

Development of Chemical Ionisation Reaction Time-of-Flight Mass Spectrometry for the Analysis of Volatile Organic Compounds in Exhaled Breath

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by

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Statement of Originality

This thesis is based on work conducted by the author in the Department of Chemistry at the University of Leicester mainly during the period of October 2005 and September 2008. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

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Kerry Ann Willis

Breath is a mixture of nitrogen, oxygen, carbon dioxide, water vapour, inert gases, and a small fraction of trace volatile organic compounds (VOCs) in the parts per million by volume to parts per trillion by volume range. VOCs can be produced anywhere in the body as a result of physiological and pathophysiological processes, and are transported via the bloodstream to the lungs where they are exhaled in breath. On the basis that VOCs in breath are representative of VOCs in the blood and therefore biochemical processes occurring in the body, the analysis of exhaled breath may become a noninvasive tool for use in clinical practice. This thesis documents the development of the analytical technique of chemical ionisation reaction time-of-flight mass spectrometry (CIR-TOF-MS) for the analysis of VOCs in exhaled breath, exploring the challenges associated with breath sampling to its application in clinical studies. Initial work focused on the design of a suitable breath sampling device that coupled directly to the CIR-TOF-MS instrument to allow the on-line, real-time analysis of breath. The analysis of exhaled breath from healthy individuals allowed a common group of breath VOCs to be identified and quantified. The CIR-TOF-MS system was applied to a number of clinical trials examining the breath of individuals with cystic fibrosis (9 cystic fibrosis children, 4 healthy children), asthma (35 asthmatic subjects, 5 COPD, 28 healthy controls) and cancer (4 female cancer subjects, 10 healthy female controls), for which the latter study required the investigation of off-line breath collection. The analysis of VOCs emitted from bacterial and fungal cultures in vitro was also explored, as a means to support the hypothesis that the measurement of VOCs in exhaled breath could be used to identify infection status in vivo. Within these applications, CIR-TOF-MS was able to demonstrate that the chemical profile of breath has the potential to identify the presence of infection or disease.

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Chapter 1. Introduction to breath analysis

1.1 Introduction and thesis outline

Breath is a mixture of nitrogen, oxygen, carbon dioxide, water vapour, inert gases, and a small fraction of trace volatile organic compounds (VOCs) in the parts per million by volume (ppmv) to parts per trillion by volume (pptv) range. VOCs can be produced anywhere in the body as a result of physiological and pathophysiological processes and are transported via the bloodstream to the lungs where they are exhaled in breath. It has been known for many years that breath odour can be an indication of disease, a common example being the characteristic fruity smell of the breath of diabetic patients owing to high levels of acetone in the body. On the basis that VOCs in breath are representative of VOCs in the blood and therefore biochemical processes occurring in the body, analysis of exhaled breath may become a non-invasive tool for use in clinical practice.

The potential for non-invasive diagnostics has prompted the search for a unique marker or set of markers ('fingerprint') for given medical conditions. The non-invasive nature of breath analysis means that it is safe and repeatable, making it equally suitable for both diagnostic and monitoring applications such as monitoring the progression of a disease or the response to treatment. The early 1970s saw the start of the 'modern era' of breath analysis, with the publication of some key studies of the trace components of breath and changes brought about by disease [1-6]. Since then breath analysis has been a growing area of research and has recently resulted in the regular conference of the International Association for Breath Research (IABR) [7] and the publication of the Journal of Breath Research (JBR) [8]. This thesis documents the development of the analytical technique of chemical ionisation reaction time-of-flight mass spectrometry (CIR-TOF-MS/CIR-MS) for the analysis of VOCs in exhaled breath. The CIR-TOF-MS instrument central to this thesis was originally designed to measure trace VOCs for atmospheric applications [9-11]. In this role CIR-TOF-MS was shown to be effective in the simultaneous, real-time monitoring of VOCs in complex mixtures with low ppbv detection on one minute timescales. The versatility of the CIR-MS technique has been demonstrated through the application to alternative fields of research [12, 13], and the new application of breath research represents a further expansion of the technique's capabilities. This thesis details the steps taken to apply the CIR-MS technique to breath analysis, examining the challenges associated with breath sampling and performing clinical studies. The main aims of this research are outlined as follows:

- To develop a method for sampling exhaled breath to design an apparatus and sampling protocol for breath collection.
- To determine 'normal' breath composition to examine the range of VOCs that are typically observed in breath during CIR-MS analysis and the concentrations in which they are present, and to investigate how VOCs vary both within and between individuals.
- To search for exhaled markers of disease to examine how breath VOCs vary in disease states through the application of the CIR-MS instrument to clinical studies and to see if exhaled markers of particular conditions can be identified.

