activated carbon**

#### 4.2.5.2. Initial purification filter designs

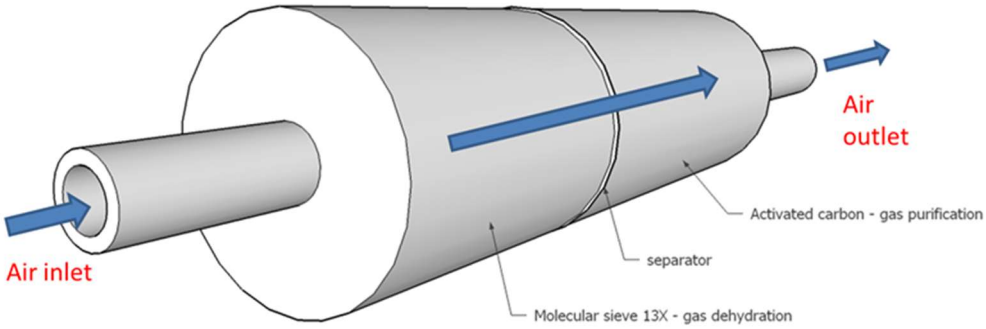


**Figure 4.2.5.2.1. Filter design 1**

Combine molecular sieve 13X (gas dehydration unit) and activated carbon (gas purification unit) together in same container, separating them by glass wool (or another type of separator), enabling both to be regenerated together.

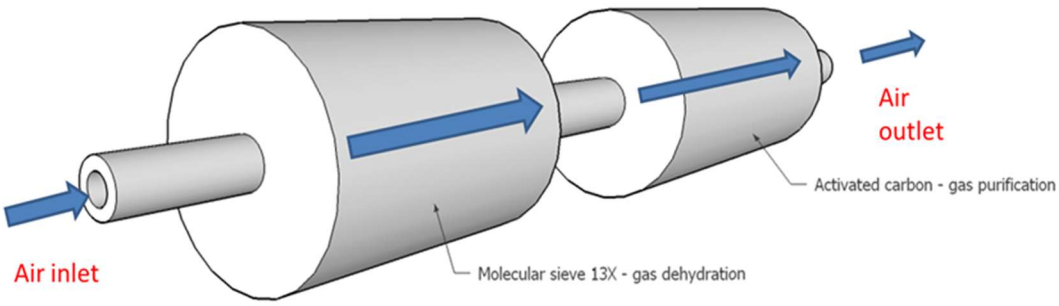


**Figure 4.2.5.2.2. Filter design 2**

Since molecular sieve 13X and activated carbon have very different adsorption capacities they will have different service intervals. This design enables them to be regenerated separately.

## 4.3. Instrumentation

### 4.3.1. Portable breath sampler setup

The portable adaptive breath sampler consists of two subunits: breath sampling unit and air supply unit. The main components of the breath sampling unit are: a sampling control box and breathing masks. The air supply unit consists of and air supply pump, a dehydration filter, a VOC purification filter and a dust particulate filter. The schematic diagram listing all main components of portable adaptive breath sampler is shown in Figure 4.3.1.1.

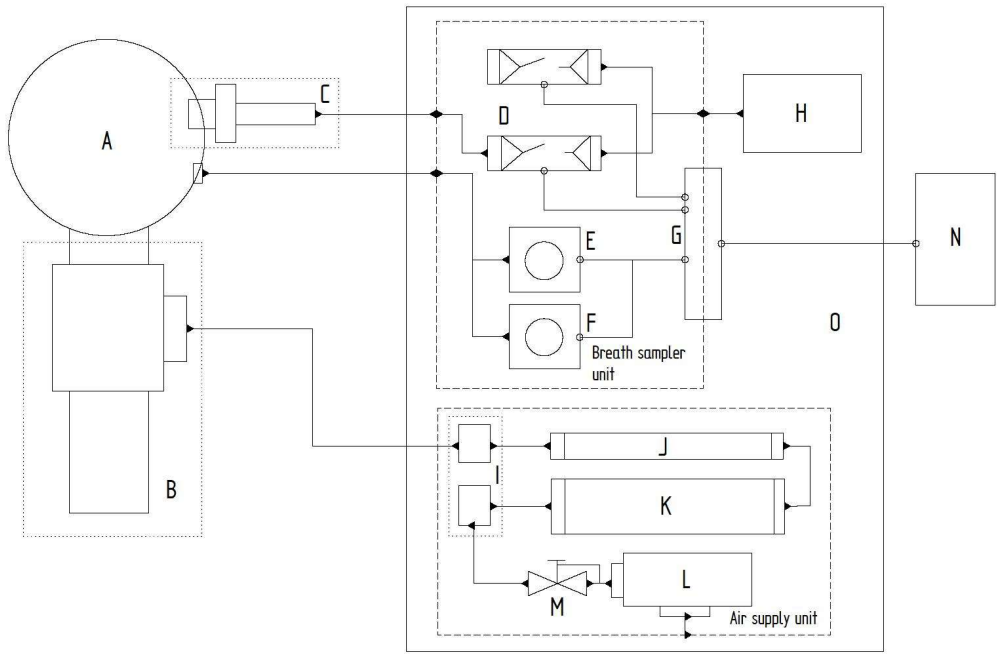


**Figure 4.3.1.1. Schematic diagram of portable adaptive breath sampler illustrating the main components: breath mask (A), mask air supply assembly (B), adsorbent sampling assembly (C), micro-control valves (D), pressure transducer (E, F), electronic interface (G), precision air sampling pump (H), dust filter (I), activated charcoal filter (J), molecular sieve 13X filter (K), air supply pump (L), pressure regulator (M), laptop containing DAC card and labview software (N) and portable suitcase (O)**

All of the portable breath sampler components were housed inside a heavy-duty steel cart. The cart was fitted with lifting handles, a telescopic drag handle, and wheels. The prototype adaptive breath sampler built was assembled in three sections stacked one on top of the other. At the bottom was an air supply pump, in the middle was the air purification unit and at the top was the breath sampling unit, placed there for easy access. Gas connections between the sections were made with 6.4 mm bore silicone tubing. The connections between components in the air purification stage were ¼ inch stainless-steel tubing and connectors (Swagelok, USA); these can be disconnected easily for cleaning and servicing purposes. Figure 4.3.1.1. illustrates prototype portable adaptive breath sampler at each level.


**Figure 4.3.1.2. The prototype portable breath sampler setup at each individual level.**

### 4.3.2. Breath sampling unit

The breath sampling unit consisted of a sampling control box and a breathing mask for the participant. The micro-control valves within the sampling control box, controlled by a Labview virtual instrument, switched the sampling pump between a sampling and vent mode depending on the phase of breathing cycle of the participant. As the participant inhaled, the micro-valve switched to vent mode to avoid sampling, inspired air and when the participant exhaled, the micro-valves switched to sampling mode at the point set in the breathing cycle. A pressure transducer tracked inspiration and exhalation and was connected to the mask with a silicone tube. The breathing profile and controls were displayed on Labview software program. The user was able to analyse and exhaled breath profile and set the system to sample a specific portion of the breath to collect. The targeting sampling of exhaled breath is essential feature for sampling variable breaths of participants with impaired lung function.

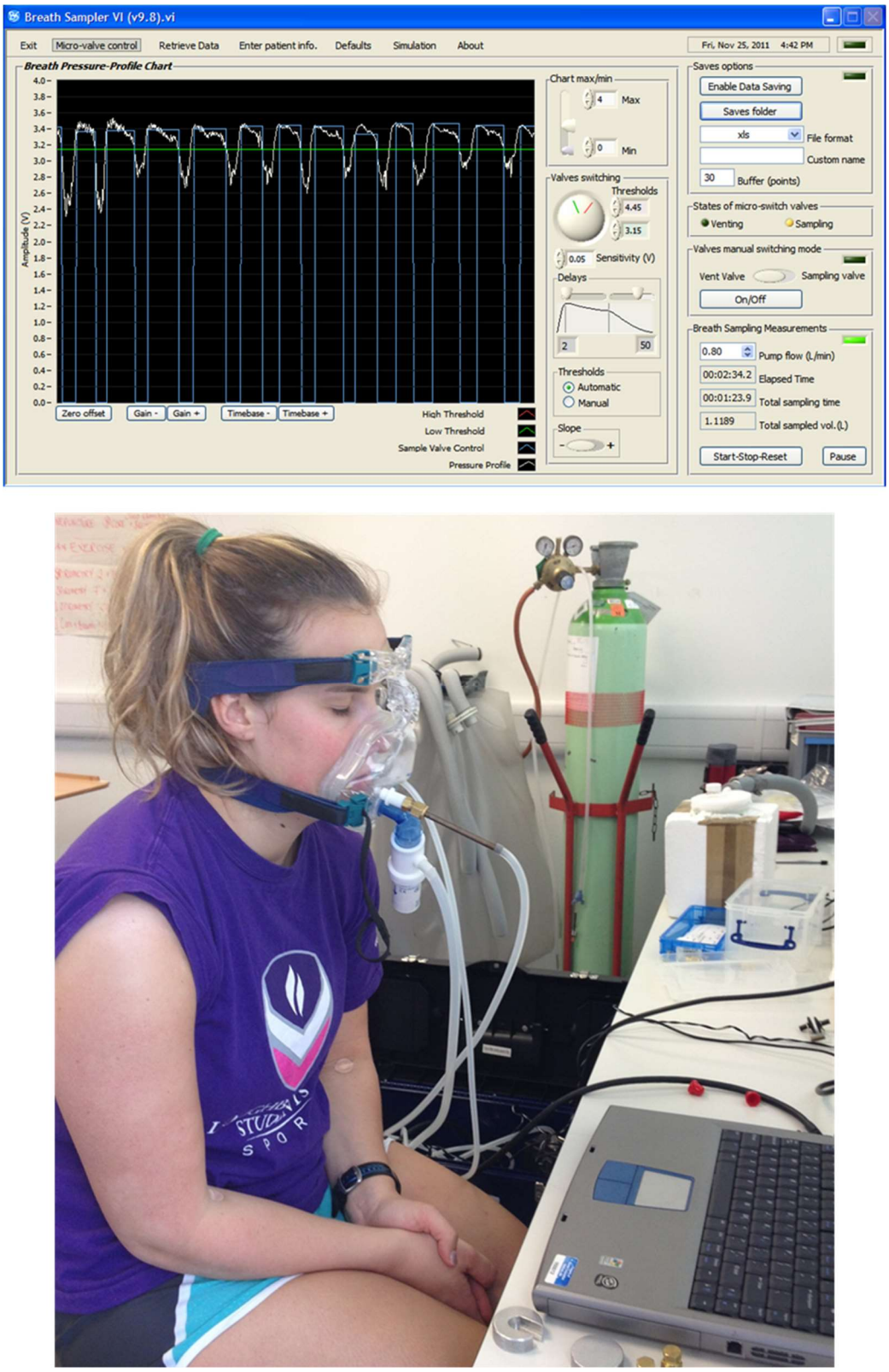


**Figure 4.3.2. (Top) Screenshot of virtual instrument Labview used to collect a participant's breath (Bottom) Photograph of collection of a breath sample from participant.**

The medical breathing mask (ResMed, Mirage Full Mask Series 2) was carried with the sampling unit at top level of the carrier and was prepared for use at the sampling location. The mask was fitted with a custom made detachable PTFE valve which was connected to the air purification unit. The PTFE valve was fitted with a single use one-way valve with to exhaust exhaled breath and surplus air supply while preventing the accidently ingress of ambient air into the mask and sampling zone. The mask also had two Luer fittings: One fitting was connected to the pressure transducer inside the sampling control box, and the other was connected to the adsorbent sampling assembly. The adsorbent sampling assembly contained a custom made PTFE interface which connected a multi-adsorbent adsorbent sampling tube (Tenax® TA/ Carbograph 1 TD, Markes International, UK) for breath VOC collection. The exhaled breath was drawn from immediately beneath the nostrils into the adsorbent tube by a 5 cm × 0.53 mm MXT stainless steel capillary guard column (Thames Restek, UK) fitted inside the PTFE interface. The other end of the adsorbent tube was connected to the micro-valve switching ports on the breath sampling unit which in turn were connected to an Escort ELF air pump (MSA, USA) with silicon tubing.

### 4.3.3. Air supply and purification

A linear air pump (HP-200, HIBLOW, Techno Takatsuki ltd, Japan) was housed at the bottom of the portable carrier. Surrounding room air was drawn through its in-built coarse filtered inlet and up to 200 L $min^{-1}$ was delivered to its outlet. 9 air holes around 1 cm in diameters were cut out at the bottom level to ensure sufficient air flow and cooling for the air pump. A custom made PTFE fitting connected the pump outlet to 6.4 mm bore silicon tubing. The room air was supplied into the three filters at the middle level of the portable carrier.

A three-stage process dried, purified and filtered the air. The first-stage was a custom built stainless steel molecular sieve 13X 10A filter (L260 mm × O.D.100 mm, 100psig pressure rating, 5351 cc). It is made airtight using custom made soft silicon gasket and mesh screen fitted at both ends of the filter. The primary function of the molecular sieve 13X desiccant filter was to dry the air supply. It has a very stable water capacity from around 20% to maximum of 28.5% at relative humidity range of 10% to 100% and ambient temperature of 10°C to 50°C (76). The dehydrated air was then passed through the custom built stainless steel activated carbon filter (L278mm × O.D.25.4mm) via ¼ inch stainless steel tubing. Since activated carbon is a stronger adsorbent than molecular sieve, the air inlet went through the weaker molecular sieve adsorbent first to remove water and relatively small polar compounds, before the relatively larger non-polar compounds were removed by activated carbon. The molecular sieve serves both a drying and low Carbon number purification adsorbent protecting the activated carbon filter from overloading. Additionally, the larger

molecular sieve filter also cools down pumped air from around 30°C to 20°C, increasing the capacity of the carbon filter to absorb and more comfortable for participants to breath.

The last filter in the purification assembly was a stainless steel dust and particulate filter with ¼ NPT in-line port (3.2 cm × 7.9 cm, 5000psig pressure rating, Model 97S6, Parker Baslton, USA). It contains a replaceable microfiber cartridge (Model 050-50-BQ, Parker Baslton, USA) which filters gas at 0.01 µm of more than 99.99% of its particulate (79).

After drying and purification the room air, was supplied to the breathing mask at flow of 30 to 40 $L.min^{-1}$. The purified air met or exceeded the air quality requirement of ISO8573-1:2010 Class 1 with a particulate count not exceeding 20,000 particles in the 0.1 - 0.5 micron size range[4]. All three filters contain re-generable adsorbent or replaceable cartridges which were regenerated at regular intervals.

## 4.4. Experimental

### 4.4.1. Exhaled breath sampling method work-flow

#### 4.4.1.1. Preparation and validation

Figure 4.4.1.1 illustrates the detailed breath sampling work-flow using the portable adaptive breath sampler. The work-flow can be separated into three stages: preparation, validation and sampling. It is based on the method adopted using the original breath sampler with enhanced method stages and checklist for a more rigorous process (80).

Preparation is an essential stage for portable adaptive breath sampling since the adsorbents filters require regeneration after use to create purified air. Additionally, thorough cleaning of all portable breath sampler parts in between samples minimise exogenous VOCs in breath samples.

All three filters in the purification assembly can be readily disconnected and removed from portable breath sampler for regeneration. The molecular sieve 13X filter can be cleaned using the thermal desorption method. The filter regeneration process consists of heating and cooling stages. Heated took place inside a conditioning oven while being back-flushed with dry high-purity nitrogen gas at 20 $L.min^{-1}$ for 5 hours at 288°C. It was then cooled to 22.5°C and left to stabilise for a further 2 hr. It was important to ensure that the filter was allowed to cool completely to room temperature before sampling to avoid variation in adsorption capacity. The regeneration temperature of 288°C was based on industrial desiccant tower regeneration temperature (78), and the regeneration conditions was calculated based on maximum sampling duration of 4 hours and stripping gas flow rate equation (81). The activated carbon trap can also be regenerated by the thermal desorption method (82). The filter was heated to 300°C while back-flushed with dry high-purity nitrogen at 10 L $min^{-1}$ for 5

hours followed by cooling and thermal stabilisation for 1 hr at 22.5°C. The fine particulate filter cartridge was also checked periodically for dust accumulation and replaced accordingly. All stainless steel parts and silicon tubing were baked out in vacuum oven at 150°C overnight before use to eliminate any VOCs residuals

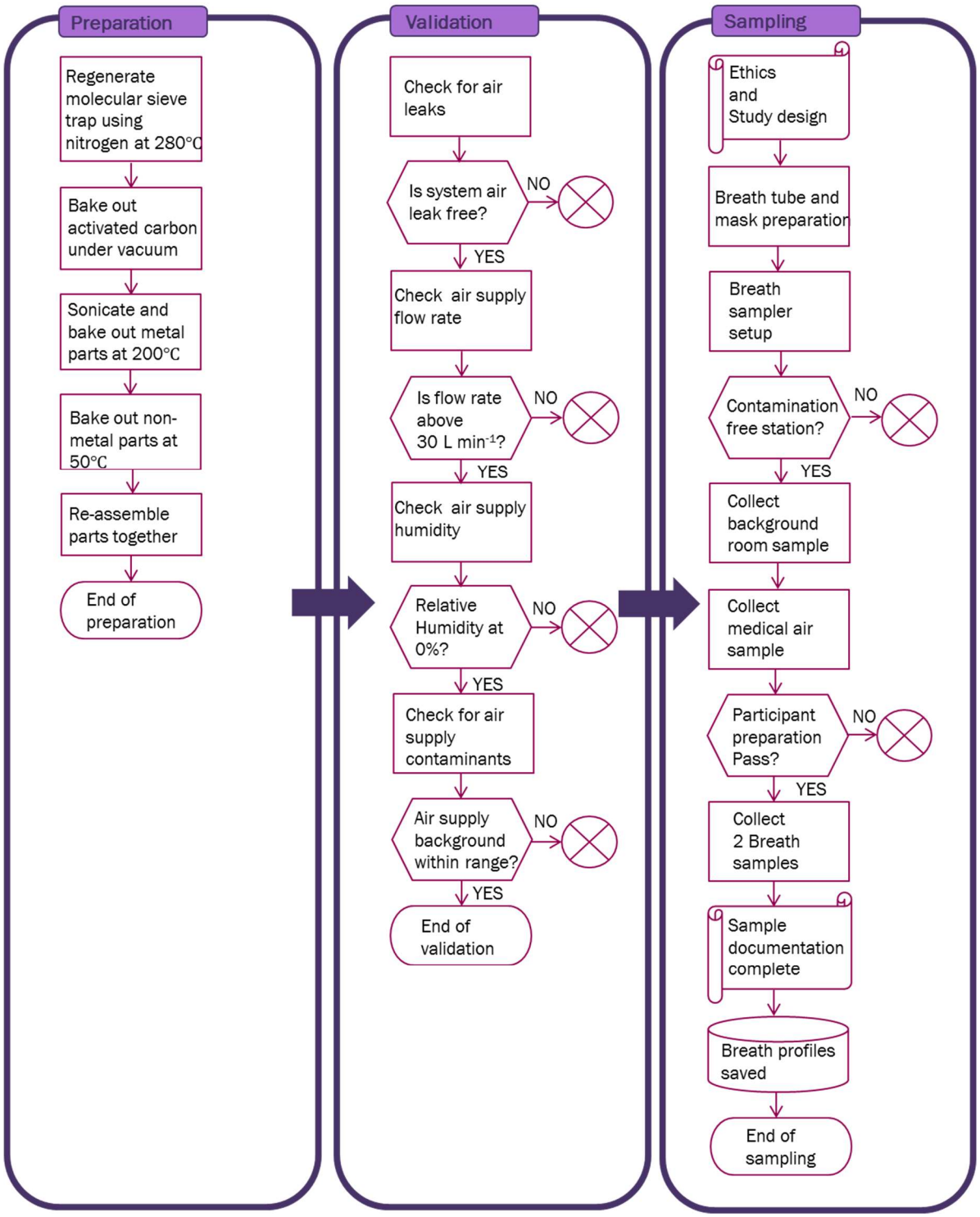


**Figure 4.4.1.1. Work-flow chart detailing the exhaled breath analysis method using the portable adaptive breath sampler including preparation, validation and sampling stages**

**Table 4.4.1.1. TD-GC-MS system instrumental parameters**

| Thermal desorption | | Gas chromatography | | Ion trap mass spectrometer | |
|---|---|---|---|---|---|
| Parameters | Setting | Parameters | Setting | Parameters | Setting |
| Pre-desorption purge | 1 min | Initial temperature | 40°C | Mass range | 40 m/z to 445 m/z |
| Tube desorption | 5 min at 300°C | Final temperature | 300°C | Scan mode | EI auto |
| Pre-trap purge | 1 min | Hold temperature/time | 300°C for 8 min | Emission current | 10 µAmps |
| Trap desorption | 5 min at 300°C | Rate | 5°C/min | Target TIC | 20000 counts |
| Heating rate | Maximum °C/min | Initial pressure | 27.3 psi | Maximum ion time | 25000 µseconds |
| Flow path temperature | 180°C | Final pressure | 56 psi | Scan time | 0.38 seconds/scan |
| Trap low temperature | -10 °C | Hold pressure/time | 56 psi for 7.83 min | Data rate | 2.63 Hz |
| HV temperature | 180°C | Rate | 0.55 psi/min | Trap temperature | 150°C |
| Cold trap flow rate | 50 $cm^3$/min | GC column | DB-5 60m length × 0.25mm i.d. × 0.25 µm film thickness | Manifold | 50°C |
| Cold trap packing | General purpose hydrophobic | Total analysis time | 60 min | Transferline temperature | 300°C |
| Split ratio | Splitless | Carrier gas | Helium | | |
| | | Carrier gas flow rate | 2 $cm^3$/min | | |

Finally all regenerated and cleaned filters, connectors and tubing were assembled together using 9/16” and ½” spanners to form air tight seals. The connected purification unit were checked for air leaks with a leak detector while pressurised with high purity nitrogen or helium gas supply at around 10 psig. Any leaking connections were tightened with spanners. After re-connecting the air purification unit to the air pump, the flow rate of the purified air supply was checked with a rotameter (50 L $min^{-1}$ maximum flow). The minimum flow required for relaxed breathing at resting state was 30 L $min^{-1}$.

The purification efficiency of the filter assembly was checked at regular intervals. The drying efficiency was tested using a humidity detector (Environics, Finland), and the background exogenous VOCs level were also be checked by collecting an air sample (2 L) from the air outlet for GC-MS analysis.

The coarse filter to the inlet of the air pump was periodically checked every 6 month according to manufacture specification. If the pump was used intensively, then frequency of inspection was increased to a check every 3 month. A dirty filter might introduce high levels of contaminants into the air supply, or reduce the flow-rate. The portable breath sampler should be cleaned with high purity water periodically to avoid dust accumulation.

The medical breath mask was sterilised by soaking it in 0.55% aqueous ortho-phthaldehyde solution for one minute, followed by careful scrubbing with a soft brush for thorough clean. The sterilised masks were then rinsed with clean distilled water and air dried away in enclosed fume cupboard. Finally it is conditioned in a vacuum oven at 0.1 kPa at 50ºC for a minimum of 2 hours.

#### 4.4.1.2. Sampling

All exhaled breath sampling experiments were conducted in accordance with the ethical principles of good lab practice and the Declaration of Helsinki. The research method followed non-invasive exhaled breath sampling protocol coded G09-P5 that was approved by the Ethics Advisory Committee at Loughborough University. Participants gave written consent and were screened by a health questionnaire.

The portable breath sampler was moved to the sampling location where a 220 V AC supply was required. The air purification had been setup and tested during preparation while the face mask and its connections to the sampling control box were transported in an air-tight container within the cart to be assembled on-site to avoid contamination in transit. The sampling room was also checked for any sources of VOC contamination. The sampling location was specified to be well-ventilated by temperature controlled to about 20°C. The participant(s) was (were) asked to refrain from using make up or strongly scented personal

care products on the day of sampling. The flow rate from the air purification unit was checked after setup and then connected to the sampling face mask air inlet. The face mask was fitted to a seated participant with straps and tightened gently until the mask was air tight. The participant was then encouraged to relax and get used to wearing the face mask while breathing in a relaxed manner through their nose. During this acclimatization, which lasted up to 10 min, the participant's breath profile was recorded and tracked on Labview software. The sampling parameters were adjusted to only collect exhaled breath from their distal airways. 2.5 L breath samples were then collected from the participant onto multi-adsorbent tubes and stored in 4°C fridge. Additionally, 2.5 L samples were taken of the purified medical air supply and the room air. The collected samples were transported back to the analytical laboratory at 4°C using cool packs in a well-insulated container. They were analysed within 72 hours. For large scale studies where immediate sample analysis is not possible, the sample may also be stored at - 80°C for up to 6 week (See Chapter 3 for more details).

### 4.4.2. Air supply drying test

The aim of the drying test was to determine the capacity of the molecular sieve 13X desiccant. This was achieved by identifying the equilibration and break through points for the molecular sieve filter.

The HiBlow HP200 air pump was connected to molecular sieve filter with a flow rate of 40 $L.min^{-1}$ recorded using a rotameter. The outlet from the molecular sieve 13X filter was connected to a gas-sensing array that included relative and absolute humidity ($g\ m^{-3}$) sensors (VAMPPI Environics oy, Finland) using a ¼" inch stainless steel T-union. A portion of the outlet air flow was split into the VAMPPI sensor array at 1.3 $L\ min^{-1}$. The monitoring continued until the molecular sieve trap appeared equilibrated with water. The RH% and absolute humidity data measured by VAMPPI was monitored and recorded on ChemPro-100 software (Environics oy, Finland). The recorded data was exported into Excel format for data analysis.

### 4.4.3. Air supply purification test

The air supply purification test determined the effectiveness of the activated carbon filter unit in reducing the levels of air contaminants supplied to the face mask.

Ten 2.5 L air samples were collected from purified air supply along with three 2.5 L background room air sample, to act as comparisons. The air supply to the portable breath sampler was pumped from surrounding room air and a comparison between these two types of air samples enabled the efficiency of the activated carbon filter to be estimated. Room air samples were collected using Elf Escort (flow rate range: 0.5 to 3 $L\ min^{-1}$, MSA, USA) air

sampling pump at flow rate of 0.5 L $min^{-1}$. Purified air supply samples were collected via a custom made sterile stainless steel needle valve at a flow rate of 0.5 L $min^{-1}$. All air samples were collected over 2 hr and analysed within 48 hr using two-stage thermal desorption Unity Series 1 (Markes international, USA) interfaced with Varian 3800 GC coupled to Varian 4000 ion-trap mass spectrometer. Retention index mixture of 18 hydrocarbons compounds with known concentration was injected before and after sample analysis as quality control.

### 4.4.4. Regeneration study

The regeneration study sought to establish the regeneration efficiency of the air purification unit. This included testing the proposed thermal desorption regeneration methods to dry and clean the exhausted molecular sieve 13X and activated carbon filters.

After approximately 4 hr with 20 samples collected the air supply and purification unit was nearly exhausted. The whole unit was disassembled and regenerated according to the protocol described in Section 4.4.1.1. After regeneration and re-assembly, the performance efficiency of the regenerated components was tested by collecting a 2.5 L air sample from the purified air supply and monitoring humidity with the VAMPPI device. The air sample was analysed immediately by TD-GC-MS method.

### 4.4.5. Trial with human participants

The portable breath sampler was tested extensively through a series of 10 purified air supply sampling and regeneration cycles, following the breath sampling work-flow. After the testing phase, the sampler was then trialled with 10 volunteer participants who completed consent forms and health questionnaires before sampling began. Some participants had mild asthma. Two 2.5 L breath samples were collected from each participant in under 30 min, and all 10 participants were sampled over one week period. They were closely monitored for any signs of discomfort during sampling along with the air-supply flow rate; measured at around 40 L.$min^{-1}$. One breath sample was collected using the portable breath sampler and the second sample was collected using the original adaptive breath sampler described in Chapter 2. This enabled comparison of breath samples collected using the two methods. Breath profiles were also recorded to assess if any changes in the breathing pattern with the two methods were discernible. The collected samples were stored at 4°C and analysed within 48 hr.

The reproducibility of breath samples was also assessed by collecting 10 replicate breath samples from the same individual over a 2 hr interval. The collected samples were stored at 4°C and analysed within 48 hr.

## 4.5. Results and discussion

### 4.5.1. Air drying test

The air inlet had an average relative humidity of 22.9% at 20°C calculated from 10 minutes of data. Once this initial measurement has been acquired experiment was set up and run, and Figure 4.5.1. illustrates the RH% plotted against time. %RH was less than 0.1 % (T= 20°C) for 4.02 hours with breakthrough detected at that time after approximately 9.6 $m^3$ of air had been processed. The %RH increased until at 40.7 hours whereit reached the %RH level of the inlet air.

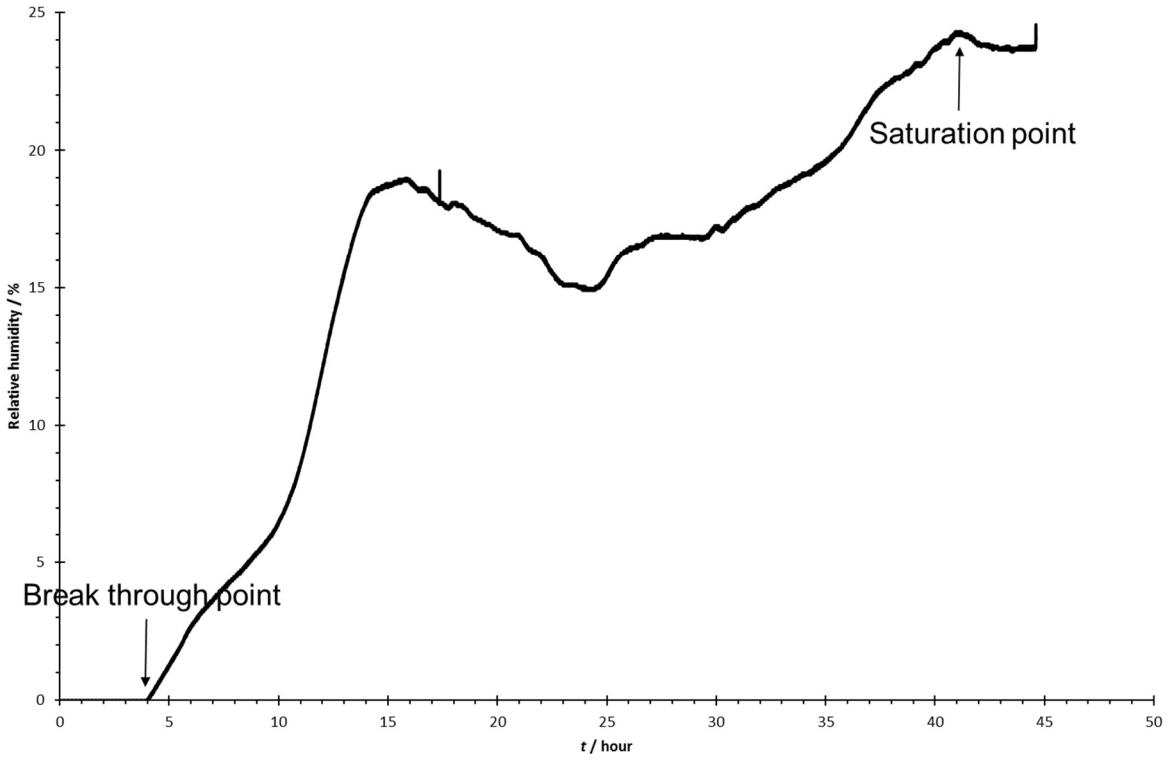


**Figure 4.5.1. Line plot of RH% against dehydration duration using molecular sieve 13X desiccant filter. Both break through point and saturation point are labelled on the graph**

### 4.5.2. Air supply purification test

#### 4.5.2.1. Purification level comparison with background air

The air supply unit not only dried the air supply, it also purified it by trapping entrained VOCs. The purification capacity of air supply unit was dependent on the adsorption capacity of activated carbon filter for VOCs in combination with the adsorption capacity of the molecular sieve 13X filter. The adsorption capacity of the air supply unit was demonstrated by comparing the levels of 12 common indoor airborne contaminants in purified air samples

and background room air samples (Table 4.5.2.1.). The average combined peak area of the 12 room-air sample compounds was measured at 637 kcounts ± 223 kCounts. The average combined peak area of these compound in the samples of purified air was 216 kCounts ± 58 kCounts. The adsorption efficiency varied significantly depending on the chemical and physical properties of any specific compound. The halogenated compounds were determined to have the highest adsorption rate among targeted compounds, chloroform was reduced most significantly by 97.8%, followed closely by trichlorofluoromethane (Freon 11) whose level in the air supply was reduced by 97.5%. In contrast, the air supply unit has shown significantly lower adsorption capacity for methylated benzene compounds, ethyl benzene level comparison shown no reduction between room air and purified air samples, xylene level was only reduced by 27.7% in the purified air samples.

**Table 4.5.2.1. The adsorption capacity of 12 common indoor air contaminant compounds by portable breath sampler air purification unit.**

| Compound name | Purified air (kCounts) | | Background room air /Counts | | Reduction % |
|---|---|---|---|---|---|
| | Average | RSD% | Average | RSD% | |
| 1,4-dichlorobenzene | 7 | 62.9 | 15 | 80.3 | 56.5 |
| 2-Butanone | 62 | 49.1 | 269 | 46.9 | 77.0 |
| Acetone | 507 | 29.9 | 1103 | 32.5 | 54.0 |
| Benzene | 183 | 11.7 | 1149 | 17.3 | 84.1 |
| Chloroform | 16 | 48.8 | 730 | 75.7 | 97.8 |
| Ethyl benzene | 407 | 32.3 | 387 | 37.0 | 0.0 |
| Heptane | 11 | 79.2 | 265 | 71.1 | 56.9 |
| Tetrachloroethylene (PERC) | 8 | 41.1 | 22 | 64.3 | 64.4 |
| Toluene | 688 | 19.2 | 2633 | 20.6 | 73.9 |
| Trichlorofluoromethane (Freon 11) | 9 | 58.2 | 352 | 72.8 | 97.5 |
| Trimethylbenzene | 39 | 24.8 | 55 | 52.5 | 29.8 |
| o-Xylene | 656 | 29.9 | 907 | 45.0 | 27.7 |

RSD% variation of compound peak area from 10 replicate purified air sample was measured at 40.6%. Similar to the variation in adsorption rates, the RSD% also ranges significantly from 11.7% to 79.2%. The variation appears to be largely dependent on the compounds peak intensity, generally compounds with greater peak intensity seem to have lower RSD% variation.

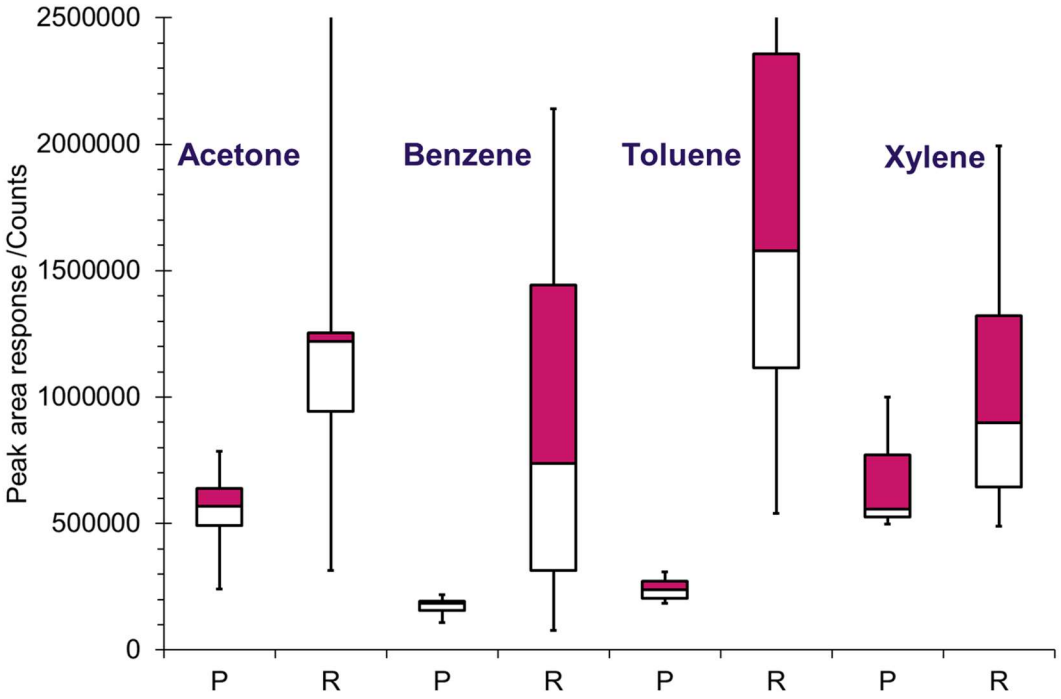


**Figure 4.5.2.1. Comparison of common air contaminants levels found in room air and purified air from the portable breath sampler. P denotes purified air supply and R denotes background room air.**

### 4.5.2.2. Comparison with original adaptive breath sampler

The original adaptive breath sampler used three stage filtration with Grade DX, Grade BX and Grade-000 high capacity purifiers (2000 Balston series, Parker Hannifin Corporation, USA). The air supply was generated from an air compressor and a wall mounted membrane drier. The purification efficiency of this air supply unit setup has been described in detail previously (41). The levels of volatile common room air contaminants were greatly reduced and less variable in the purified air. For example, average acetone concentration were 14 times higher than 14 times more variable compared to room air sample. Volatiles generated from personal care and cleaning products were also significantly reduced (4 times reduction) in purified air.

The average signal responses of the 12 compounds are listed in Table 4.5.2.1. For the original breath sampler was 272 kCounts ± 48 kCounts compared to 216 kCounts ± 58 kCounts. The contamination levels in the air from portable breath sampler was lower although more variable. The RSD variation for 10 portable breath sampler generated air sample was measured at 40.6% compared to variation only 20.8% obtained from original adaptive sampler. This variation may be due to be the variability of the intake air to the portable sampler due compared to air in the plant room intake. Some variability may also be attributed to the packing of the adsorbents inside the portable breath sampler. An example

chromatogram comparing purified air supply from the two sources is illustrated in Figure 4.5.2.2.

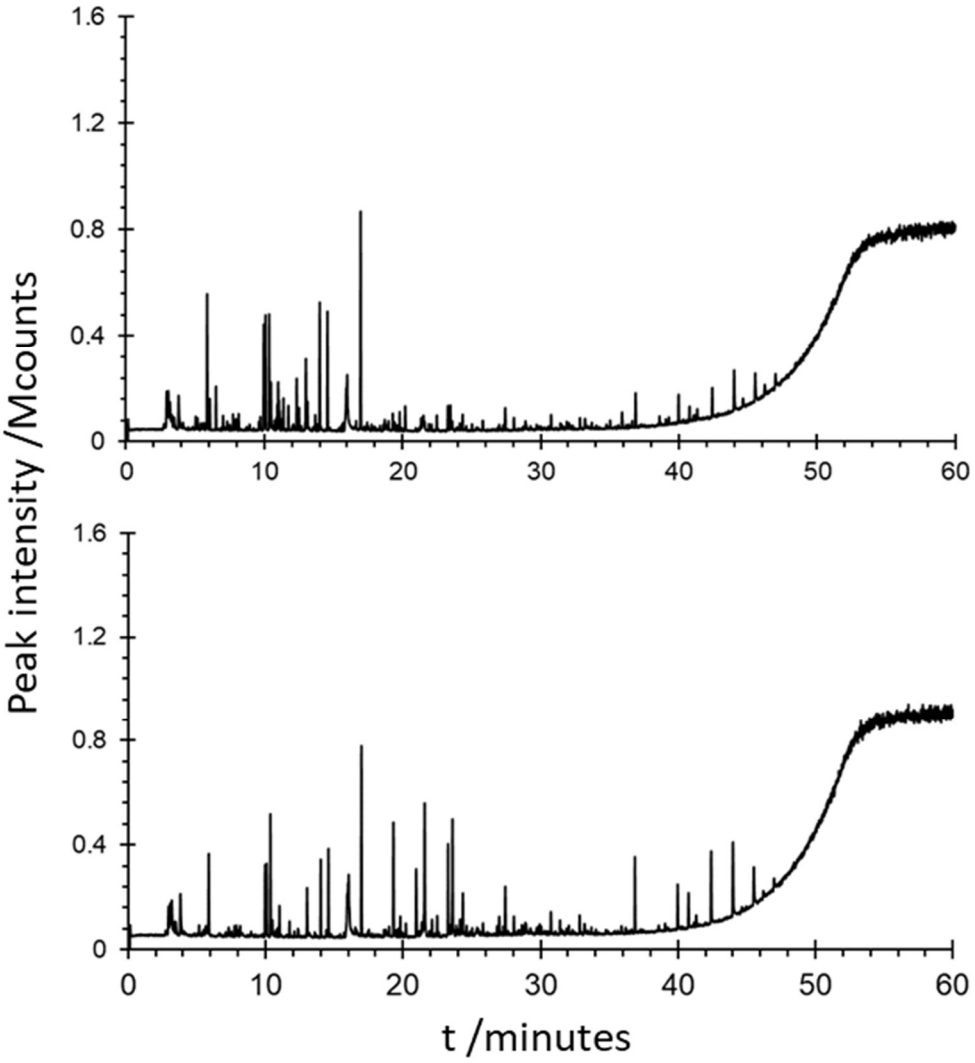


**Figure 4.5.2.2. TIC chromatogram of purified air supply from two sources on the same day: purification elements from original adaptive breath sampler (top) and custom built purification unit from portable breath sampler (bottom)**

The adsorption rate of 12 compounds by original breath sampler purification unit was determined by comparison with their respective background room air level, and it was measured with average at 42.7%. The portable breath sampler air purification unit measured purification efficiency at 59.5% against its own background air inlet. The inlet air to the two devices are from different sources, the portable breath sampler uses room air as inlet source, whereras original adaptive breath sampler uses generator air as its own air inlet source, the purification efficiency calculated is just to show the portable breath sampler is able to achieve comparable purification efficiency compared to the original adaptive breath

sampler. Also this is highly compound dependent and a significant adsorption capacity variation can be observed across the 12 compounds. Similar to portable breath sampler, the original adaptive sampler also has the highest adsorption capacity for chloroform compounds with 92.7%. The original adaptive also achieve similar adsorption capacity for other compounds such as heptane, acetone and benzene, however the adsorption capacity differ significantly for other compounds such as tetrachloroethylene which it shown poor adsorption capacity (0%) compared to significantly higher adsorption at 64.4% by portable breath sampler. For other compounds the original adaptive sampler seems to perform more efficiently, portable breath sampler demonstrated poor adsorption for ethyl benzene (0%), but the original adaptive sampler was able to remove around 53.7% of contaminant present in pumped air. The significant difference in adsorption capacity for these specific compounds is likely to be due to the specific adsorbent material used inside the filter. The high capacity Balston filter used in original adaptive breath sampler partly consists of Grade 000 CI type cartridge to remove trace quantities of oil vapour. The cartridge itself is made of activated carbon adsorbent sandwiched between two microfiber materials, but no details can be found on the specific type of activated carbon used (83) (84). Although the portable breath sampler also uses activated carbon as trace VOCs adsorbent, but there are various and complex types of activated carbon adsorbent available on the market, and their adsorption capacity differ significantly due to huge range of criteria such as surface characteristics, size and preparation methods. Hence it is important to test and establish the adsorption capacity of activated carbon adsorbent for target compounds or applications, and the purification test carried out on the 12 common indoor contaminant compounds has determined that the portable breath sampler three stage filtration process and multi-adsorbent combination of molecular sieve 13X and activated carbon can successfully remove the majority of trace contaminant VOCs present in the air supply to produce purified air for breath sampling, additionally its comparison with a commercial filter assembly has established it can achieve the same level of adsorption capacity with smaller and lighter setup, adding a new aspect of portability.

### 4.5.3. Regeneration test

The air purification unit was regenerated periodically depending on frequency of sampling, generally the maximum sampling time was 4 hours, based on sampling time of 10 minutes per breath sample, around 20 breath samples can be taken before it needs to be regenerated. The thermal desorption regeneration used was described in Section 4.4.1.1. The average VOCs adsorption capacity % of air purification unit just after regeneration was determined to be 95.1% compared to 59.5% before regeneration. The adsorption capacity was calculated from the adsorption rate of the 12 common airborne VOCs. The increase in

adsorption capacity demonstrates that the thermal desorption cleaning procedure for the purification filters adopted was successful in regenerating the desiccants. Additionally after the thermal desorption regeneration procedure, the dehydration capacity of the desiccants was too successfully recovered to full, the humidity level % after regeneration was measured at 0% compared 100% just before. The adsorption capacity of molecular sieve and active carbon is limited and decline as a function of the number of regeneration cycles. The life time of the purification unit depends very much on frequency of breath sampling, therefore it is essential to keep monitor and measure the adsorption capacity of the purification unit after sampling, and replace the adsorbent beds readily if a reduction in adsorption capacity is observed.

### 4.5.4. Trial with human participants

All 10 participants were asked about the experience with portable breath sampler and the original adaptive breath sampler after sampling, none reported any difficulties with breathing during sampling. None reported any major differences in experience between the portable breath sampler and original breath sampler. One participant reported purified air supply in portable breath sampler was very relaxing and was falling asleep during sampling. Although most (8 out of 10) participants reported that breathing the purified air supply felt very different to breathing room air and some reported that it took time to acclimatised, but all participants felt they were able to breathing comfortably and relaxingly during sampling.

**Table 4.5.4.1. Participant portable breath sampling trial summary.**

| | |
|---|---|
| Participants number | 10 participants |
| Study duration | 4 days |
| Breath samples on portable breath sampler | 10 samples |
| Breath samples on original breath sampler | 10 samples |
| Background air samples for breat samples | 8 ( 2 per day) |

The sample of these 10 participants were carried out in 4 separate days, 2 breath samples were collected per participant, additionally room air and air supply samples were collected after and before breath sampling (See Figure 4.5.4.1.). Sampling time for each sampling session is between 3 to 4 hrs. The desiccant air filters were regenerated by thermal desorption process in between the sampling days. Air humidity tests carried out after the

session have all shown 0% humidity indicating the filters have not reached breath through point.