This chapter continues with a brief description of the respiratory system to provide a basis for understanding the area of breath analysis. This is followed by a review of breath research to date, including a summary of the commonly detected VOCs that have previously been identified in breath and the main analytical methods currently involved in exhaled breath analysis.

This leads into Chapter 2, which focuses on the CIR-TOF-MS technique. The theoretical principles and instrumental arrangement are described, along with an evaluation of the instrument performance towards the conditions encountered during breath measurement. Chapter 3 then focuses on the practical requirements of breath sampling, detailing the development of a suitable breath collection device and sampling protocols. During the development the breath collection methods were tested with small trial studies, initially with a group of cystic fibrosis children to investigate the use of breath analysis for the identification of bacterial lung infection, and later monitoring the metabolism of alcohol in healthy adults.

The capabilities of the CIR-MS instrument for breath research were demonstrated with a series of exploratory, cross-sectional clinical studies. Chapter 4 details the relocation of the CIR-MS instrument to a hospital setting to carry out the on-line analysis of exhaled breath from a group of asthma patients. This also allowed the determination of concentration distributions for a small number of common VOCs in a reasonably large group of subjects. Chapter 5 discusses the use of off-line breath collection, detailing the testing of the suitability of Tedlar bags for the containment of breath samples and their application to the study of the exhaled breath from a group of female cancer patients.

In Chapter 6 the focus moves away from the direct analysis of breath to investigate the emission of VOCs from bacterial and fungal cultures in vitro, as a means to support the hypothesis that the measurement of VOCs in exhaled breath could be used to identify infection. Chapter 7 summarises and discusses the future direction of this work.

1.2 The respiratory system

The lungs are the vital organs of respiration. The cells of the body need to be supplied with oxygen which is essential for aerobic respiration to occur, and the carbon dioxide produced by this process must be removed. The main function of the lungs is to provide a surface for gas exchange, over which oxygen and carbon dioxide pass between inspired air and capillary blood. The respiratory system is a network of airways that carry air between the surrounding environment and the lungs. The upper part of the respiratory system acts as the 'conducting portion' which warms and moistens inspired air while also providing a passageway to the 'respiratory portion', the site of gas exchange. The respiratory portion of the lungs begins with the first generation of airways that contain the alveoli in their walls. Each alveolus is surrounded by pulmonary capillaries, which together form alveolar-capillary membrane over which the respiratory gases diffuse (Figure 1.1). There are thought to be about 300 million alveoli in an adult human lung [14], creating an extremely large surface area that along with the very thin alveolar-capillary membrane allows for rapid and efficient gas exchange.



Figure 1.1 The alveolar-blood capillary membrane.

As air enters and leaves the lungs by the same airways, the last fraction of inhaled air that fills the conducting airways is the first air to be exhaled. This air, which never reaches the alveoli and does not take part in gas exchange with the capillary blood, is referred to as the anatomical dead space and forms approximately the first 150 mL of an exhaled breath [14]. Additionally, some alveoli are ventilated but not perfused, providing an additional volume of air that has not participated in gas exchange; this is

called the alveolar dead space. The alveolar dead space is negligible in healthy subjects, but can be increased in disease states. The anatomical and alveolar dead spaces together form the overall physiological dead space.

The amount of air breathed in and out at rest is called the tidal volume and is approximately 500 mL in a normal adult [14]. The lungs are capable of taking in more air than the tidal volume. If a subject inhales to total lung capacity, the maximum volume of air that the lungs can hold, and provides a maximum exhalation, this is known as the vital capacity. Lung volumes, which are summarised in Figure 1.2, vary depending on gender, age and height. Lung volumes are often measured clinically, usually by spirometry, as they are affected by disease and provide information about the physical condition of the lungs.



Figure 1.2 A summary of lung volumes and capacities. Tidal volume (TV) is the volume of air inhaled and exhaled at rest. Inspiratory reserve volume (IRV) and expiratory reserve volume (ERV) are the volumes of air that can be forcibly inspired or expired beyond tidal volume respectively. Vital capacity (VC) is the total amount of air that can be expired after a maximum inhalation. There is always a volume of air which remains in the lungs even after a forced exhalation which is the residual volume (RV). The total lung capacity (TLC) is the amount of air the lungs contain after a maximum inhalation.

1.3 Trace constituents of exhaled breath

1.3.1 Breath VOCs

It is not only the respiratory gases that are exchanged between air in the lungs and the blood, as trace VOCs also diffuse across the alveolar-capillary membrane (Figure 1.1). VOCs can be produced anywhere in the body and are transported via the bloodstream to the lungs, where they rapidly equilibrate with alveolar air and are exhaled in breath. In addition to endogenous VOCs that are produced by the body, breath also contains exogenous VOCs that originate from outside the body, the main source being inspired air. The pathways of VOCs through the body are summarised in Figure 1.3. The rates of excretion of breath compounds are related to rate of ventilation rate and cardiac output, and also the properties of the compound (solubility, lipid solubility) [15, 16]. The total exhaled profile will reflect those VOCs present in the blood, those in inspiratory air, and also those originating from the airways, nose or mouth [16].