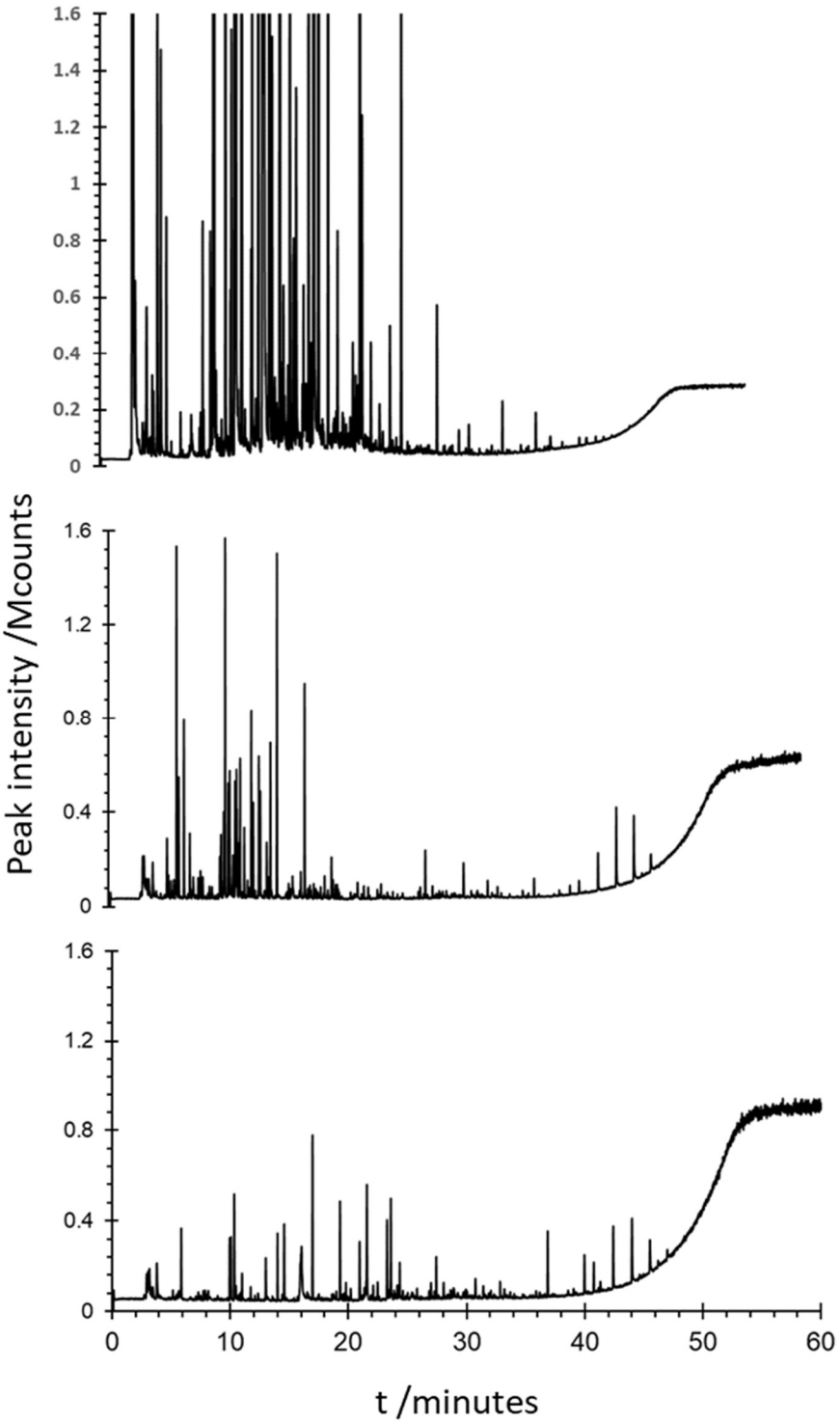


**Figure 4.5.4.1. TIC chromatogram illustrating participant breath sample using the portable breath sampler (top), room air (middle) and purified air supply (bottom)**

The breath profiles of the 10 participants recorded during breath sampling sessions were observed for breathing patterns. Each breath sample generated from the portable breath sampler took on average 5.39 ± 0.19 minutes (RSD = 3.49%) to collect while passing 2.52 ± 0.02 L (RSD = 0.70%) into the adsorbent trap. Average breath amplitude was calculated at 1.71 V ± 0.68 V (RSD = 39.8%). The average exhaled sample comprised of 89 ± 33 breaths (RSD = 37.2%) with a breathing rate of 17 ± 2 breath per minute (RSD = 38.8%) and frequency of 0.058 ± 0.006 minute per breath (RSD = 58.4%). Statistics summarising breath amplitude, breath rate and numbers of breaths are dependent on the physical status of the participant; hence significantly greater variation was observed for these factors compared to the sampling volume and duration.

Breath samples were also collected from the same 10 participants using original breath sampler. The breath profiles were analysed and compared to those collected from the portable breath sampler. Table 4.5.4.2. illustrates comparison of breathing patterns of the same 10 participants collected from two different type of sampling devices. Using paired two-tailed t-test demonstrated no significant changes in breathing profiles can be observed with calculated p value at 0.20 (P = 0.05). Thus showing high degree of similarity between the breathing patterns using two type of sampling devices. Figure 4.5.4.2. shows an example of breath profile observed from one of the healthy participants.

The 12 common airborne VOCs compounds from the 12 participants were analysed. The signal responses for these 12 compounds were hugely varied between the participants. The RSD% ranged from 28.2% to 165.7%. Trimethylbenzene had the lowest RSD of 28.2% and while 1,4-dichlorobenzene had the highest RSD of 165.7%. Additionally many compounds were recovered from one participant but completely absent in the next sample. The significantly varying RSDs between the participants is to be expected due to individual absorption and elimination metabolic rate. These values are summarised in Table 4.5.2.1.

**Table 4.5.4.2. Comparison of breath profiles of 12 participants generated from portable breath sampler and original adaptive breath sampler**

| | Portable breath sampler | | | Original adaptive breath sampler | | |
|---|---|---|---|---|---|---|
| Breath profile statistics | Average | Standard deviation | RSD% | Average | Standard deviation | RSD% |
| Total sampling time (sec) | 323.2 | 11.3 | 3.5 | 321.6 | 14.5 | 4.5 |
| Total sampled vol. (L) | 2.5 | 0.0 | 0.7 | 2.5 | 0.0 | 0.6 |
| Total number of breath | 89.0 | 33.1 | 37.2 | 86.2 | 33.2 | 38.6 |
| Breathing rate (Breath per min) | 13.9 | 5.4 | 38.8 | 13.8 | 5.6 | 40.5 |
| Single breath duration (min) | 0.09 | 0.05 | 58.4 | 0.09 | 0.04 | 50.5 |
| Average breath amplitude* | 1.71 | 0.68 | 39.84 | 1.87 | 0.82 | 43.80 |

*Average breath amplitude is the average height of exhaled breath measured from the baseline

A comparison between breath samples taken from portable breath sampler and original adaptive breath sampler shown that no significant difference between the two methods. Paired two-tailed t-test was performed based on the two set of signal responses of 12 VOCs compounds from the two samples, p value was calculated at 0.96 (P=0.05).

Overall the analysis of breath profile and breath VOCs samples have shown great system stability and reproducibility (RSD of 3.49% for breath collection duration) , thus demonstrating that the augmented portable breath sampler could be deployed in large scale breath VOCs studies for extensive sampling period. Additionally when compared with the original adaptive breath sampler, the two datasets have shown no significant difference, since the original adaptive breath sampler has already been successfully deployed in numerous large scale clinical studies, the comparable datasets the portable breath sampler can generates indicates that it can successfully replace the current system with no impact on data quality.

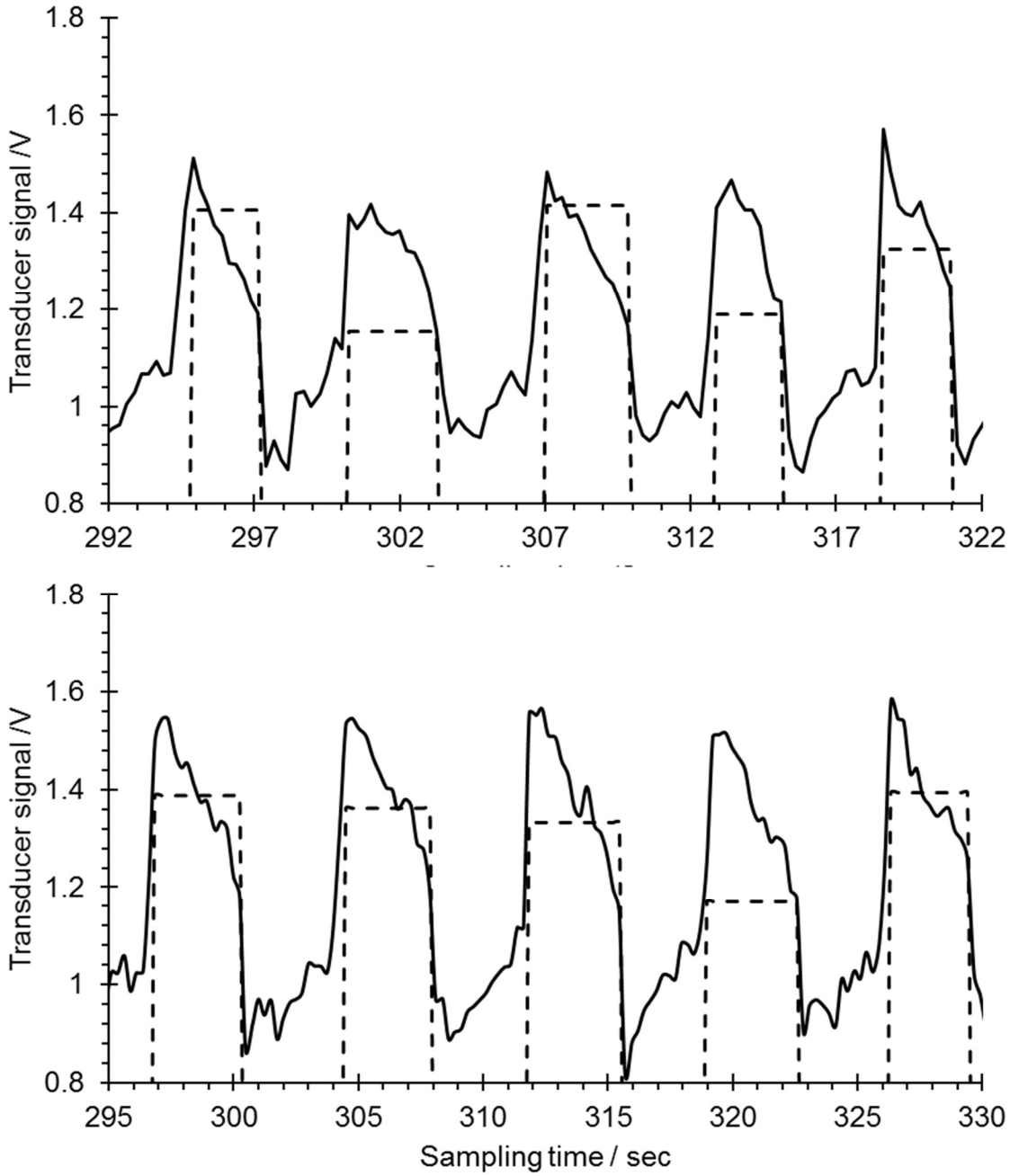


**Figure 4.5.4.2. An example of breath profile from a healthy participants collected from portable breath sampler (Top) and original adaptive breath sampler (Bottom)**

## 4.5.5. Breath reproducibility study

The stability of portable breath sampler was determined by assessing the reproducibility of 10 replicates breath sample collected from the same participant within a short time frame. The 10 replicates breath samples were collected in 1.5 hr and the participant reported no changes in the breathing experienced during this period. The air supply had a stable flow rate of 40 L $min^{-1}$ throughout. Each sample took on average 5.47 ± 0.03 min (RSD 0.47%) to collect an average sample volume of 2.51 ± 0.01 L (RSD 0.27%). Breathing rate was stable

at 22.8 ± 1.4 (RSD 5.39%) breath per min with total number of breath per sample at 135.4 ± 3.7 (RSD 2.72%). High level of reproducibility can be observed from breaths profile of the 10 replicate breath samples, thus determines that the portable breath sampler can be used reliably to collect large amount of breath samples with high degree of stability.

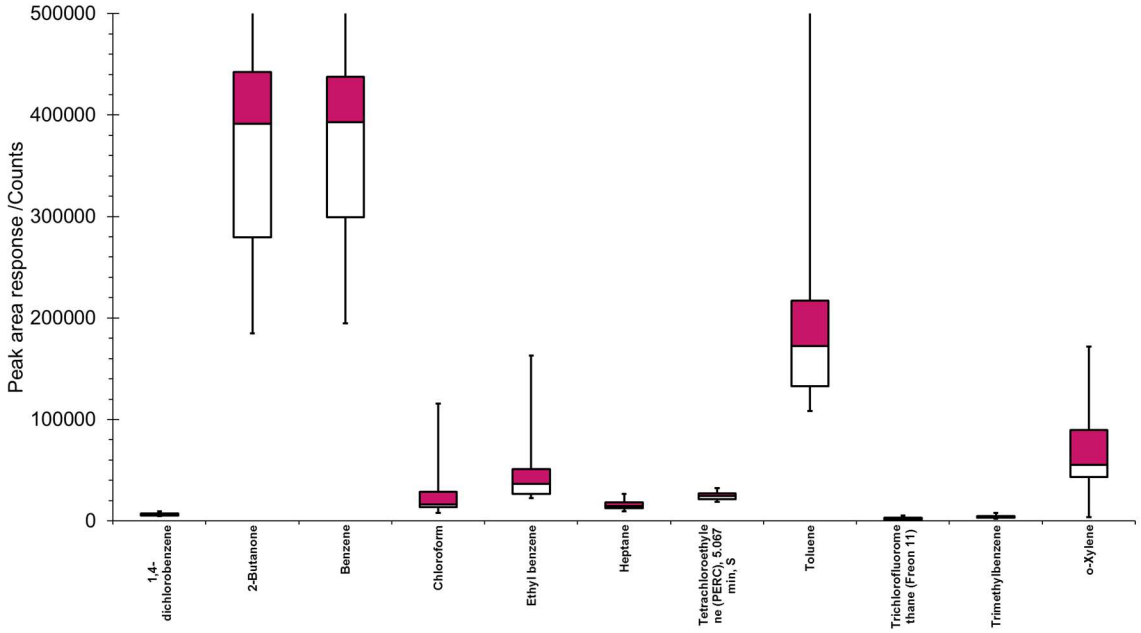


**Figure 4.5.5. The variability of 11 common room air contaminants found in breath sample using the portable breath sampler.**

The 12 common airborne room air contaminants in the 10 replicates breath samples were analysed. The average RSD variation between the 12 compounds was determined to be 34.6%, however there is a significant RSD variation between the 12 compounds ranging from 16.2% for tetrachloroethylene (PERC) to 111.8% for toluene. Trichlorofluoromethane (Freon 11) level was not detected in most breath samples. Compounds with the lowest variation were tetrachloroethylene (PERC) (16.2%), acetone (20.8%) and 1,4-dichlorobenzene (23.6%). Compounds with the highest variation were chloroform (110.8%), toluene (111.8%) and ethyl benzene (85.5%). The RSD variation of signal responses for different compound species is potentially due to varying wash out rate for different compounds, certain exogenous VOCs would be absorbed, metabolised and eliminated out of system slower than others leading to those compounds level during sampling to vary more significantly.

## 4.6. Summary

A portable breath sampling system was designed and developed from the original adaptive breath sampler. Its concept originated from the limitation where breath VOCs studies could only be carried out in specific locations. This significantly hindered participant recruitment,

especially in clinical facilities where participants may have difficulties reaching designated facilities. The earlier design of the adaptive breath sampler required space and time for initial set up, and most importantly it required a medical air supply. These requirements have been found difficult to meet in many facilities, so this work investigated and explored the different aspects required to provide a portable breath sampling system to achieve reproducible and standardized breath samples.

In order to replace a static air generator with a wall mounted drying and purification assembly, a smaller bespoke air supply unit was developed built and tested. It was portable and delivered up to 40 L $min^{-1}$ and required regular regeneration. A thermal desorption regeneration procedure for the purification media was developed and successfully tested.

The capability of the portable breath sampler to dehydrate and purify air supply was also investigated. The VOCs adsorption rate of its air purification unit based on signal responses from 12 common airborne room air contaminants was measured to be approximately 59.5%. While its air supply dehydration rate was measured to be 0% which is complete dryness for up to 4 hours. Maximum sampling period of around 4 hours was measured based on maximum air dehydration capacity of molecular sieve 13X desiccant filter.

After rigorous testing of portable breath sampler, it was then trialled with human participants. In participant experience feedback, no participants reported any discomfort or difficulties during breath sampling session. The stability of portable breath sampler was tested when 10 replicate breath samples were taken from the same individual continuously for extended sampling period, reproducible breath profile and signal responses from targeted VOCs were achieved. The results from 13 participant's trial study had shown more variation for both breath profiles and targeted compounds responses. This variation between participants can be down a number of factors. Such as individual metabolic adsorption and elimination rate for VOCs, their lung capacity or breathing actions. However when compared with their counterpart results collected using original adaptive breath sampling system, the two datasets were highly comparable ($p = 0.96$), this indicates the enhanced portable breath sampler can achieve the same level of breath sampling capability and reproducibility.

The portable breath sampler described was subsequently used in breath VOCs studies at various locations (chemistry laboratory, swimming pool side and sports centre laboratory) with participants with asthma, see Chapter 5. It is important to note that the portable breath sampling system described here is only the first prototype, and it can be improved further. One aspect is the limit of an air supply flow rate of 40 L $min^{-1}$. Although this flow rate is enough for relaxed breathing at rest, a higher flow rate is required for breath sampling during exercise when the breathing rate is much higher; elite athletes will breath at up to

300 L $min^{-1}$. Another aspect that could be developed further is the size of the current system. The current system can be wheeled to a remote location for sampling or transport to further afield. It is much too big to be incorporated with a handheld sampler. Further redesigns to the next generation prototype should be focused on reducing its size and weight further to make it even more portable. The goal is a clinically compatible user friendly adaptive breath sampler for diagnostic purposes that can be used anywhere enabling research in breath analysis in a much wider range of locations.

# CHAPTER 5: VOCS PROFILING OF EXERCISED INDUCED BRONCHOCONSTRICTION (EIB) IN YOUNG ATHLETES USING PORTABLE BREATH SAMPLER

## 5.1. Introduction

### 5.1.1. Brief description of exercise-induced bronchoconstriction (EIB)

Exercise-induced bronchoconstriction (EIB) is a common pulmonary disorder characterized by airflow obstruction occurring after several minutes of physical activity. Typically short period (4 – 10 minutes) of intense exercise can trigger constriction of bronchial smooth muscles typically 5 to 15 minutes following the completion of the exercise. It may also occur after 30 minutes of continuous exercise. EIB is recognized as an indirect measure of non-specific bronchial responsiveness and severity of asthma. It has been used as an epidemiological tool for diagnosing asthma. Common symptoms of EIB may include coughing, wheezing, chest pain and shortness of breath.

EIB is a common form of asthma and occurs in 70% to 80% of untreated asthmatics and 35% to 40% of those with allergic rhinitis/hay fever symptoms if challenged under appropriate exercise conditions. This can represents approximately 12% to 15% of populations. Exercise is the most common trigger of bronchospasm in asthmatics with 50%-90% of all individuals with asthma have airways that are hyper-reactive to exercise.

EIB can also occur in up to 10% of the population who are not known to be asthmatic, and up to 40% of children with documented EIB have no symptoms of asthma. The health consequence of unrecognized or inadequately treated EIB are significant. In a review of over 30 years of Isreali military recruit data, it was found that asthma was the main risk factor for unexplained death (85). Another research has identified 61 deaths was caused by asthma occurring in close association with sporting event or physical activity (86). Out the 61 deaths, 81% occurred in subjects who were younger than 21 years of age and 57% occurred in subjects who were considered to be elite young athletes. It was also found that 10% of these deaths occurred in subjects who were not known to be asthmatics. These research suggest that all individuals participating in organized sports should be made adware the risk of EIB.

### 5.1.2. EIB in athletes

The prevalence of exercise related bronchospasm in athletes range from 11% to 50% (87), and up to 90% of subjects with asthma with have EIB (88). Recent research has demonstrated EIB is also commonly found in elite sports athletes. Athletes who compete in high ventilation or endurance sports are more likely to experience symptoms of EIB

compared to low ventilation sports. Athletes in endurance sports such as cross country skiing, swimming and long distance running with long period of elevated ventilation are especially prone to EIB (89). EIB is also prevalent in winter sports which is thought to be due to cooling of airway at high ventilation rate for extended period of time (90). One study found that 18% to 26% of Olympic winter sport athletes and 50% of cross-country skiers were shown to have EIB (90). Another study has shown out of a group of both asthmatic and non-asthmatic 50 elite summer athletes studied, 50% were found to have EIB. The US Olympic Committee reports an 11.2% occurrence of EIB in all athletes who competed in the 1984 summer Olympics (91).

Correctly diagnosing and treating EIB in elite athletes is essential as it can have significant impact on their performance and health. Inappropriate treatment of EIB can lead to tremor, tachycardia, increase in EIB, poor response to inhaler and even death. From a performance point of view, EIB can significantly reduce their exercise capacity and speed. However, accurate diagnosis of EIB in athletes has proven to be difficult in the past, mainly because spirometry is poor indicator of EIB in athletes and physicians therefore rely heavily on athletes' own symptoms description to form diagnosis. But the relationship between symptoms and objective evidence of EIB was found to be poor in athletes leading to many misdiagnosis. Eucapnic voluntary hyperpnoea (EVH) using dry air was proven to be the most sensitive and accurate bronchial provocation test to identify EIB in elite athletes. One study has found that out of 65 football players with physician diagnosis of asthma/EIB, only 51% of these subjects tested had a positive bronchial provocation test result, thus demonstrating the difficulty of accurately diagnosing EIB in athletes.

### 5.1.3. Origin of EIB

The first theory regarding EIB was proposed in the 1970, it suggest that respiratory evaporative water loss induced airway cooling trigger bronchoconstriction (92). Later theory suggests EIB is the result of rapid warming of airway at end of exercise (93). However other studies have demonstrated the dehydration of airways is the main contributing factor to EIB (94) (95).

The physiological response to asthma exacerbation which includes a cascade of mediator events leading to smooth muscle hyperresponsiveness, mucus hypersecretion, and changes in mucociliary function. Antigen introduction into the pulmonary system stimulates the releases of mast cells, eosinophils, leukotrienes, prostaglandins, T lymphocytes, and other cellular mediators, leading to the physiological stress of bronchoconstriction. EIB may be unique in its pathophysiological response to exercise. During exercise, catecholamine

release initiates a bronchodilatory response in both healthy subjects and asthmatic patients. The results include progressive bronchoconstriction (96).

### 5.1.4. Pathology of EIB

The pathogenesis of EIB is associated with following factors: minute ventilation ($V_E$) (tidal volume times the respiratory rate per minute), the temperature and humidity of the inhaled air, and the baseline airway reactivity of the patient. Airway obstruction is more pronounced when the inhaling air is dry and cold, and less pronounced when the air is warm and humid. High levels of $V_E$ is required for more vigorous exercise and is associated with a greater degree of airway obstruction. Therefore the degree of $V_E$ and climate conditions associated with specific sport is considered the difference in asthmogencity. Sports such as golf, baseball and boxing is considered of low asthmogenicity. High asthmogenicity sports include downhill skiing, basketball, cycling and football. EIB sufferers are recommended to carry out activity of low asthmogencity associated with a warm and humid climate condition. Patients carrying out higher asthmogencity activity associated with colder and drier climate conditions would be more prone to EIB requiring medical intervention.

### 5.1.5. Cardiopulmonary exercise testing (CPET) and assessment of EIB

Cardiopulmonary exercise testing (CPET) is non-invasive and sensitive stress test that is widely used to assess the limits and mechanisms of exercise tolerance of human body. The test participant participating a CPET test will be required to perform exercise challenge in a controlled manner to measure oxygen uptake ($V_{O2}$), carbon dioxide output ($V_{CO2}$), ventilation, breathing pattern as well as other pulmonary and cardiovascular functions. CPET assesses profile and performance of the responses of the heart, lungs and muscles individually as well as in union. Pulmonary and cardiovascular conditions can cause symptoms such as shortness of breath and exercise intolerance, thus can lead to reduction in lung, heart and muscles responses during CPET testing. It can be used for the diagnose conditions such as exercise induced bronchoconstriction/asthma (EIB/EIA) and cardiac ischeamia. CPET can be used to provide information on dysfunction, monitoring and prognostic value in a wide range of other conditions such as chronic lung diseases and cardiac diseases. In recent years, CPET has become a popular testing method in pulmonary medicinal field due to its non-invasive nature, wide range of clinical applications and additional information it provides compared to lung function testing at resting state (97).

The aim of CPET is to induce exercise stress to organs involved in the exercise. The exercise chose for CPET must therefore involve exercising large muscle group. The exercise usually used for CPET is running on a treadmill or by cycling on an ergometer. However, cycle ergometer is generally the preferred method for CPET due to a few reasons; It is

easier for attach a breathing valve to participant's mouth or nose during exercise. Cycling on ergometer introduces less movements and noise making measurements easier. Also the work rate is largely dependent on body weight. The key requirement for exercise used for CPET is that the exercise stimulus must be quantifiable and is directly proportional to participant responses measured. For ergometer cycle, the workload can be added on incrementally as a stimulus to increase pulmonary and cardiovascular responses such as heart rate and breathing rate.

There are two types of standard clinical exercise protocols that is widely used for CPET; One is incremental test and other one is constant work rate exercise testing. In incremental exercise testing, the exercise stimulus is increased continuously or uniform amount each time for a set duration. The aim is to provide a smooth gradational stress to participants so the entire exercise can be carried out in a short period of time. For constant work rate exercise testing, the exercise stimulus is increase continuously before constant steady state exercise for a set duration. Constant work rate exercise testing is useful for measuring steady state responses at a moderate intensity work rate. At a high intensity work rate, it can be used to assess whether the exercise testing is sustainable by the participant. For the assessment of EIB, constant work rate testing is preferred to achieve a high level of ventilation to trigger bronchoconstriction.

## 5.2. EIB study design

Exhaled breath collected from alveolar region contains endogenous VOCs contains aerosolized airway lining fluid and volatile compounds that provide non-invasive indications of biochemical and inflammatory information. Volatile exhaled breath compounds had been used as biomarkers of lung inflammation. Potential correlation between biomarkers of EIB between systemic circulation and those found in exhaled breath had been indicated in recent research. A panel of healthy athletes and EIB prone athletes participate in an intense exercise challenge to investigate cellular & molecular mechanisms of EIB. In order to achieve comprehensive CPET, an array of additional testing including blood, nitric oxide, and spirometer will be used to combine different aspect of interest together to explore biochemical and inflammatory information of EIB. The hypotheses is that CPET will trigger bronchoconstriction in EIB positive participants which will lead to a change in VOC profile compared to healthy participants post exercise.

**Table 5.2. List of testing for EIB challenge**

| Number | Name | Run time /minute | Description |
|---|---|---|---|
| 1 | Breath VOC collection | 5 | Exhaled breath VOCs collection from alveolar region |
| 2 | EBC collection | 5 | Exhaled breath condensate collection at -10℃ |
| 3 | Breath temperature measurement | 3 | Measurement of exhaled breath temperature |
| 4 | Nitric oxide measurement | 10 | Exhaled breath nitric oxide measurement |
| 5 | Blood sample collection | 3 | Collection of blood to be analysed for airway inflammation, remodelling and gene information |
| 6 | Respiratory muscle pressure test | 5 | Testing for muscle stress |
| 7 | Breath by breath analyser measurement | 8 | Measurement of participant CO2, O2, and lung function during exercise challenge |
| 8 | Spirometry | 10 | Lung function test |

The exercise testing used for the assessment of EIB is high intensity ergometer cycling at a constant work rate in order to achieve a high ventilation rate. The constant work rate exercise protocol adopted in this study is a slightly modified version of the recommended procedure (97). The procedure consists of a rapid increase in work rate within 3 to 5 minutes to 85% to 95% of the participant's maximal heart rate, and sustaining the work rate and 85% to 95% maximal heart rate at the same work load for 6 minutes. The recommended procedure suggests 4 minutes of high intensity constant work rate exercise, this has been extended in our protocol to 6 minutes to increase the probability of triggering EIB. The workload (W) of ergometer is used as the exercise stimulus to increase work rate. At the first, second and third minutes of cycling the work rate was set at 60%, 75% and 90% of target value. In EIB positive participants, the $FEV_1$ (forced expiratory volume in 1 second) generally falls to minimum level within 10 minutes after exercise, with substantial recovery to the baseline level by 30 minutes. To assess the participant response to exercise, forced spirometry measurements are to be performed before and then at 2, 5, 7, 10 and 20 minutes after exercise. The post exercise $FEV_1$ is subtracted to baseline $FEV_1$ pre-exercise and

expressed as percentage of pre-exercise $FEV_1$. A greater than 10% fall in $FEV_1$ post exercise is regarded as abnormal and greater than 15% fall is regarded as diagnostic of EIB. The work flow chart for constant work rate exercise testing and forced spirometry measurements is shown in Figure 5.2.1.

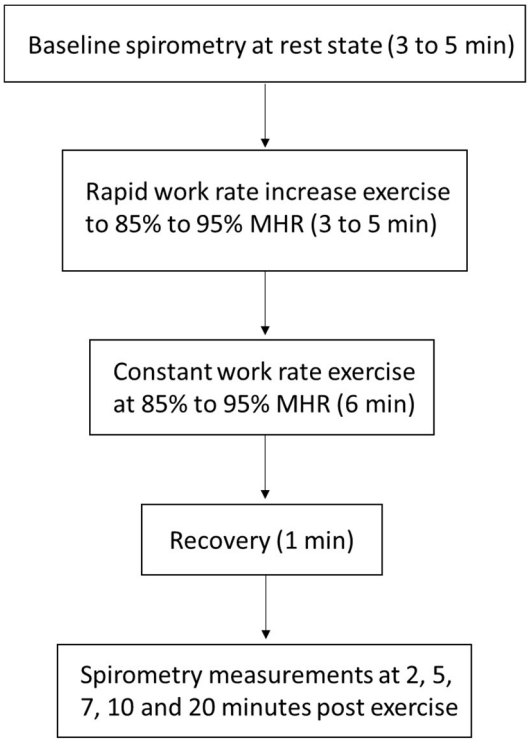


**Figure 5.2.1. Constant work rate exercise testing and diagnostic spirometry using an electric cycle ergometer**

A key aspect of the CPET is the interpretation of gas exchange measurement. The conventional technique of measuring gas exchange involves directing the expired air into a collection bag. $V_{O2}$ and $V_{CO2}$ values are then measured and calculated from the oxygen and carbon dioxide mixed gases in the bag. In recent years, on-line digital computer analysis of physiological transducer signals has become widely used, it has become practical to measure $V_{O2}$ and $V_{CO2}$ values breath by breath. The breath is broken down into a number parts and $V_{O2}$ and $V_{CO2}$ values were computed for each interval. Individual intervals are added together over the expiration to compute total volume. The main advantage of digital breath by breath analyser is high resolution compared to bag collection where gas exchange can only be measured every couple of minutes. Although bag collection is still a much cheaper way of measuring gas exchanges.

Breath by breath analyser is only capable of detecting oxygen and carbon dioxides species in the expired breath. Separate analysers can be added on for measuring other gas species. Nitric oxide (NO) is widely reported as marker of exercise stress which can be monitored by breath analyser. During exercise stress, endothelial cells produces vasodilator and

vasoconstrictor compounds such as NO for the regulation of vascular tone. Other exercise and asthmatic species needs to be monitored by more complex means of measurements. A wide range of exhaled breath VOCs relevant to exercise such as isoprene can be monitored using TD-GC-MS method. Other metabolic fluids such saliva and blood collected pre and post exercise are used to monitor other large non-volatile species.

Other non-compound specific breath measurements were also carried out, respiratory muscle strength can be assessed by measuring maximal inspiratory pressure, it determines the strength of diaphragm and other inspiratory muscles. It can be used to establish any respiratory muscle weakness caused by EIB. Participant breath temperature before, during and after exercise challenge can also be measured. The hypotheses is that participant suffering from EIB can cause elevation in breath temperature. Core temperature is measured before and after exercise challenge.

A total of 8 analysis are to be carried out on the participant before, during or after exercise. Due to the high number of analysis, it is essential to have comprehensive planning and time management to keep total experimental time to a minimum to avoid excessive strain on the participant. A complete EIB time schedule is shown in Figure 5.2.2. The estimated total experimental time for one participant is around 1 hour 30 minutes. However, actual testing time can actually go up to 2 hours per participant. The testing setup time prior to start of the experimental is estimated to be around 3 hours. This study will focus mainly on participants exhaled breath VOCs profiles from before and after exercise challenge.

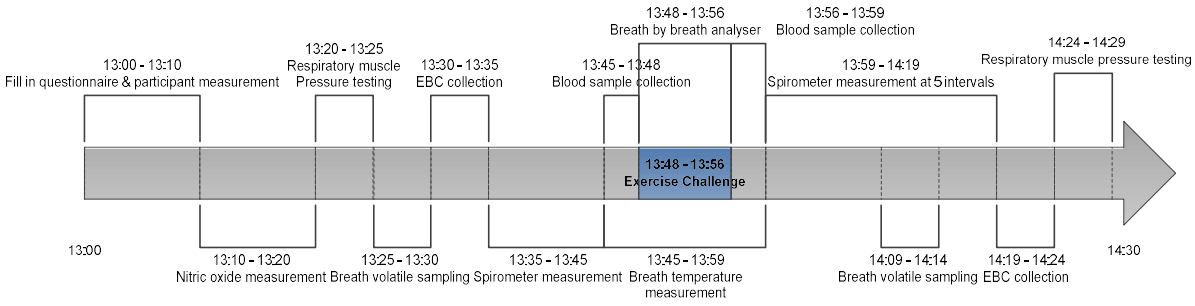


**Figure 5.2.2. Participant schedule for EIB ergometer exercise challenge with estimated total duration of 1 hour 30 minutes**

## 5.3. Experimental

### 5.3.1. Ethics

The study was conducted in accordance with ethical principles of good clinical practise and Declaration of Helsinki. All research protocols used in this study was reviewed and approved

by the Loughborough University ethics advisory committee; Exhaled breath VOCs sampling protocol G09-P5, Exercise challenge protocol G09-P8, Spirometry protocol G99-P5 and Ventilated gas volume measurements protocol G04-P2.

### 5.3.2. Participant preparation

All participants were given information sheets explaining the study when they were first recruited. Before the start of study, the participants were given healthy screen questionnaire, exhaled breath collection questionnaire and consent form to fill in. For participants with history of asthma, an additional asthmatic questionnaire was also filled in to assess the severity of their asthma. The participant information sheet and questionnaires used in this study are attached in the Appendix section.

Twelve male and twelve female participants aged between 19 and 23 years participated in the study. Nine participants have reported to be suffering from asthma. All participants were hockey or rugby athletes who trained regularly on a daily or weekly basis. Some of the participants which including healthy and asthmatic participants have reported to have experienced EIB during outdoor training sessions.

All participants were also asked to avoid any medications, alcohol, caffeine and abstain from strenuous exercise for 24 hours prior to the start of the procedure. Asthmatic participants were asked to bring rapid acting inhaled bronchodilators to reverse a severe bronchospasm episode.

Participants were also asked to wear sportswear or loose cloth for the exercise challenge. Just before the procedure, participants were asked to have a short warm up session.

Prior to the start of the procedure, all participants had their weight, height and body temperature measured. A heart rate monitor with wet electrodes were fitted around participant's chest.

Room air temperature, humidity and pressure were also measured and recorded.

### 5.3.3. Clinical exercise challenge and ventilation measurement protocol

A sport cycle ergometer (Lode Excalibur, Netherlands) was used to conduct the exercise challenge. It uses an electromagnetic eddy current braking system and is intended to be used as a stress test device in a medical environment. It can measure workload (W), Revolutions per minute (rpm), time (min and sec) and distance (km). The testing capacities of the Ergometer include a workload range between 8 to 2500 W, minimum load increments of 1W, maximum continuous load of 1500 W and pedal speed range between 25 to 180 rpm. The seat height and handle bars were adjusted to the correct level for individual participant.

Breath by breath analyser (Ultima CPX™ metabolic stress testing system, MGC Diagnostics, USA) was used in this study for cardiopulmonary exercise (CPX) testing. CPX measures the body's ability to perform gas exchange during an stress test by measuring the performance of the respiratory, cardiovascular, and circulatory systems all in one test. This testing detects the causes of symptoms such as shortness of breath, chest pain, and fatigue. The key component of a CPX test is the measurement of instantaneous gas exchange-oxygen uptake and carbon dioxide output of each breath. The concentration of these gases is measured by in-built oxygen and carbon dioxide analyzers. The volume of air moving in or out of a person's lungs per minute (minute ventilation or $V_E$) is measured by a flow sensor or volume-measuring device. These signals are then phase-aligned by the computer to produce breath-by-breath measurements of gas exchange.

The breath by breath analyser and its pneumotach was calibrated prior to start of the procedure to ensure stable signal value and breath measurements. A clean pneumotach was connected to the breath by breath analyser via an umbilical line (Figure 5.3.3.). The pneumotach was fitted into participant’s mouth and its head straps were fitted on participant’s head. The participant was asked to wear a nose clip during the procedure to ensure mouth breathing.

After the participant is ready to start, he was asked to first to gently pedal for one minute with low work load specific for that individual. A timer with 10 minutes count down was started, workload was increased by 1kg every 30 seconds according to participant’s heart rate. The target was gradually increase the participant’s heart rate to 85% to 95% of maximum heart rate (MHR) at around 4 minute. The participant’s $V_E$ and $V_{CO2}$ values were constantly monitored via breath by breath analyser throughout also used to assess workload increase. After participant’s heart rate has reached 85% to 95% of MHR, the workload was then kept constant for 6 minute. Throughout the exercise challenge, the participants were encouraged to keep their work rate (rpm) constant at around 60 to 70 rpm, participants were asked to give an exercise stress score from 0 to 10 every minute to assess their condition, if the participant feels too strained during the continuous exercise stage to maintain the same work rate, workload can be reduced to continue the exercise challenge.

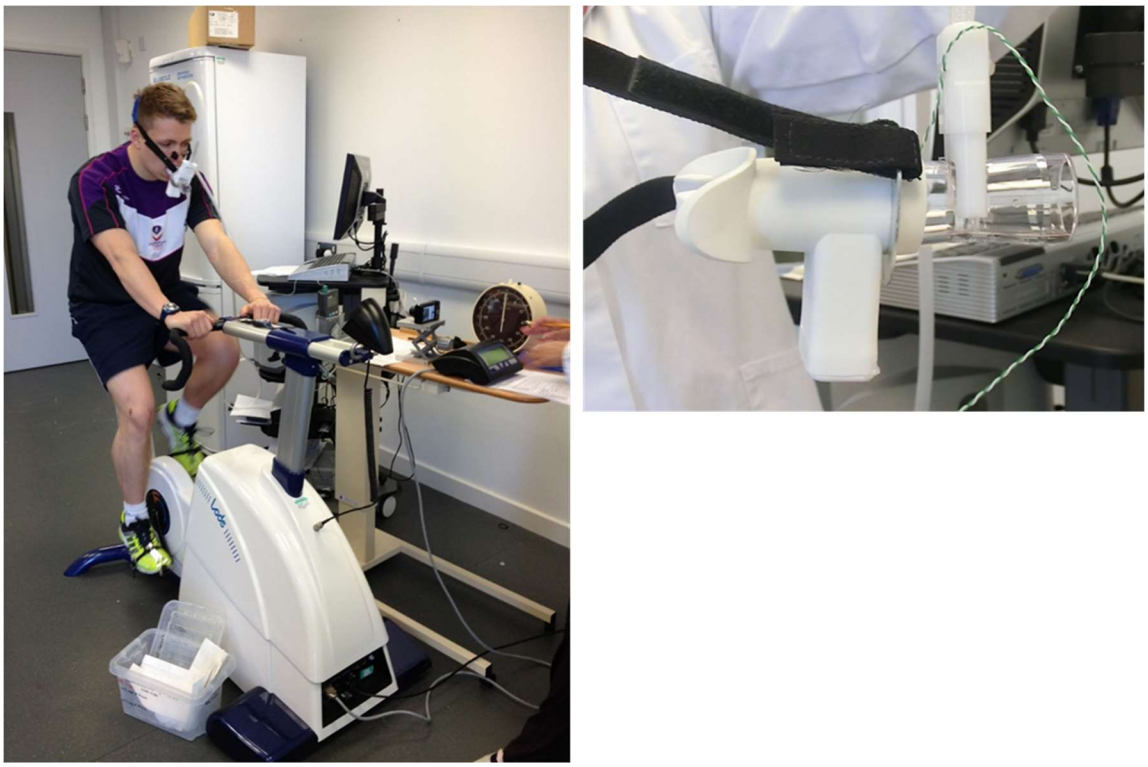

**Figure 5.3.3. (Left) Photo of participant during EIB ergometer exercise challenge (Right) Photo of breath by breath analyser pneumotach used by participant during exercise challenge**

## 5.3.4. Spirometry measurements

Micro Loop spirometer (CareFusion, USA) consisting of hand-held instrument, battery charger and turbine transducer was used to carry out spirometry during this study. The spirometer was calibrated on the day of measurement as per American Thoracic Society (ATS) guidelines for spirometry testing (98). A series of baseline lung function measurements was carried out prior to start of exercise challenge; Forced expiratory volume ($FEV_1$, L), forced vital capacity (FVC, L), percentage forced expiratory volume to forced vital capacity ($FEV_1$/FVC, %), peak expiratory flow (PEF, L $min^{-1}$), forced expiratory flow related to 25% to 75% of FVC curve ($FEF_{23-75}$, L $s^{-1}$), maximal voluntary ventilation (MVV, L $min^{-1}$). Participants were asked to wear nose clip and coached on the correct technique of forced spirometry manoeuvre. At least 3 repeat forced spirometry blows meeting ATS/ERS criteria were measured and recorded. The highest FVC and $FEV_1$ value from the 3 or more repeats was used as the baseline spirometric value.

After exercise challenge, participant was asked to repeat forced spirometry measurement at a series of time intervals; 2, 5, 7, 10 and 20 minutes. Same sets of pulmonary function measurements were taken as baseline measurement with 3 acceptable repeats blows. The highest $FEV_1$ and FVC was used at each time point.


**Figure 5.3.4. Photo of participant undertaking spirometry after exercise challenge**

### 5.3.5. Exhaled breath VOCs collection using the portable breath sampler

The portable breath sampling system was used to collect 2 L of participant exhaled breath onto multi-sorbent tube (Markes international, UK). The samples were collected from participants around 15 minutes before the start of exercise challenge and around 20 minutes after exercise challenge, and the sampling takes around 5 to 10 minutes. 2 L of air supply and background room air samples were also collected prior to the start of exercise challenge. The collected samples were stored at stored at 4°C fridge prior to sample analysis on TD-GC-MS. After sample analysis, thermal desorption tubes are conditioned at 300°C for re-use. The activated carbon and molecular sieve filters are conditioned at 200°C under high purity nitrogen. Breathing mask and other connections are cleaned and dried under vacuum before re-use. A detailed sampling work flow and procedure for exhaled breath VOCs collection using the portable adaptive breath sampler can be found in Chapter 4.

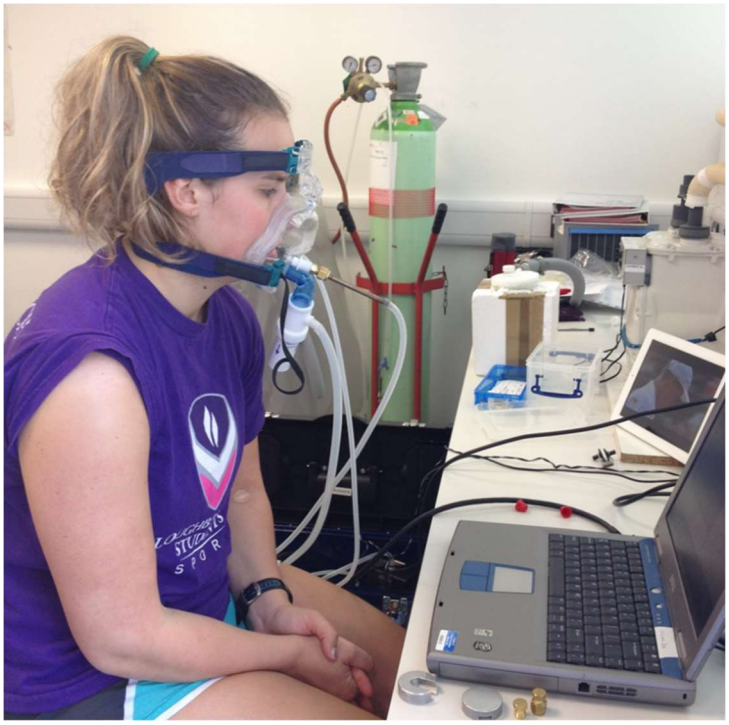

**Figure 5.3.5.1. Photo of exhaled breath VOCs sample collection using portable breath sampler**

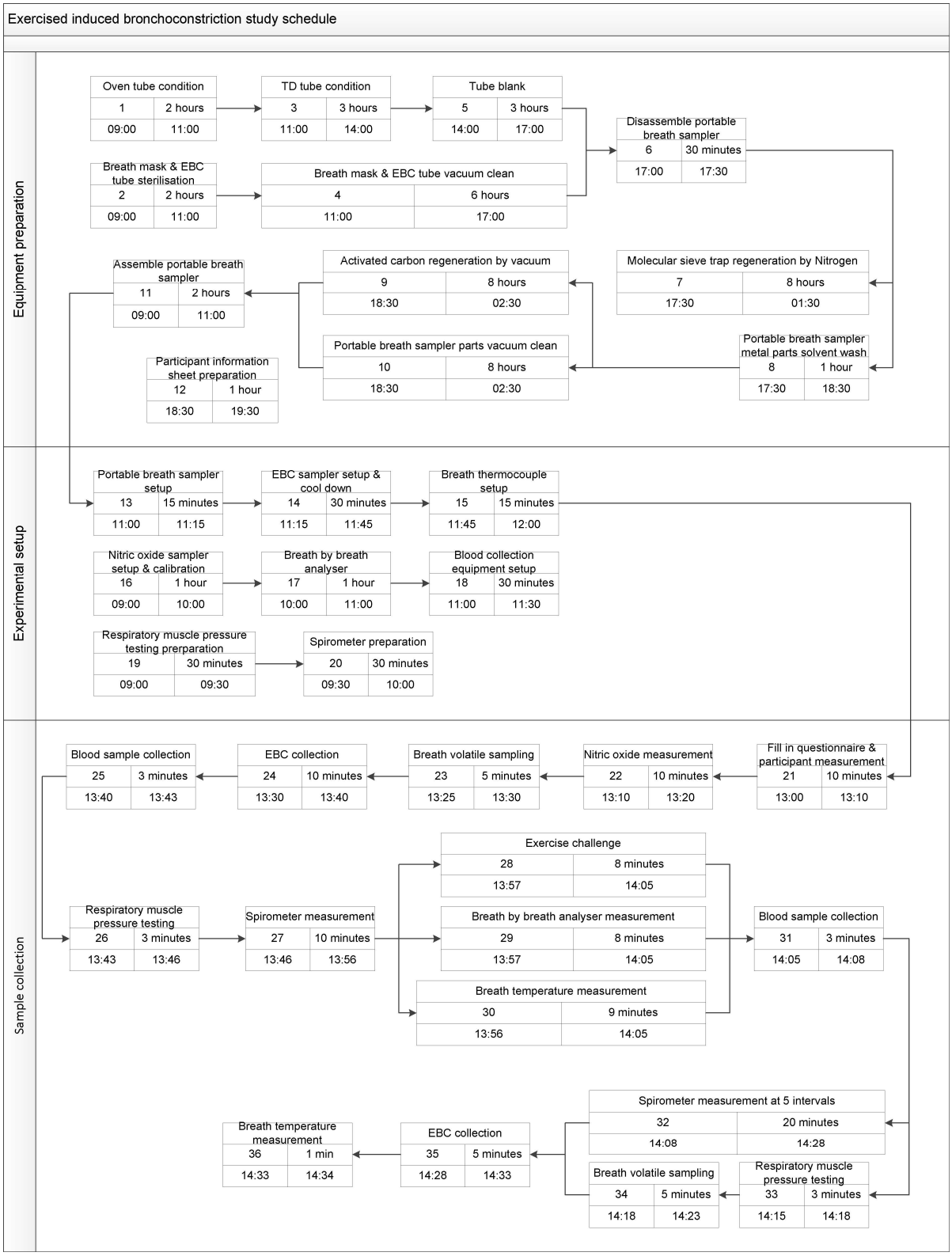


**Figure 5.3.5.2. The 3 stages of EIB study design plotted by PERT chart**

## 5.4. Results and discussion

### 5.4.1. Direct exhaled breath measurements

#### 5.4.1.1. Spirometry

Each participant is assessed by their percentage fall in $FEV_1$ post exercise ($\Delta FEV_1$). $\Delta FEV_1$ is calculated by taking the each participant's highest $FEV_1$ result from post exercise spirometry subtracted from the pre exercise baseline $FEV_1$ value. The difference is then calculated as percentage of the pre exercise $FEV_1$. At least 3 repeats forced spirometry blows meeting ATS/ERS criteria needs to be recorded at each time point. A greater than 10% fall in $FEV_1$ after exercise is regarded as abnormal, and a greater than 15% fall is diagnostic of EIB.

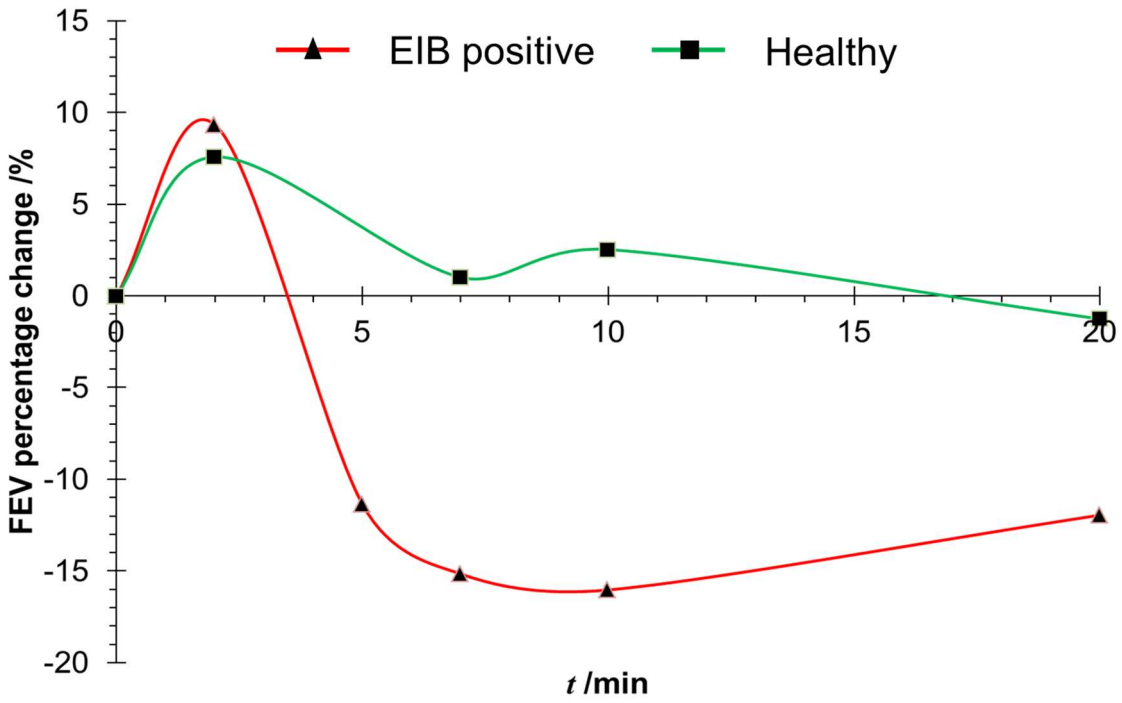


**Figure 5.4.1.1. $FEV_1$ percentage change pre and post exercise challenge between EIB positive participant (red line with triangle symbol) and EIB negative participant (green line with square symbol)**

Out of all 24 participants, 4 participants had shown a greater than 15% drop in post exercise $FEV_1$ and can be diagnosed as EIB positive. Out of these 4 participants, only 2 were reported to be asthmatic. One of the other 2 participants didn't report to be asthmatic, but had previous experiences of EIB symptoms during outdoor exercise. The last participant was

not reported to be asthmatic and has no previous experience of EIB symptoms, but did suffer from flu the previous week and had variable spirometry results, so this participant's result could be a false positive.

2 participants had shown a greater than 10% drop in $FEV_1$ post exercise and is regarded as potentially EIB positive. One of these participant is reported to be asthmatic and had often experienced EIB symptoms during outdoor exercise. The other participant wasn't asthmatic and had no previous EIB experience.

It to be noted that there are 6 asthmatic participants who had shown no significant $FEV_1$ drop post exercise. Some of them had reported to have had previous experience of EIB symptoms while exercising outdoor, thus could mean there are some false negative in the spirometry results.

When compared to EVH testing results that was carried out separately on a different day, there are 4 participants who had positive EVH results (greater than 15% drop in $FEV_1$ during EVH testing), but had negative spirometry results post exercise challenge. 3 out of the 4 participants who were diagnosed to be EIB positive from the exercise challenge were also EVH testing positive. The other EIB positive participant didn't take the EVH test, but was asthmatic and regularly takes bronchodilator inhaler before training to avoid EIB symptoms. This does seem to shown that it is possible the exercise challenge couldn't trigger EIB in some potentially EIB positive participants.

#### 5.4.1.2. Breath by breath analyser

The breath by breath analyser accurately measures the responses of $V_{O2}$, $V_{CO2}$ and $V_E$ from participant during exercise in every inspired breath, thus generates real-time comprehensive data for ventilation and gas exchange during exercise. Figure 5.4.1.2. demonstrates the ventilation rate, $V_{O2}$ work load and heart rate during the 10 minutes constant work rate exercise challenge. Participant carbon dioxide/oxygen output, ventilation rate is directly proportional to heart rate and work load during exercise.

Lung diseases usually causes an increase in ventilation rate and heart rate, therefore y comparing participants $FEV_1$ drop against heart rate and ventilation rate for any correlation. Participants who shown greater than 5% drop $FEV_1$ post exercise have an average ventilation rate of 127.3 L $min^{-1}$ and heart rate of 177.8 beats $min^{-1}$. Participant who shown no significant change in $FEV_1$ post exercise have an average ventilation rate of 126.6 L $min^{-1}$ and heart rate of 125.7 beats $min^{-1}$. It does seem to shown an elevated ventilation rate and heart rate in participants with significant drop in $FEV_1$ post exercise.

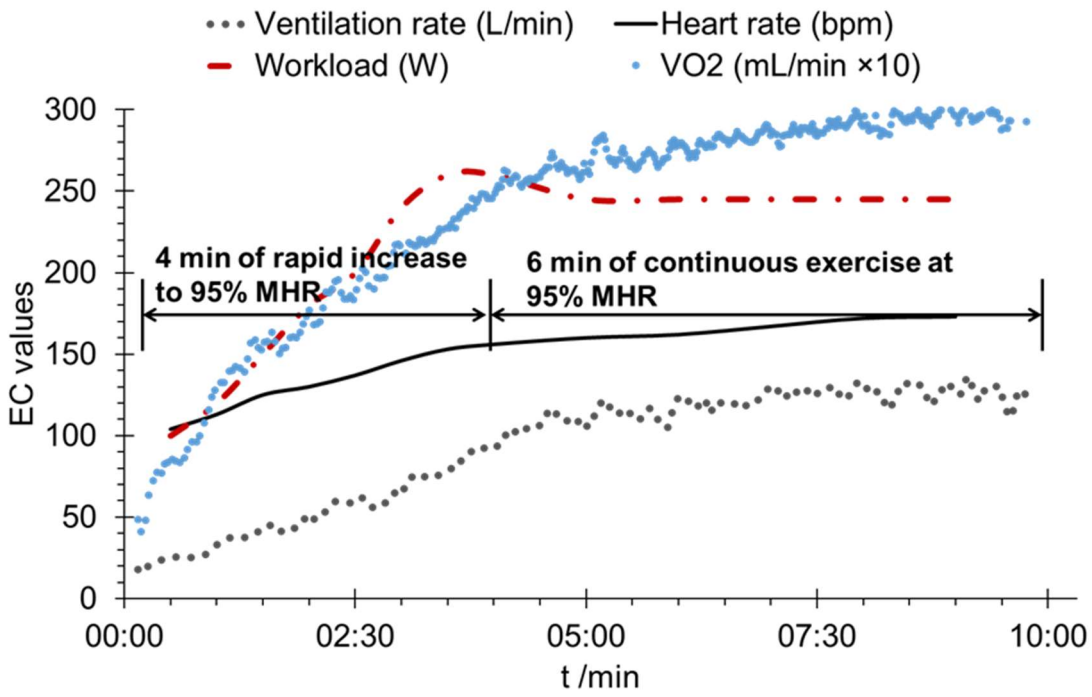


**Figure 5.4.1.2. Standard clinical ergometer exercise challenge to induce bronchoconstriction by maximising participant exercise workload (red line) to 95% of maximum heart rate (black line).**

## 5.4.2. Exhaled breath VOCs multivariate analysis (MVA)

### 5.4.2.1. Multivariate analysis

Exhaled breath GCMS data collected from the EIB study was first deconvoluted and retention indexed using AnalyzerPro (Spectralworks, UK) into breath components identified, all the found breath components from all participants pre and post exercise challenge were then pooled into a single breath components matrix separated by participants against identified breath components. Each breath component was referenced using unique breath library ID code consisting of its RI value and de-convolved mass spectrum, the breath library ID code was written in the format of BRI-XXX-YY-YY-YY-YY-YY, where XXX is the calculated retention index valve and YY is the 5 most abundant fragmentation ions found in its mass spectrum. Also, individual integrated peak area was generated for each breath component in the breath matrix. The breath matrix was then manually checked through against raw GCMS data to eliminate any replicates and noise data using Varian MS workstation software (Varian, UK). A more detailed description of data processing method used for exhaled breath analysis can be found in Chapter 2.

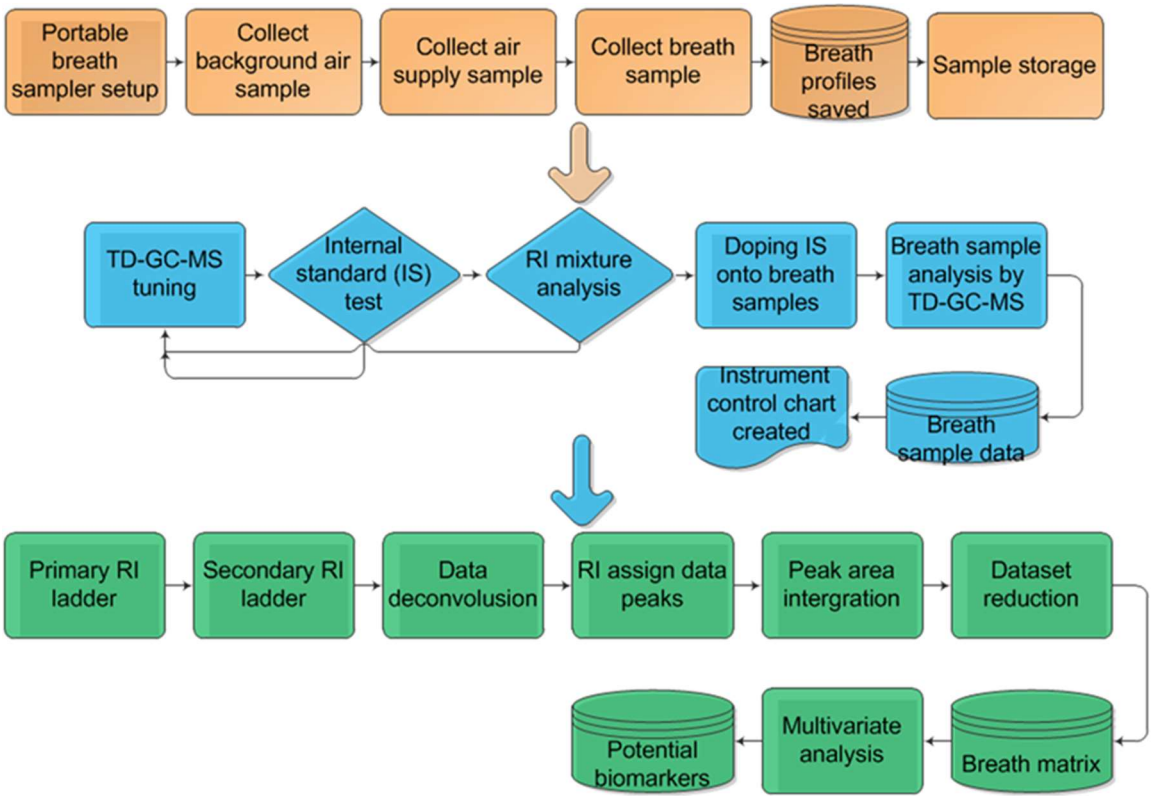


**Figure 5.4.2.1.1. Exhaled breath VOCs sampling work-flow using the portable breath sampler for EIB study**

The breath matrix constructed contained identified and peak integrated 692 breath components based on the 24 participants data pre and post exercise. Background air and air supply samples data were analysed using the same method as participant breath samples, the resultant background air matrix was compared against breath matrix to determine the origin of each individual breath component (See Figure 5.4.2.1.2.). If the breath component was to be found in the background air or air supply matrix to a comparable level, that specific compound was said to be an exogenous component that originated from a source outside human body. These compounds will be excluded from subsequent MVA. The breath matrix was separated into 226 endogenous and 466 exogenous breath components.

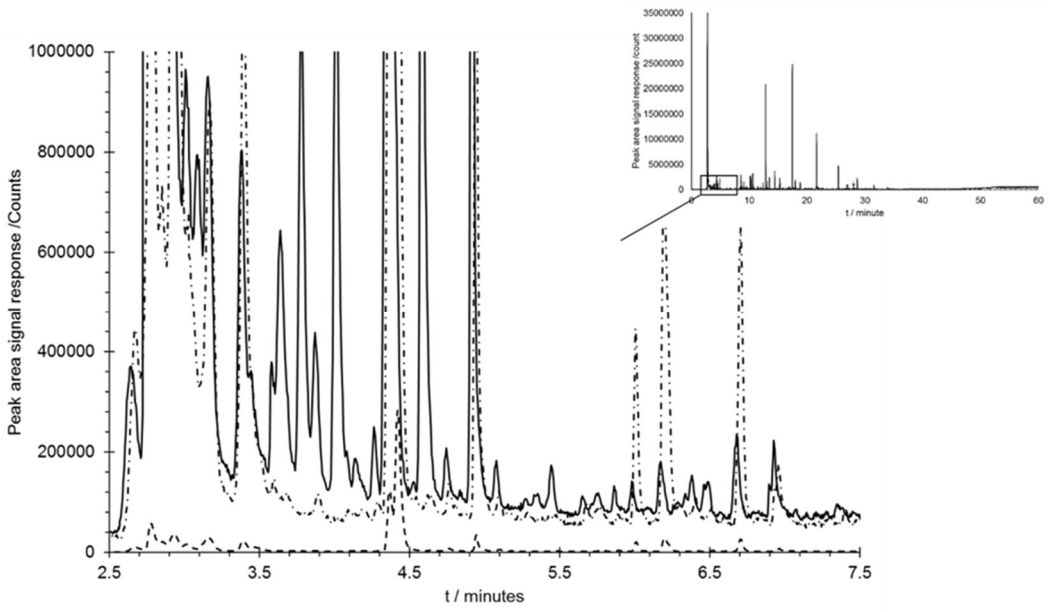


**Figure 5.4.2.1.2. TIC chromatogram of participant breath sample (bold line), purified air supply (dash line) and background room air (dash dot line) illustrating the comparison between endogenous and exogenous VOCs**

Multivariate analysis (MVA) was applied to the resultant endogenous breath matrix using data analysis software SIMCA-P+ (Version 12.0.1.0, Umetrics, UK). Each individual breath samples pre and post exercise challenge were assigned as observations and each breath components were assigned as X variables. Subsequently, all variables were assigned to single block weight of 1(1/SQRT) and Pareto base scaling (Par). Depending on the classification required, all breath samples were assigned individual classes prior to modelling, eg, to observe effect of exercise, pre and post exercises classes were assigned to each sample. Initial unsupervised principal components analysis (PCA) was then performed on the scaled breath matrix to determine the correlation between pre and post exercise challenge.

Exercised induced bronchoconstriction (EIB) is unique type of asthma in which the symptoms of bronchoconstriction is only onset by vigorous exercise. Many mild EIB sufferers aren't aware of the disease since there is no symptoms occurring in their daily lives. Unlike pure exercise or asthmatic studies where the MVA modelling is purely based on two distinct classes (ie pre or post exercise, healthy or asthmatic participants), the modelling for EIB study is dependent upon both exertion of exercise and participant health status. Therefore prospection for markers of EIB was focused on biomarkers that were onset after vigorous exercise challenge and in participants where the symptoms of EIB occurs. Two types of modelling were used to achieve this, initially unsupervised and supervised MVA modelling classed by pre and post exercise was used to determine correlation and upregulated

markers of exercise. Subsequent MVA modelling was applied to the small group of upregulated exercise markers to prospect for markers with high correlation between exercise and asthmatic symptoms hence potential markers of EIB. Upregulated biomarkers here refers to potential biomarkers that were produced in the human body as result of exercise. The concept of determination of EIB biomarkers is illustrated in Figure 5.4.2.1.3.

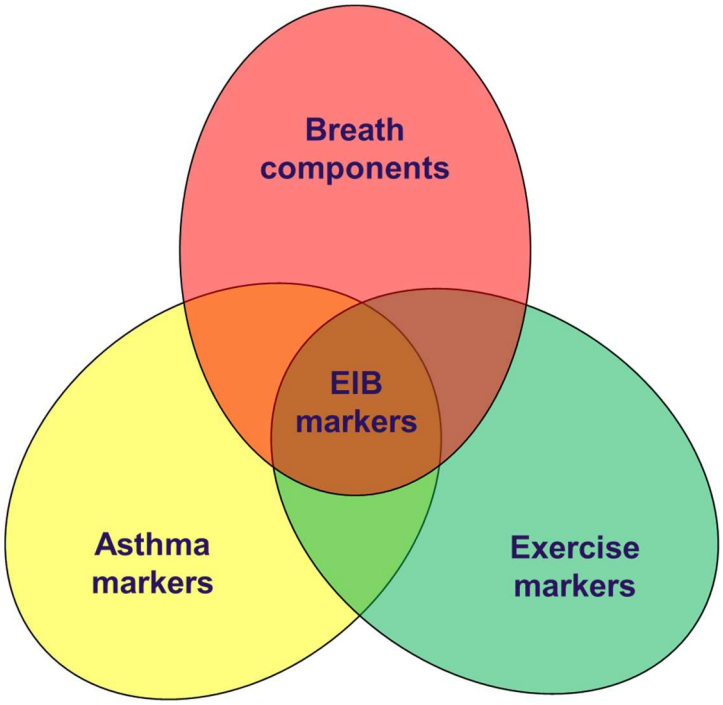


**Figure 5.4.2.1.3. The concept of determination of EIB biomarkers based on both exercise and asthmatic modelling**

## 5.4.2.2. The effect of exercise

Initial unsupervised PCA model classified by exertion of exercise challenge on participants using endogenous breath components had shown separation between the pre (blue dot) and post (green dot) exercise datasets (Figure 5.4.2.2.1.). In order to determine breath components which shown high correlation to exercise, supervised MVA modelling orthogonal partial least square – discriminant analysis (OPLS-DA) was applied to the breath matrix. In both the unsupervised PCA model and supervised OPLS-DA model, the pre and post exercise participants were separated through the first principal component. The unsupervised PCA consists of three principal components with combined $R^2X$ value (fraction of variability of the X variables explained by the PCA model) of 56.7% which explained majority of the data variability. The supervised OPLS-DA model due to discriminatory focus on exercise, has a reduced $R^2X$ value of 27.01% for the two principal components, with first the principal component (t1) which the exercise classification separates on showing a value of 9.81%. As shown from the supervised OPLS-DA model, pre-exercise participant dataset was dispersed on the right side of the scatter plot (blue dots) and post-exercise participants

dataset dispersed on the left side of the scatter plot (green dots) shown clear separation between the groups. One participant was out of the 95% confidence defined by the Hostelling region.

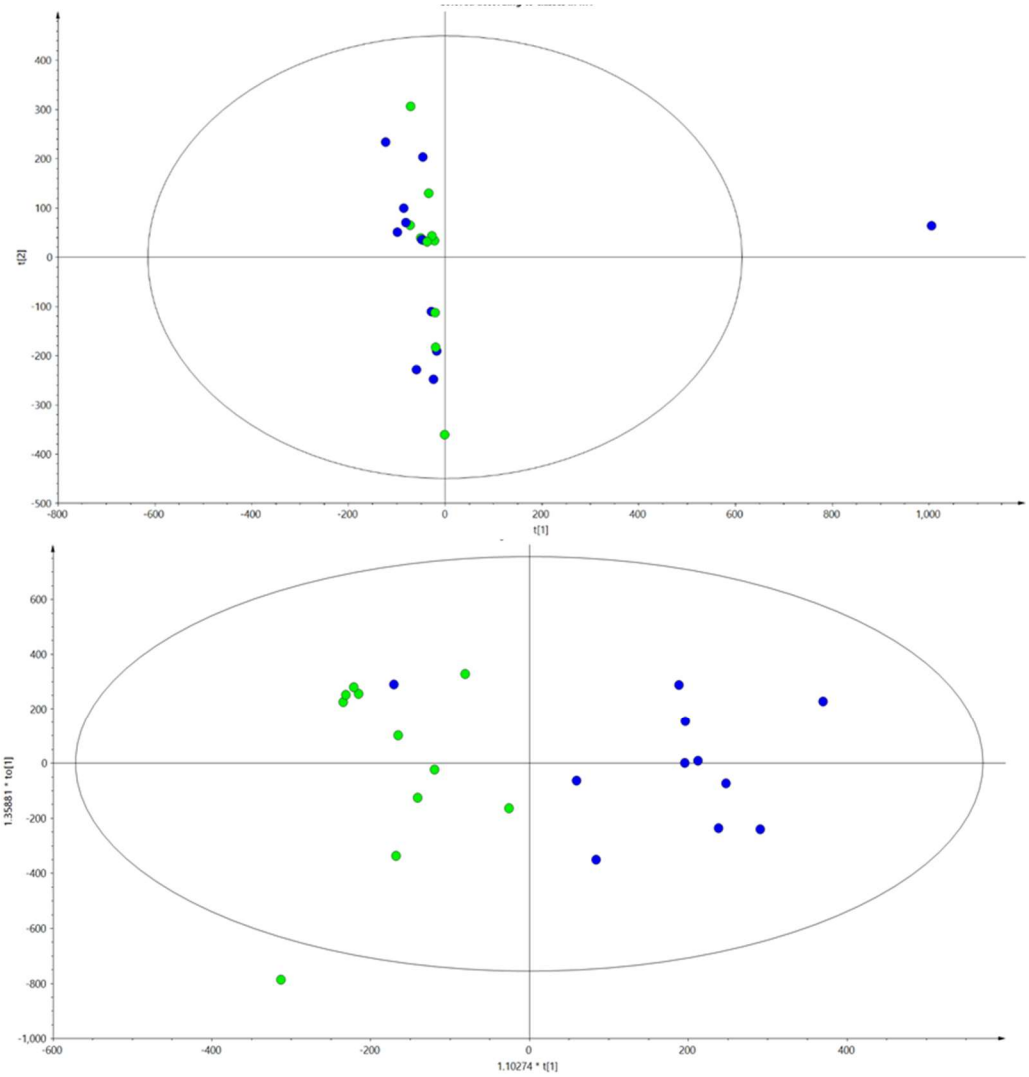


**Figure 5.4.2.2.1. (Top) Unsupervised PCA model (Bottom) supervised OPLS-DA mode are classified by exertion of exercise challenge on participants using endogenous breath components had shown separation between the pre (blue dot) and post (green dot) exercise datasets.**

After plotting the S-plot and loading plot for the supervised OPLS-DA model, a list of discriminating VOCs between pre and post exercise challenge can be determined (Figure 5.4.2.2.2.). The variables located on the right hand side of the S-plot with positive p1 (weight combining the original X variables to form t1) value correlates to pre-exercise challenge observations and the ones located on the left hand side of S-plot with negative p1 value correlates to post-exercise challenge observations. Variables located in the center of the plot with p1 value close to 0 has low correlations to both observations groups and doesn't

contribute to the first principal component. As observed from the S-plot, most of the discriminating VOCs that contribute to OPLS-DA model are compounds with high p1 value that correlate with pre-exercise observations, these compounds are potential downregulated biomarkers which has shown reduced levels after participant undergo vigorous exercise challenge. Most of these downregulated breath components are cyclosiloxane derivative as result of water content from participant breath, this shows participant exhaled breaths were much drier after exercise challenge. Although there are potential downregulated markers correlating to post-exercise observations, they are located much closer to the centre of the S-plot which means they shown lower correlations to observations compared to downregulated markers, hence they aren't as discriminatory. A list of top discriminating VOCs was created from the S-plot. The same unsupervised and supervised approach was repeated to determine the best discriminator of exercise.

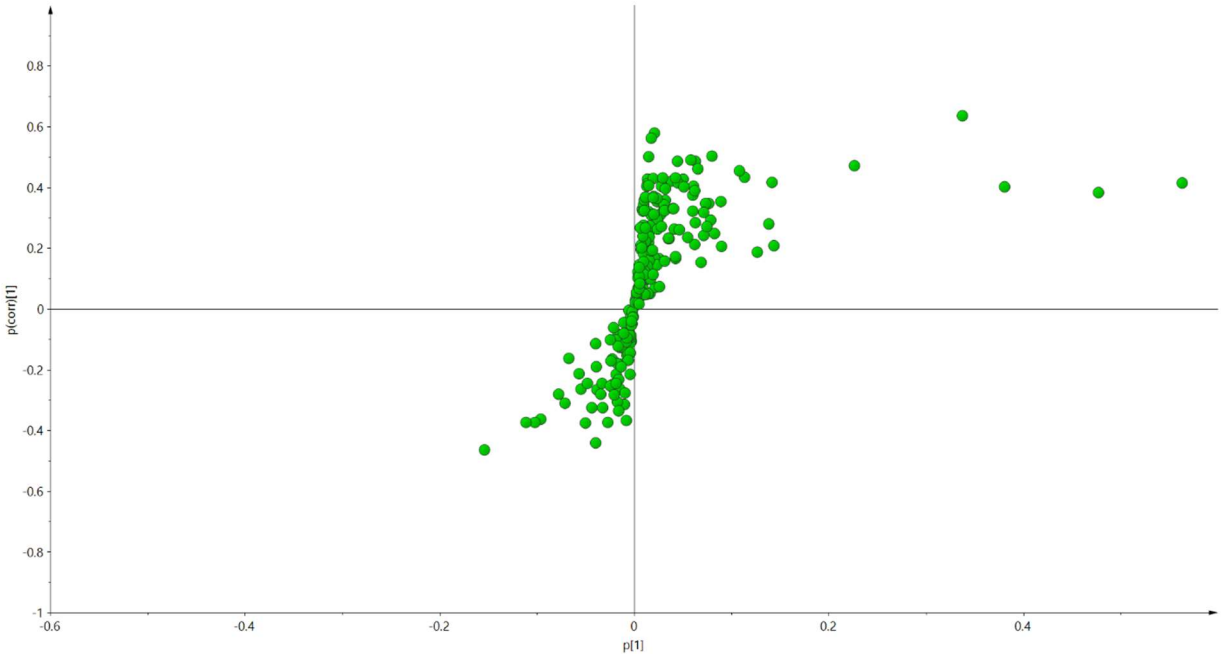


**Figure 5.4.2.2.2. S-plot and loading plot for the supervised OPLS-DA model, a list of discriminating VOCs between pre and post exercise challenge.**

A new PCA model with 4 variables (Figure 5.4.2.2.3.) was created, the unsupervised model shows a much clearer separation between pre and post exercise observations than the initial model through the first principal component. The first two principal components explained 76.6% of the data variability. All 4 variables used in the model are upregulated breath components that correlate strongly with post-exercise observations. This is demonstrated by the closely clustered pre-exercise observations and much more dispersed post-exercise observations demonstrating the much greater level of variability post participant undergoing exercise challenge.

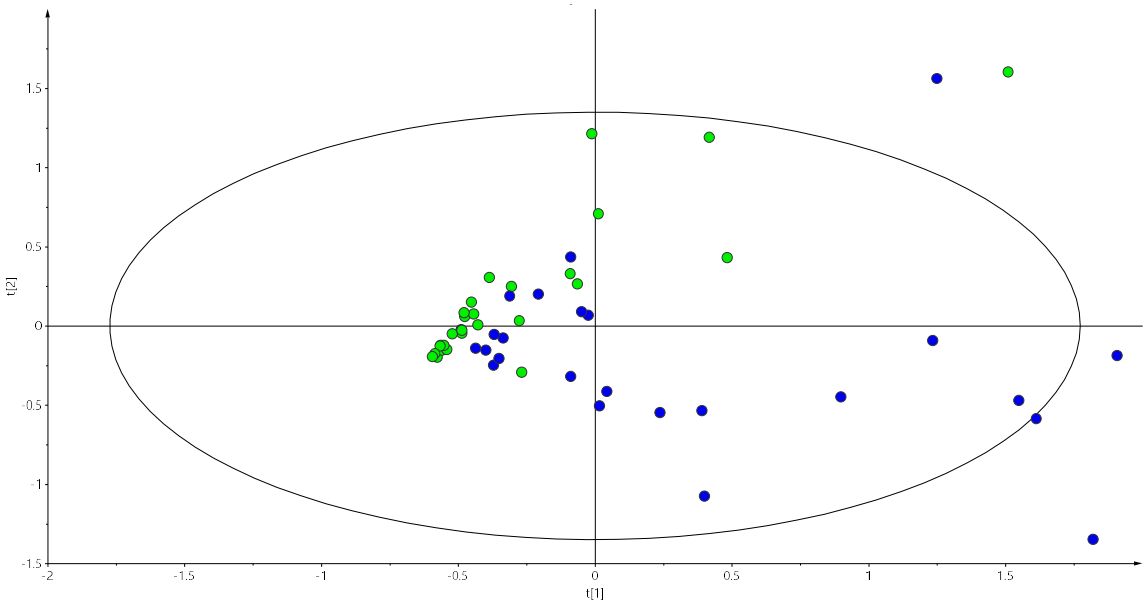


**Figure 5.4.2.2.3. Unsupervised PCA model with 4 most discriminating variables between pre and post exercise observations**

The list of potential markers of exercise is summarised in Table 5.4.2.2. All four breath compounds had shown high correlation to post-exercise observations and its spectra has been compared in NIST library for identification. However only compounds with forward and reverse library match score higher than 750 were identified in the summary table, compounds with low NIST library match score were only given proposed functional groups description based on the list of matches and its mass spectra. Out of the four compounds, butylated hydroxytoluene (BHT) and indole have been successfully identified with forward and reverse match of 835/835 and 893/893. The other two compounds were identified as terpene or ketone or benzoic acid derivatives.

**Table 5.4.2.2. Summary of upregulated markers of exercise**

| Name | Library entry | MS F/R | RI O/E | CAS No. |
|---|---|---|---|---|
| BRI-1568-205-57-220-206-145 | Butylated Hydroxytoluene | 835/835 | 1568 | 128-37-0 |
| BRI-1331-117-90-89-116 | Indole | 893/893 | 1331 | 120-72-9 |
| BRI-1537-161-203-175-218-189 | Terpene or benzoic acid derivative | N/A | 1537 | N/A |
| BRI-1449-147-119-91 | Methylated aryl ketone or benzoic acid derivative | N/A | 1449 | N/A |

Box whiskers plots summarising the difference between pre and post exercise dataset were shown in Figure 5.4.2.2.4. Although all four compounds are upregulated in the post-exercise participant gourp, out of the four compounds butylated hydroxytoluene (BHT) has shown to have highest weight value (*p1* = 0.63)) due to the significantly increased level after exercise

challenge. The unknown terpene or benzoic acid derivative (BRI-1537-161-203-175-218-189) has the second highest weight score (*p1* = 0.52), the compound was undetected in all but two participants pre-exercise data. The other upregulated breath component indole (p1 = 0.48) is present in majority of pre-exercise participant dataset, but a significant increase after exercise challenge can be observed for all participants. For the unknown methylated aryl ketone or benzoic acid derivative (BRI-1449-147-119-91), the increase in the post-exercise participant dataset is much less apparent compared to the other three compounds due to huge variation in the pre-exercise data. Example selected ion chromatograms of each upregulated discriminating VOCs that differentiate participants pre and post exercise challenge are shown in Figure 5.4.2.2.5.

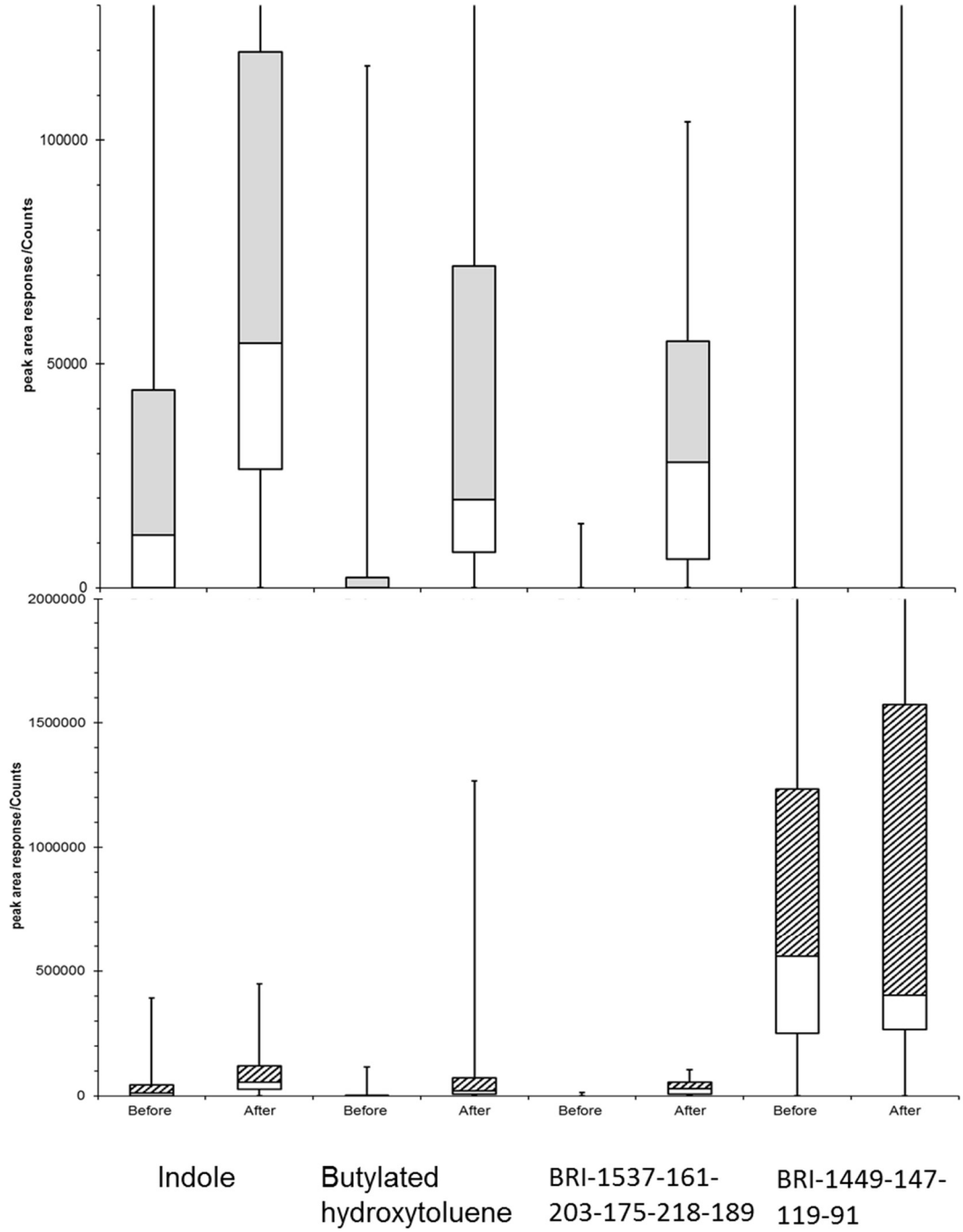


**Figure 5.4.2.2.4. Box whiskers plots summarising the difference in before and after exercise challenge datasets The top figure is the zoomed in version.**

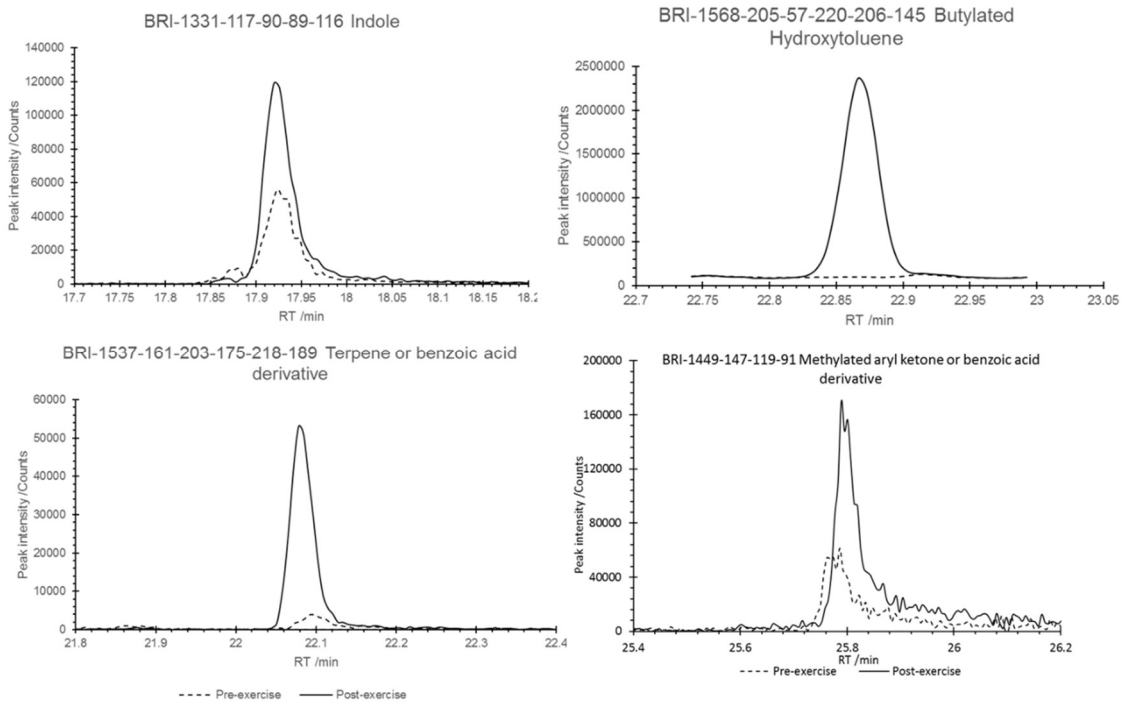


**Figure 5.4.2.2.5. The 4 highest discriminating VOCs for separating pre and post exercise participants.**

## 5.4.2.3. Markers of exercise induced asthma (EIB)

After establishing the discriminating VOCs determining the effect of exercise on participants, the 4 variables are then re-classed using forced expiratory volume in 1 second ($FEV_1$) measurements. As discussed in previous sections, $FEV_1$ values were measured using spirometer pre and post exercise challenge at fixed time intervals, percentage variation at $FEV_1$ value before and after exercise challenge was calculated. The lowest $FEV_1$ response recorded after exercise was subtracted from pre-exercise value and the differenced was expressed as a percentage of pre-exercise $FEV_1$ value. A drop in $FEV_1$ value of more than 10% is seems as abnormal and >15% drop can be diagnosed as EIB positive. Participants with a significant post-exercise >5% drop in $FEV_1$ value were separated into one class (red dot), participants with no significant $FEV_1$ drop (<5%) after exercise were separated into a second class (green dot).

A new unsupervised PCA model with new percentage $FEV_1$ drop classifications was created for both pre and post exercise (Figure 5.4.2.3.1.). As observed from the pre-exercise dataset, there is no clear separation between the EIB positive and healthy participants before exercise based on the 4 upregulated exercise-dependent breath compounds. For the post-exercise dataset, clustering of mostly healthy participant's data was observed onto the left side of the plot. The participants' data with significant $FEV_1$ drops and some healthy

participants data were dispersed onto right side of the plot. This difference shows there is correlation between the upregulated discriminating VOCs for exercise and EIB positive participant data. The specificity and sensitivity of the post exercise dataset only model was calculated at 68.8% and 50%. High rate of false negatives is common for EIB exercise testing in a laboratory, thus could be influenced by many factors during the exercise challenge, such as the duration and intensity of exercise challenge, the temperature and humidity of inspired air, and the time since last exercise induced airway narrowing all affect the percentage $FEV_1$ drop post exercise (24). It been reported that high intensity exercise with extended duration can also trigger the release of catecholamine and may induce bronchodilation resulting in false negative diagnosis (25). Previous journal has reported some participants who was involved in repeated exercise challenge on different days, but was diagnosed negative for one test and positive for the second one, which indicates the level of variability participant is responding to duplicate exercise challenge (99).

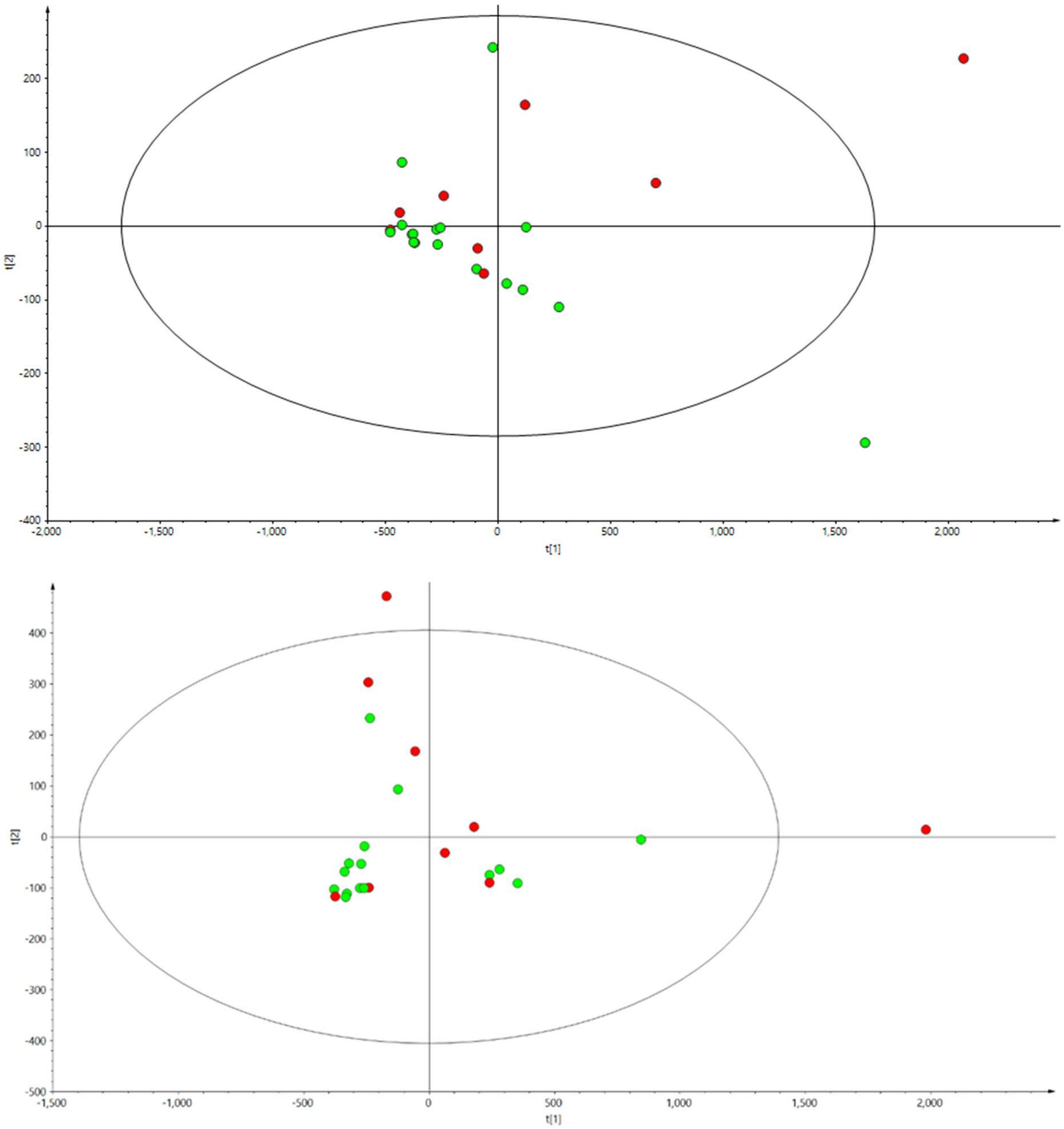


**Figure 5.4.2.3.1. PCA of 4 markers of exercise listed in previous section re-classified using spirometry result and meta data from participants, (Top) pre-exercise (Bottom) post-exercise**

It is observed that 6 participant datasets with no significant difference after exercise were mixed with the participants with high $FEV_1$ drop on the scatter plot, as well as two participants with significant $FEV_1$ drop clustering with healthy participants. A closer look at the participants' metadata show some of the participants with no post exercise $FEV_1$ drop were diagnosed to be asthmatic or were shown to have significant $FEV_1$ drop during EVH testing, this indicate that some of the participants who had shown no significant $FEV_1$ drop

could have shown signs of bronchoconstriction post exercise at physiological and biochemical level, but it was not detected by spirometry measurements.

By combining different types of breath diagnostic measurements performed on participants and their metadata from participants healthy questionnaires. The dataset was re-modelled on 4 classes (Figure 5.4.2.3.2.): participants who were shown >5% drop in $FEV_1$ post exercise (red dots), participants who shown significant $FEV_1$ drop (>10%) during EVH testing (blue dots), participants who have indicated they been diagnosed with asthma in the past (yellow dots) and participants who were negative of three previous classes (green dots). The combination of different diagnostic data were used to reveal if there is any possible mild lung inflammation which was not significant enough to be detected by spirometry, but were detected in other forms of testing. EVH testing is widely regarded as gold standard for diagnosing EIB, the participant breathes in cold and dry air at very high ventilation rates which simulates outdoor sports conditions better than indoor exercise challenge, hence more likely to detect EIB positive participants.

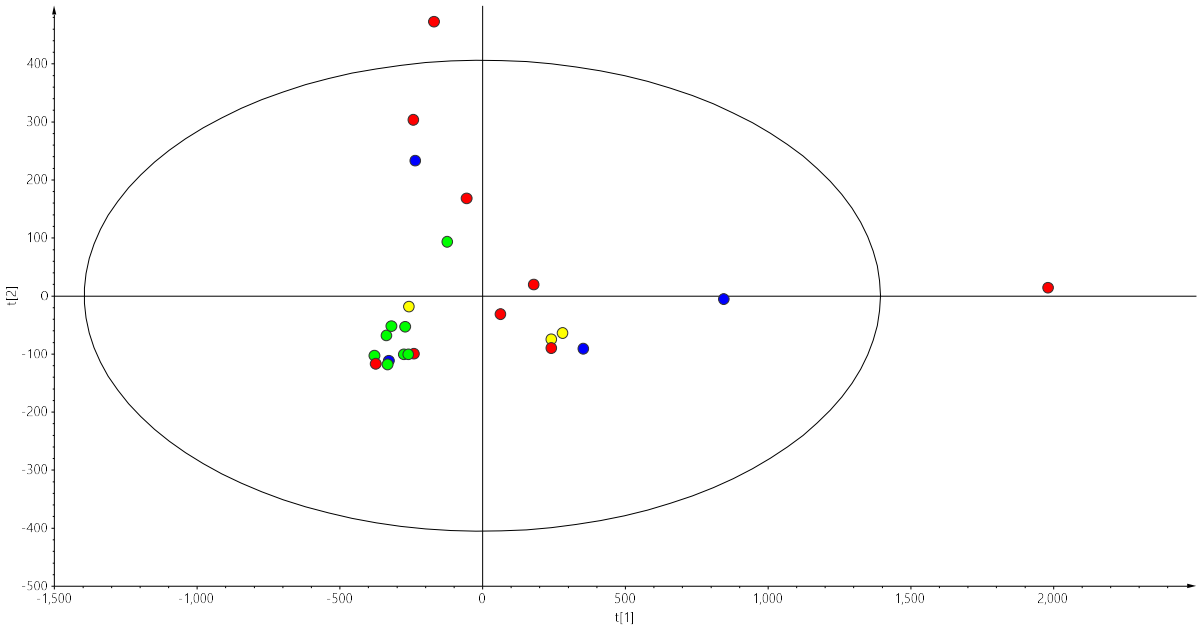


**Figure 5.4.2.3.2. Unsupervised PCA model with combined diagnostics; participants who were shown >5% drop in FEV1 post exercise (red dots), participants who shown significant FEV1 drop (>10%) during EVH testing (blue dots), participants who have indicated they been diagnosed with asthma in the past (yellow dots) and participants who were negative of three previous classes (green dots).**

For the new PCA model with combined diagnostics, the specificity and sensitivity of the model was calculated to be 50% and 87.5% respectively. Compared to the spirometry only model, the sensitivity of the model has increased by 37.5% after EVH and past medical

asthma diagnoses were added, but specificity has reduced by 18.8%, this indicating there is indeed possible mild asthmatic symptoms after exercise challenge that was not detected by spirometry. This could be due to a number of reason; The exercise challenge although was vigorous enough to increase participant heart rate to 95%, but the indoor condition was too warm and humid to trigger significant bronchoconstriction, the average room temperature and humidity was measured at 21.5 ± 0.92 °C and 33.5 ± 14.3% for all participants, although the variation to temperature was not significant, significant variability to room humidity can be observed with minimum humidity measured at 22% and maximum humidity at 65%, the temperature and humidity directly affects the stress level in the airway, hence the prevalence of EIB is higher among winter athletes compared to summer athletes. The indoor air where exercise challenge took place was air conditioned, but humidity of the room couldn't not have been controlled. Research has demonstrated that the temperature of inhaled air was less important, and no difference in response between cold and dry air challenges, but EVH testing which has been the gold standard analysis does specify the use of dry air that contained eucapnic carbon dioxide content for inducing asthma, therefore it is possible the variable humidity in the testing room could have made triggering significant asthma difficult after exercise challenge, but during EVH testing, the condition of the inhaled air was much more controlled was able to trigger an asthmatic reaction for the same participant.

As shown by the loading plot for combined diagnostics PCA model (Figure 5.4.2.3.3.), the unknown methylated aryl ketone or benzoic acid derivative was found to have the highest weight value (p1 = 0.99) for the principal component (t1), its location toward lower right hand side corner shows it is strongly correlated to EIB positive participants. Both indole and BHT also show positive correlation towards EIB positive participant's datasets post exercise, but they also show significantly reduced level of correction compared to the unknown ketone or benzoic acid. The other unknown terpene or benzoic acid derivative which had been the highest discriminating VOC for the exercise model, is now shown to have a negative weight value, this indicates it had no correlation towards EIB positive datasets.

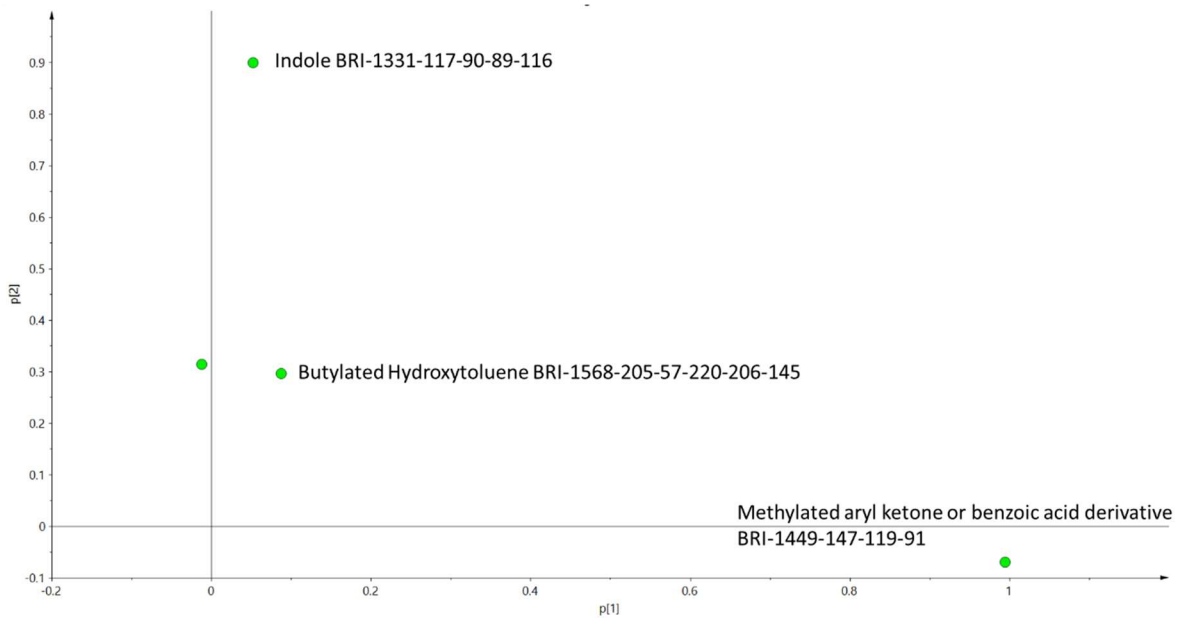


**Figure 5.4.2.3.3. Loading plot for combined diagnostics PCA model**

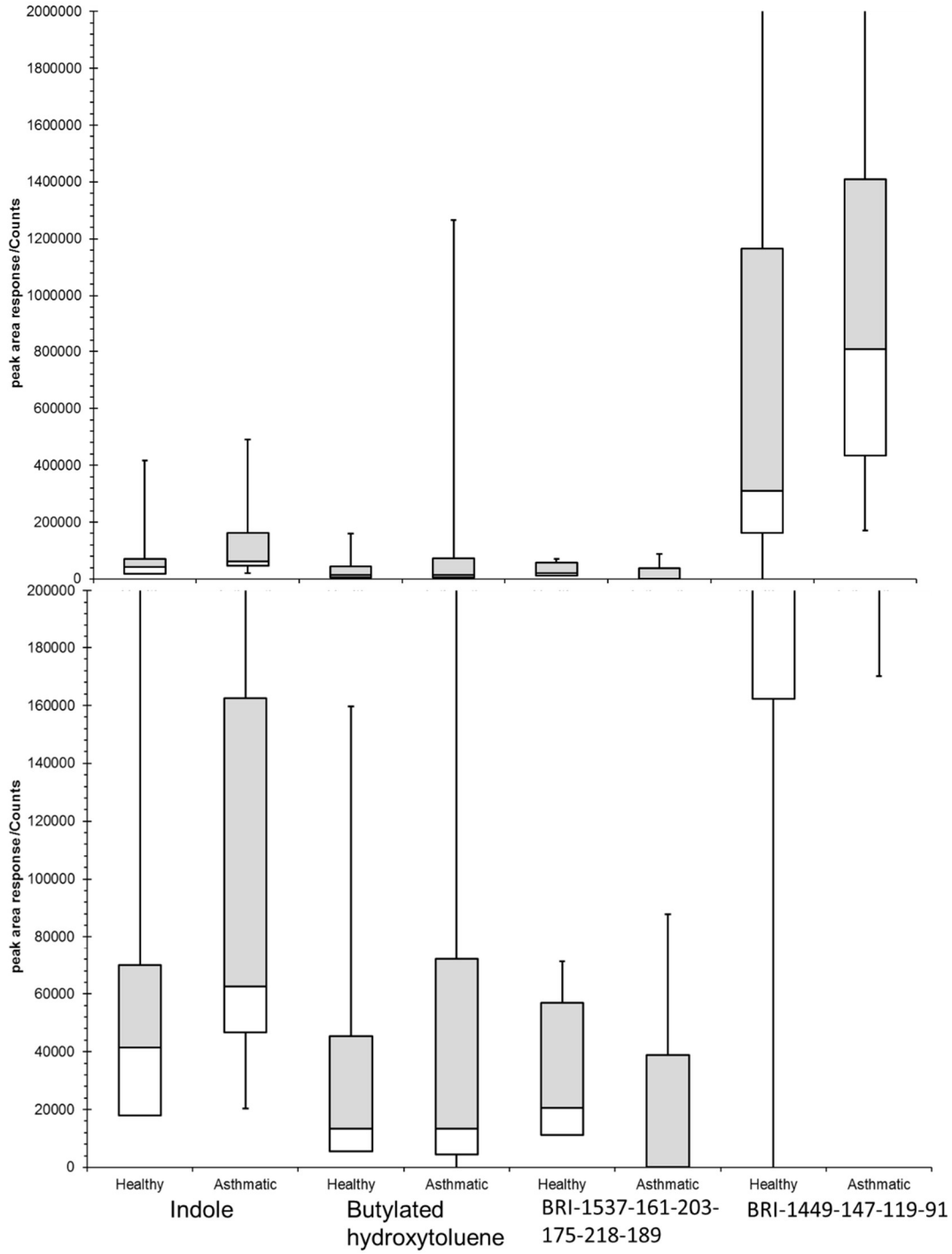


**Figure 5.4.2.3.4. Box whisker plots of the four variables for separating healthy and participants with significant drop in $FEV_1$ post exercise.**

### 5.4.2.4. Biomarkers origin

All four compounds were found to have high p1 value in the MVA for separating pre and post exercise participants, but only indole, butylated hydroxytoluene and unknown ketone or benzoic acid derivative have shown positive p1 value when used in the PCA for separating healthy and EIB positive participants.

Indole is an aromatic heterocyclic organic compound. It has a bicyclic structure, consisting of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring. It is a naturally occurring compounds that can be found in many types of organisms including bacteria, plants and animals, and its signalling activity is quite dynamic in various organisms. It is an intercellular signal molecule found in many types of Gram-negative and Gram-positive bacteria regulating a variety of bacterial physiological activity including extracellular signal, drug resistance, virulence control and biofilm formation. Indole is precursor for formation of amino acid tryptophan in bacteria which is essential protein for many organisms including humans and the precursor of neurotransmitter serotonin (100). In the human body, it is believed that indole are produced by the enteric intestinal bacteria, and these bacteria are essential for human nutrient assimilation and immune system. Compounds that contain an indole ring are called indoles or indole derivatives. Research have suggested the indole derivatives such as indoxyl sulfate and indole-3-propionic acid were also produced in the human body by enteric bacteria. Derivatives of indole are widely distributed in human body, and research have suggested these derivatives may have diverse biological function in the human body. Due to its diverse biological function, indole and its derivatives have been studied closely in recent years, pharmaceutical drugs using indole derivatives treating a wide range of diseases have been developed, these applications include anti-cancer drug, anti-depressant drug, anti-viral drug, anti-emetic drug etc, and most notably application relating to this study, one of its application is anti-asthmatic drug (101).

Several of indole derivatives have known to have anti-asthmatic activities due to their effect on the leukotriene formation pathway. One of the common asthma treatment drug Zafirlukast is an indole-containing structure with lipid-like tail, acidic head region and indole-backbone. Zafirlukast is leukotriene receptor antagonist (LTRA) which blocks the action of cysteinyl leukotrienes ($LTC_4$, $LTD_4$ and $LTE_4$), thus reducing bronchoconstriction and lung inflammation (102) (103). Zafirlukast has been widely used for treating EIB and have shown to inhibit maximal bronchoconstriction response after exercise by 50% to 80%. When the drug is taken before exercise, it also promote faster normal lung function recovery and reverse EIB (104).

As well as being LTRAs, other indole derivatives also have been shown to be 5-lipoxygenase inhibitors. By inhibiting the catalytic action of 5-lipoxygenase on arachidonic acid, the biosynthesis of leukotrienes is prevented, thus preventing any asthmatic symptoms. Competitive inhibitors of 5-lipoxygenase which are based on thiopyranoindole and thiazole structures are more selective than other inhibitors (102).

In recent years, indole has been reported to be a marker of chronic obstructive pulmonary disease (COPD) (44) (105). It has been found to be in elevated level in subjects suffering from COPD compared to healthy controls. Other study has shown indole to be a product of non-physical stimuli, indole has been found to be at elevated level in participants who took stress intervention tests (75).

Indole has also been reported to be produced after exercise. Early study examining muscles tissue after exercise, a wide range of compounds have been found to be produced during exercise which include indole as well as lactic acid, oxybutyric acid, carbon dioxide, hydrochloride, sulfuric acid, phenol and skatole etc. Indole, phenol and skatole were products of protein disintegration in the muscle and are capable of producing exercise fatigue symptoms (106).

With its extremely dynamic biological activities in organisms, indole has links to both exercise and asthmatic activity. The initial theory derived from its other reported activities in anti-asthma and exercise would be indole was produced after exercise challenge but potentially at an elevated level in asthmatic participants due to increased ventilation level and airway stress.

Butylated hydroxytoluene (BHT) is lipophilic organic compound, a chemical derivative of phenol. It is widely used as food additive due to its anti-oxidant properties. It also been used in health food supplement capsules as it was reported to have anti-viral effects. Most of the BHT found in human body is likely to come from food intake, human body is not known to produce BHT intrinsically, BHT is metabolized by liver to BHT-acid with minor amount BHT-alcohol via oxidation. BHT is mostly excreted in urine or feces in animal models, but it is believed to accumulate to a greater extent in human due to the lack of enterohepatic circulation in human (107). BHT has been reported to have biological activities in human. When administered orally, it Is said to have hepatic and gastro-intestinal tract toxicity in rats, it is found to enhance the development of spontaneously occurring liver tumours in rats (108) (109). It is also said to exacerbates chronic urticaria via Type 1 allergy (110). The link between BHT and lung inflammatory has been extensively studied over the years. In mice models, the injection of BHT causes increased DNA, RNA and lung weight due to cell injury and necrosis occurring at alveolar epithelium and interstitium with production of fibrosis

(111). Although BHT is widely used as anti-oxidant, but at a higher dose it can result in oxidative stress. Studies have shown that the oxidative stress caused by higher doses of BHT can enhance high affinity IgE receptor mediated degranulation through P13K enzyme activation and increasing intracellular $Ca^{2+}$ concentration in mast cells, thus action can enhance passive cutaneous reaction in rats, although these studies have mostly conducted on rats models or in vitro, it does suggest BHT can have the ability to affect allergic rhinitis and asthma in human as well (112).

The other discriminating variables weren't able to be determined in this study, so only speculative determination of its function groups were able to be described here based on its NIST library matches. One of the unknown BRI-1449-147-119-91 is likely a methylated aryl ketone or benzoic acid derivative, it was found to be a discriminating variable found in elevated level in both post exercise and asthmatic participants. Both ketone and benzoic acid derivatives have been reported to have biological activity in lung tissue (113) (114). Benzoic acid is found naturally in plants and acts as intermediate metabolite during biosynthesis of other secondary metabolites. It is metabolized by human body and excreted as hippuric acid in urine. Benzoic acid was also reported to be asthmatic biomarker in children (67). Domestic exposure to airborne ketone compounds were also reported to be asthma and allergy triggers (115).

The last unknown variable BRI-1537-161-203-175-218-189 is likely to be a terpene or benzoic acid derivative, this variable was a strong discriminator for exercise MVA analysis model, but it had negative p1 value for discriminating asthmatic participants. Terpene derivative has been previously reported to be a stress marker (75).

## 5.5. Summary

Constant work rate CPET was used to induce bronchoconstriction indicative of EIB in participants. The initial hypothesis was that EIB participant will show a difference in their VOC profile post exercise compared to healthy participants. Our study has found preliminary findings that are consistent with our initial hypothesis of possible existence of EIB specific response shown in human VOC profiles. Three upregulated EIB discriminating VOCs have been determined through unsupervised PCA using post exercise spirometry results with a sensitivity of 68.5% and specificity of 50%. The reduced sensitivity is due to high number of false negatives in the model. High rate of false negatives is common for EIB exercise testing in a laboratory, thus could be influenced by many factors during the exercise challenge, such as the duration and intensity of exercise challenge, the temperature and humidity of inspired air, and the time since last exercise induced airway narrowing all affect the percentage $FEV_1$ drop post exercise (99). It been reported that high intensity exercise with extended duration

can also trigger the release of catecholamine and may induce bronchodilation resulting in false negative diagnosis (116). Breath by breath analyser results does show an elevated level of both ventilation rate and heart rate during exercise in participants who shown a significant drop in $FEV_1$ post exercise. It is reasonable to assume that high intensity CPET will cause a significant physiological and biochemical change in participants who are EIB positive, thus will lead to a change VOC profile.

Two out of three VOCs were identified through NIST library matches to be indole and butylated hydroxytoluene. Both VOCs have been linked to biological activities in lung diseases. Especially indole which have been shown to increase in exercise and stress. However due to small size of this study, this result need to be established in larger scale before these VOCs can be confirmed to be indicative of metabolic or biochemical response to bronchoconstriction triggered by high intensity CPET. The quantification of these compounds was not possible at the time of the experiment, targeted studies with labelled standards with large group of participants should be the appropriate follow up study.

# CHAPTER 6. CONCLUSIONS AND FUTURE WORK

## 6.1. Chapter 1 and 2 summary

Chapter 1 explores physiology, composition and origin of exhaled breath VOCs. Then, the application of exhaled breath VOCs as diagnostic tool for lung diseases in recent years is discussed in details. Finally, it investigates the current exhaled breath VOCs collection, sampling and analysis methods used. Chapter 2 describes the current exhaled breath analysis work-flow employed in this thesis in detail. The exhaled breath analysis work-flow consists of 3 sections: exhaled breath sampling, sample analysis by TD-GC-MS and data analysis using MVA. Each section both cover the methodology adopted and the theoretical principles of the method or technique.

## 6.2. Chapter 2 summary

Thermal desorption has been used extensively in exhaled breath volatile organic compound (VOC) analysis, and in large scale studies it is often necessary to store the adsorbent tube samples before analysis. The possible introduction of storage artefacts is an important potential confounding factor in the development of standard methodologies for breath sampling and analysis.

The stability of VOCs trapped from breath samples onto a mixed bed Tenax® TA:Carbograph 1TD adsorbent tube and stored for up to 12 months at - 80°C was studied. 25 samples were collected from a participant over a 3 hr interval and then stored at - 80°C. Tubes were selected at random for subsequent analysed by thermal desorption-gas chromatography-mass spectrometry at 5 times points throughout a 12.5 month storage period. 3 deuterated internal standards were used to validate the instrument variability throughout the year. A breath-matrix consisting of 161 endogenous and 423 exogenous VOC was created using retention index and data deconvolution methods. Iterative OPLS-DA and Principal components analysis indicated that 1.5 months was the maximum storage duration with sensitivity and specificity of 100% and 94%. Individual analysis of endogenous and exogenous datasets shown endogenous compounds are more sensitive to storage. Paired two-tailed t-test on endogenous compounds has concluded optimum storage duration is at 1.5 month with 94% compounds stable and decrease to 73% at 6 month time point.

## 6.3. Chapter 3 summary

The original adaptive breath sampler for exhaled breath VOCs collection was created in 2006. It utilises pressure sensor sampling control unit to selectively collect alveolar portions of human exhaled breath onto adsorbent tube where it is analysed using TD-GC-MS system.

A portable adaptive breath sampling system was developed based on this original concept of adaptive breath sampling. It enables breath sampling to be carried out outside the designated laboratory with no location restriction. It can be setup quickly in clinical settings, sports venue or even private residents at participant's convenience. Additionally, custom build filtration system and comprehensive regeneration procedure was used to enhance and standardise background VOCs purification.

## 6.4. Chapter 4 summary

Exercise-induced bronchoconstriction (EIB) is a common pulmonary disorder characterized by airflow obstruction occurring after several minutes of physical activity. This study sought to determine if there is detectable changes in participant exhaled breath VOCs profile after high intensity cardiopulmonary exercise testing (CPET). Breath samples were collected from 24 participants (12 female and 12 male) before and after 10 minutes' constant work rate exercise testing using portable adaptive breath sampling system. 9 participants from the group were reported to be asthmatic. Heart rate, ventilation rate, oxygen output and carbon dioxide output were monitored throughout the exercise challenge. Forced spirometry was used to assess the participant response after exercise with measurements taken before and after exercise at 2, 5, 7, 10 and 20 minutes. A greater than 15% fall in post exercise FEV1 measured compared to baseline value is regarded as diagnostic of EIB. Breath samples were collected onto Tenax/Carbograph 1 thermal desorption tubes using portable adaptive breath sampler and analysed using thermal desorption gas chromatography – mass spectrometry. Multivariate analysis was performed on participant exhaled breath VOCs profiles to generate supervised orthogonal partial least squares discriminant analysis (OPLS-DA) model, thus generated three compounds which had shown to discriminating for participants with significant post exercise $FEV_1$ drop. Indole and butylated hydroxytoluene were successfully identified from mass spectral NIST library, the other compound couldn't not be determined due to low library match score, but its mass spectral is indicative of methylated aryl ketone or benzoic acid derivative.

## 6.5. Thesis summary

Around 400 to 600 VOCs were identified in a single human exhaled breath sample by studies discusses in this thesis. On average 100 to 200 VOCs were identified to be endogenous breath components and only a handful were identified to be potential biomarkers. For exhaled breath studies involving a group of participants, thousands of breath VOCs can be discovered. This demonstrates the complexity of breathomics, as well as its sensitivity to a variety of factors. The changes in metabolic profiles of participants with respiratory diseases can be trace pptv to ppbv level, thus requires a reproducible and

standardised approach to breath sampling methodology. A lack of reproducibility in the exhaled breath data can impact on multivariate modelling since small changes in participant VOCs profiles can be difficult to determine.

Many clinical studies experience difficulty in participant recruitment especially if the participant is suffering from serious illness. This means the breath samples collected from participants for one study can be spread out over several month if not years. This can introduce inter-instrumental variability to the data that can cause significant difficulties during data processing. A long term storage study was carried out assessed the stability of exhaled breath collected on multi-sorbent tubes at - 80°C, the result has determined a maximum storage duration of 6 months for endogenous breath components. Therefore study has established a storage protocol that can be used when planning and conducting other exhaled breath clinical studies.

Exposure to air borne contamination can also significantly influence the composition of exhaled breath. Air borne contaminants can also enter the body through inhalation and skin absorption, and can be released at different rates through breath or other excretion. Clinical or laboratory environments can introduce a wide range of VOCs from solvents, equipment or disinfecting solutions. A strictly controlled clean room is an ideal solution to avoid air borne background contamination. However, it is not always possible to bring participants to the designated breath collection facility, especially if the participant is suffering from respiratory diseases. Therefore, a portable breath sampling system has been developed to collect breath samples from participants at remote locations. The use of custom made filtration assembly and standardised breath sampling setup enables all participants to be sampled under the standardised conditions.

The standardised breath sampling and storage approach was then applied to a clinical study to determining detectable changes of breath VOCs profiles in participants suffering from excised induced bronchoconstriction (EIB). Breath samples were collected from 24 participants before and after 10 minutes' constant work rate exercise testing using ergometer cycles. In order to capture breath VOCs profile changes during possible bronchospasm, it is essential to be able to collect breath samples right after exercise. This means samples need to be collected at remote sports facility away from laboratory conditions, and sports facility can introduce extra air borne contaminants from human sweats and odour. The use of portable breath sampling system enabled remote breath sampling right next to the participant as well as the elimination of background room air contaminants in breath samples. The study had generated three compounds which had shown to discriminating for

participants with significant post exercise $FEV_1$ drop. Out of three compounds, Indole and butylated hydroxytoluene were successfully identified from NIST library matching.

## 6.2. Future works

### 6.2.1. Further improvement to the portable breath sampling system

The portable breath sampling system described in this thesis is only the first prototype developed. One aspect is the limit of an air supply flow rate of 40 L $min^{-1}$. Although this flow rate is enough for relaxed breathing at rest, a higher flow rate is required for breath sampling during exercise when the breathing rate is much higher; elite athletes will breath at up to 300 L $min^{-1}$. Another aspect that could be developed further is the size of the current system. The current system can be wheeled to a remote location for sampling or transport to further afield. It is much too big to be incorporated with a handheld sampler. Further redesigns to the next generation prototype should be focused on reducing its size and weight further to make it even more portable. The goal is a clinically compatible user friendly adaptive breath sampler for diagnostic purposes that can be used anywhere enabling research in breath analysis in a much wider range of locations.

### 6.2.2. Further development of data processing method

Currently the data processing method employed for analysing exhaled breath GCMS data involves the use of multiple data processing and analysis softwares, as well as extensive manual checking against raw data files. This is extremely time consuming and significantly limits the sample turnaround time. One of the most important future works will be to develop an alternative simplified standardised approach to data processing for breathomics data that can still preserve the integrity of comprehensive data analysis approach used now.

### 6.2.3. Development of exhaled breath condensate (EBC) method to compliment exhaled breath VOCs

The metabolic pathways of many VOCs identified as biomarkers for various diseases are poorly understood. The endogenous VOCs identified are usually by-products of biochemical mechanisms occurring in the human body. Exhaled breath condensate (EBC) collected from exhaled breath contain aerosols particles that reflect the composition of bronchoalveolar extracellular lining fluid. Larger non-volatiles compounds that cannot be collected using VOCs sampling method can be analysed using EBC method. Important biochemical mediators such as cytokines that have been identified in EBC can be used to reveal important information regarding the origins of potential biomarkers and developing a comprehensive breathomics approach.

## Appendix 1. EIB study information sheet

**INFORMATION FOR PARTICIPANTS**

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**Exercise challenge testing**


| Principal Investigator: | | Study co-ordinators: |
|---|---|---|
| Dr Martin R Lindley | Ms Nilam A Khan | Shuo Kang |
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| Contact tel: 01509 226349 | 01509 226352 | 01509 564100 |

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**PURPOSE OF STUDY**

As part of my PhD studies, I am looking into Exercise Induced Bronchoconstriction (EIB) which is the term used for airway narrowing that occurs after physical exertion in some individuals. As EIB is very frequent among young athletes and little is known of the reasons about the condition, we wish to conduct a research study to analyse the presence of EIB in athletes at Loughborough University.

In this study we will look closely at symptoms occurring after an exercise challenge in athletes with EIB (as confirmed by a previous eucapnic voluntary hyperventilation test).

**WHO IS DOING THIS RESEARCH AND WHY?**

This study is part of a Student research project supported by Loughborough University.

**EXCLUSION CRITERIA**

- Male and female participants with no history of exercising
- Participants under 18 and above 25 years of age
- Participants taking caffeine-related products (beverages, chocolate etc.) 24 hours before testing
- Smokers

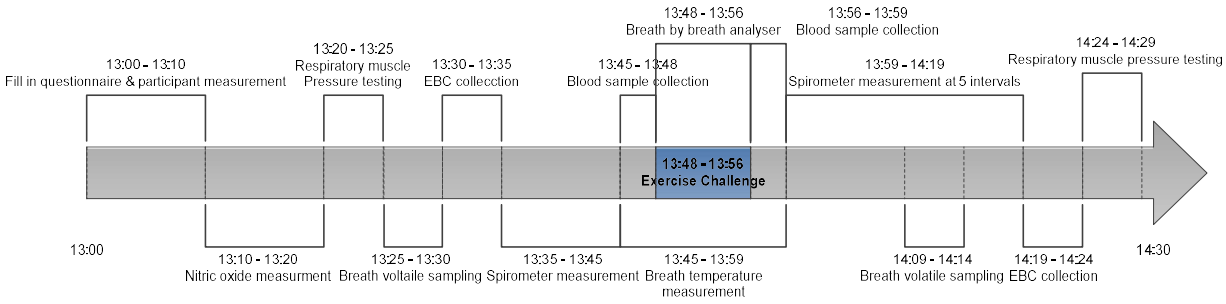
**RIGHT TO WITHDRAW**

After you have read this information and asked any questions you may have we will ask you to complete an Informed Consent Form, however if at any time, before, during or after the sessions you wish to withdraw from the study please just contact Nilam Khan. You can withdraw at any time, for any reason and you will not be asked to explain your reasons for withdrawing.

**STUDY DEMANDS**

You will be asked to visit the Exercise & Physiology laboratory at Loughborough University once.

In short, this will involve questionnaire filling, an exercise challenge test eliciting a heart rate response of 85-90% of maximal heart rate, blood sample collections before and after the exercise, collection of a cheek swab, exhaled breath condensate and performing spirometry. The following diagram highlights the sequence of events on the day of testing:




**DETAILS**

At the **start**, we will ask You to fill in three questionnaires:

1. A Health Screen questionnaire – to determine Your level of physical activity
2. A Juniper Asthma Control questionnaire – to determine whether you have a previous diagnosis of asthma or not (so we can bear this in mind when interpreting the test result)
3. A symptom score sheet – to find out what symptoms, if any, you experienced before and after the test
4. For exhaled breath collection; a questionnaire asking for details of diet, lifestyle and exposure to chemicals (make-up) in the last week.

You will then perform a lung function test, or forced spirometry, ***before the EVH*** test. This will also be performed at several time points ***after*** the test, as this helps us to find out if the results are positive or negative for exercise-induced bronchoconstriction.

Fig 1: Spirometry test

We will also perform a Fractional exhaled nitric oxide assessment which a non-invasive test involving slow breathing into a valve to detect airway inflammation. This will also be done ***before and after*** the EVH test. It will be followed by an exhaled breath condensate (EBC) collection, before and after the exercise session. During the EBC collection, you will be asked to breathe at normal pace into a tube and then put on a mask and breathe at normal pace for 5 minutes.

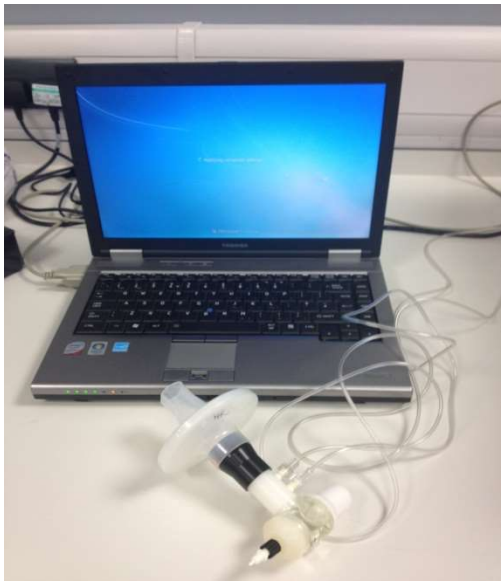
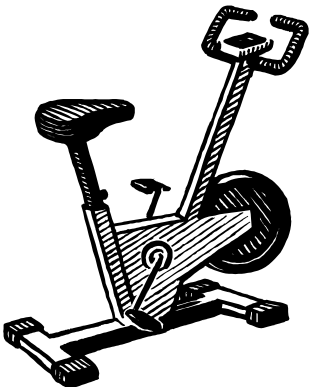


Fig 2: Nitric oxide (NO) test

Fig 3: Exhaled breath collection



Blood will be drawn from your arm and collected into two tubes, immediately prior to the exercise session. When you complete the exercise session, blood will be drawn again into two tubes.

Whilst exercising, we need tp collect the air that you exhale, so that we can measure your ventilation and your use of oxygen. To do this, we will ask you to:

- breathe through a **mouthpiece** which is connected to some valves and tubing. This is light, but you will notice it in your mouth. However, it will not cause you any difficulty with breathing (you may notice a slight dryness at the back of the throat).
- wear a **heart rate monitor** which continuously records your heart rate data.
- wear a **nose clip**, so that we do not loose air this way.

**HOW MUCH TIME WILL IT TAKE?**

| | |
|---|---|
| Questionnaires: | 10 minutes in total |
| Exercise challenge: | 10 minutes |
| Lung function: | 10 minutes before, and 20 minutes after exercise |
| $FE_{NO}$ assessment: | 10 min before and 10 min after exercise |
| EBC collection: | 10 min before and 10 min after exercise |
| Blood collection: | 5 min before and 5 min after exercise |

This amounts to **1hour and 40 min in total**, BUT could go up to **2 hours**.

**PREPARATION FOR THE TESTS**

- Exercise: No vigorous exercise should be undertaken 4 hours prior to testing.
- Medication: We ask you to refrain from medication 12 hours prior to testing. However, if you need to use your medication you should and then inform us so that we can reschedule your testing. You **must** bring your medication with you when you arrive for testing.
  NB: Antihistamines should be withheld for 48 hours.

- Food intake: Alcohol should not be consumed 12 hour prior to testing. Caffeine products (coffee, tea, cola, chocolate) should not be consumed on the morning of testing. These points are very important in order to prevent external variables influencing the study findings and we will discuss this with you in depth prior to the main trials.

**TESTS AND TRIALS INVOLVED**

Exercise challenge: 10min ergometer exercise including a 4 min 'climbing' period where we try to increase heart rate to 85-90% of maximum by increasing work load and 6 min exercise maintaining the 90% maximum heart rate.

Spirometry: Full lung function test that will measure how well your lungs are working by asking you to blow at different times into a machine. You will be asked to take a maximum breath in and a maximum forced breath out into a machine. You will be asked to do this at least three times before the EVH test, and immediately, at 5, 10, 15 and 20 minutes after the EVH test has been completed. Three blows will be required at each of the time points.

Exhaled nitric oxide: A blowing test to measure the amount of a gas that you naturally produce in your air passages, called nitric oxide,that you release when you breathe out. This provides us with some information of how active your asthma is. You will be asked to take a normal breath in and then breathe out slowly into a nitric oxide analyser, connected to a computer. You will do this three times to get an average. You will do this before and after the EVH test.

Exhaled breath collection:

1. Exhaled breath volatile organic compounds (VOCs) collection: Human breath contain trace amount of VOCs which reveal important information on status of human health. An adaptive breath sampler will be used to collect specifically for breath VOCs. You will asked to breath normally for 5 to 10 minutes while wearing an air tight breathing mask with purified air supply before and after intense exercise. Collected breath VOCs will be analysed in laboratory later.

2. Exhaled breath condensate collection (EBC): EBC collects both volatile and non-volatile part of exhaled breath. You will be asked to breath into a mouth piece for around 5 minutes before and after intense exercise. Exhaled breath breathed into the mouth piece is quickly condensed into liquid by freezing it at -10C. Collected EBC will be analysed in laboratory later.

***NB: Please note that the tests will be carried out by trained individuals, and in accordance with international/European guidelines and by strictly adhering to Loughborough University's policies.***

**POSSIBLE RISKS**

There are risks of worsening asthma symptoms in relation to refraining from medication 12 hours prior to testing or during testing. This is why you must bring your asthma medication with you. If you wish, your GP can be informed in case symptoms worsen or cause exacerbation.

Risk with spirometry includes lightheadedness. The researchers performing spirometry are fully familiar with the procedures and have carried them out before.

*Please note that all tests will be carried out under standard operating procedures and include careful monitoring of participants. There will always be more than one person in the laboratory whilst tests are taking place.*

## BENEFITS OF THE STUDY

The study should lead to greater understanding of the respiratory problems occurring after exercise in young athletes. The respiratory problems arising from exercise-induced bronchoconstriction can have quite marked effects on the general health, quality of life, mental health and social interactions of young people.

We will give you feedback on your results and direct you to support services relating to respiratory symptoms. Our researchers have years of experience and expertise in Exercise Physiology & Health and will be able to provide information on exercise training for you.

## CONFIDENTIALITY

Although information will be stored on computer each participant will be entered as a number rather than a name. Data will be used for research purposes only and confidentiality will be maintained in any publications arising from the study. Participant data will be kept for a maximum of 3 years from the time of collection and deleted upon completion of thesis writing and publication of the findings. The official closure date of this study is September 2014.

## MISCONDUCT

Any issues of research misconduct should be reported to Ms Zoe Stockdale at the following e-mail address: z.c.stockdale@lboro.ac.uk

In addition, Loughborough University has a policy relating to Research Misconduct and Whistle Blowing which is available online at *http://www.lboro.ac.uk/admin/committees/ethical/Whistleblowing(2).htm*.

## Appendix 2. Participant health questionnaire for EIB study

Name/Number .....

### Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research

**Please complete this brief questionnaire to confirm your fitness to participate:**

1. **At present,** do you have any health problem for which you are:

| | | | | | |
|---|---|---|---|---|---|
| (a) | on medication, prescribed or otherwise ..... | Yes | ☐ | No | ☐ |
| (b) | attending your general practitioner ..... | Yes | ☐ | No | ☐ |
| (c) | on a hospital waiting list ..... | Yes | ☐ | No | ☐ |

2. **In the past two years,** have you had any illness which required you to:

| | | | | | |
|---|---|---|---|---|---|
| (a) | consult your GP..... | Yes | ☐ | No | ☐ |
| (b) | attend a hospital outpatient department ..... | Yes | ☐ | No | ☐ |
| (c) | be admitted to hospital ..... | Yes | ☐ | No | ☐ |

3. **Have you ever** had any of the following:

| | | | | | |
|---|---|---|---|---|---|
| (a) | Convulsions/epilepsy ..... | Yes | ☐ | No | ☐ |
| (b) | Asthma ..... | Yes | ☐ | No | ☐ |
| (c) | Eczema ..... | Yes | ☐ | No | ☐ |

| | | | | | |
|---|---|---|---|---|---|
| (d) | Diabetes ..... | Yes | | No | |
| (e) | A blood disorder ..... | Yes | | No | |
| (f) | Head injury ..... | Yes | | No | |
| (g) | Digestive problems ..... | Yes | | No | |
| (h) | Heart problems ..... | Yes | | No | |
| (i) | Problems with bones or joints ..... | Yes | | No | |
| (j) | Disturbance of balance/coordination ..... | Yes | | No | |
| (k) | Numbness in hands or feet ..... | Yes | | No | |
| (l) | Disturbance of vision ..... | Yes | | No | |
| (m) | Ear / hearing problems ..... | Yes | | No | |
| (n) | Thyroid problems ..... | Yes | | No | |
| (o) | Kidney or liver problems ..... | Yes | | No | |
| (p) | Allergy to nuts ..... | Yes | | No | |

**4**. **Has any,** otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ..... Yes ☐ No ☐

**If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)**

.....

.....

.....

5**.** Allergy Information

| | | | | | |
|---|---|---|---|---|---|
| (a) | are you allergic to any food products? | Yes | | No | |
| (b) | are you allergic to any medicines? | Yes | | No | |
| (c) | are you allergic to plasters? | Yes | | No | |

**If YES to any of the above, please provide additional information on the allergy**

……
……..

5. How many times per week do you currently engage in physical activity (e.g. gym, running)?

| | | | | | |
|---|---|---|---|---|---|
| (a) | Once a week ..... | Yes | ☐ | No | ☐ |
| (b) | Twice a week ..... | Yes | ☐ | No | ☐ |
| (c) | Three times a week or more ..... | Yes | ☐ | No | ☐ |
| (d) | I don't engange in regular physical activity | Yes | ☐ | No | ☐ |

6. Additional questions for female participants

| | | | | | |
|---|---|---|---|---|---|
| (a) | Could you be pregnant? ..... | Yes | ☐ | No | ☐ |
| (b) | Are your periods normal/regular?..... | Yes | ☐ | No | ☐ |
| (c) | Have you ever skipped your period for more than 3 months? | Yes | ☐ | No | ☐ |
| (d) | Do you use birth control pills? | Yes | ☐ | No | ☐ |

If yes, what kind?.....

If yes, for how long?.....

(e) How old were you when your menstrual periods started? **Age:**……...

(f) When was your last menstrual period? **Date:**.....

(g) How many days are there between your menstrual periods? **Days:**……

(h) How many periods did you have in the last 12 months? **Periods:**…...

Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name:……
……

Telephone Number:……Work ☐ Home ☐
Mobile ☐

Relationship to
Participant:……

Are you currently involved in any other research studies at the University or elsewhere?

Yes ☐ No ☐

If yes, please provide details of the study

……
……..

Circle the number of the response that best describes how you have been during the past week

1. On average, during the past week, how often were you **woken by your asthma** during the night?

   0 Never
   1 Hardly ever
   2 A few minutes
   3 Several times
   4 Many times
   5 A great many times
   6 Unable to sleep because of asthma

**2.** On average, during the past week, how **bad were your asthma symptoms when you woke** up in the morning?

   0 No symptoms
   1 Very mild symptoms
   2 Mild symptoms
   3 Moderate symptoms
   4 Quite severe symptoms
   5 Severe symptoms
   6 Very severe symptoms

3. In general, during the past week, **how limited were you in your activities** because of your asthma?

   0 Not limited at all
   1 Very slightly limited
   2 Slightly limited
   3 Moderately limited
   4 Very limited
   5 Extremely limited
   6 Totally limited

4. In general, during the past week, how much **shortness of breath** did you experience because of you asthma?

   0 None
   1 A very little
   2 A little
   3 A moderate amount
   4 Quite a lot
   5 A great deal
   6 A very great deal

5. In general, during the past week, how much of the time did you **wheeze**?

   0 Not at all
   1 Hardly any of the time
   2 A little of the time
   3 A moderate amount of the time
   4 A lot of the time
   5 Most of the time
   6 All the time

**6.** On average, during the past week, how many **puffs of short-acting bronchodilator** (eg. Ventolin) have you used each day?

   0 None
   1 1±2 puffs most days
   2 3±4 puffs most days
   3 5±8 puffs most days
   4 9±12 puffs most days
   5 13±16 puffs most days
   6 More than 16 puffs most days

To be completed by a member of the clinic staff

7. $FEV_1$ pre-bronchodilator: ..... 0 >95% predicted
   1 95±90%

| | | |
|---|---|---|
| $FEV_1$ predicted ..... | 2 89±80% | |
| | 3 79±70% | |
| $FEV_1$ % predicted ..... | 4 69±60% | |
| | 5 59±50% | |
| | 6 <50% predicted | |

(Record actual values on the dotted lines and score the $FEV_1$ % predicted in the next column)

## Appendix 3. Publications

Kang S, Paul Thomas CL, How long may a breath sample be stored for at - 80 °C? A study of the stability of volatile organic compounds trapped onto a mixed Tenax: Carbograph trap adsorbent bed from exhaled breath. J Breath Res. **2016** 7;10(2):026011.

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