Figure 1.3 The pathways of VOCs through the body (modified from [17]).

In a normal person, average alveolar mixing ratios are around 75% nitrogen, 14% oxygen, 6% water vapour and 5% carbon dioxide [18]. Trace compounds make up less than 100 ppmv [19], or 0.01%. These trace compounds consist of both VOCs and small inorganic molecules. Breath composition can vary widely from person to person, in

terms of both the presence and abundance of VOCs. In 1971 Pauling et al. revealed the presence of around 250 VOCs in a sample of exhaled breath [4]. Phillips et al. later identified thousands of different $C_4 - C_{20}$ VOCs in a study of the alveolar breath collected from 50 healthy subjects that included aliphatic and aromatic hydrocarbons, oxygen-containing compounds such as ketones, aldehydes and alcohols, and a number of nitrogen-containing and sulphur-containing compounds [17]. Only a small number of these compounds were common to all subjects, which were presumed to result from 'core' metabolic processes occurring in the body, while many compounds were thought to be of exogenous origin.

It is important to differentiate between exogenous and endogenous VOCs. The measurement of exogenous VOCs in breath can be used for the biological monitoring of occupational and environmental exposure [20-22], but it is the endogenous VOCs that provide the physiological information about an individual for medical diagnosis applications. Concentrations of VOCs originating from the airways or the oral cavity will be highest in the dead space air, while systemically produced VOCs originating from the blood will be highest in the alveolar air [23], which is often the target for breath analysis. The action of disease can cause changes in breath composition, although 'normal' breath composition must first be qualitatively and quantitatively determined so that deviations as a result of disease can be identified. A selection of compounds that have been identified in breath and their reported concentrations in healthy subjects are presented in Table 1.1. In order to assess the physiological significance and therefore diagnostic potential of exhaled VOCs, it is also important to understand the biochemical pathway responsible for their production. The known or suggested origins of some compounds are listed in Table 1.2, although many of the sources of VOCs remain unknown.

Compound	Concentration (ppbv)	Reference
Acetone	72 - 2744	[24-29]
Butanone	5 - 71	[30, 31]
Pentanone	0-38	[31]
Isoprene	0 - 737	[24, 25, 29, 32, 33]
Methanol	32 - 1684	[25, 26, 29, 34]
Ethanol	0 - 1663	[24-26, 29, 35]
Propanol	0 - 270	[25, 26, 28-31]
Pentanol	0 - 88	[25]
Formaldehyde	4 – 7	[23, 36]
Acetaldehyde	0 - 106	[24, 25, 29, 35]
Ammonia	68 - 2935	[24-29]
Hydrogen cyanide	0-62	[25-27]
Dimethyl sulphide	0-157	[31, 37, 38]
Dimethyl disulphide	5-6	[37]
Hydrogen sulphide	9 – 37	[37, 39]
Methanethiol	2 - 10	[37, 39]
Ethane	0 - 11	[40]
Acetic acid	30 - 60	[41]
Acetonitrile		
Non-smokers	0 - 17	[30, 42, 43]
Smokers	17 - 200	[30, 42, 43]

Table 1.1 A selection of breath compounds and their reported concentration ranges (in ppbv) in healthy subjects.

Table 1.2 Some breath compounds and their reported origins.

Compound	Source	Reference
Acetone	Fatty acid metabolism	[44]
Isoprene	Cholesterol synthesis	[45]
Ammonia	Protein metabolism	[28]
Methanol	Dietary sources	[46]
2-Propanol	Acetone metabolism	[47]
Acetaldehyde	Ethanol metabolism	[48]
Thiols/sulphides	Methionine metabolism	[49]
Ethane	Lipid peroxidation	[50]
Pentane	Lipid peroxidation	[50]
Ethene	Lipid peroxidation	[50]
C1 – C5 Alkanes	Colonic bacteria	[51]
C1 – C5 Alcohols	Colonic bacteria	[51]
C1 – C5 Carboxylic acids	Colonic bacteria	[51]

Even though a large number of VOCs have been identified in breath, few breaths tests have found use outside of the research setting. The most well known breath test is the 'breathalyzer' used by police officers for the measurement of breath alcohol levels. In terms of clinical applications breath tests fall into two categories; those carried out after the administration of a substrate and those that measure endogenous compounds [16]. Examples of substrate-based tests include the measurement of exhaled hydrogen following the ingestion of carbohydrate for the assessment of gastrointestinal disorders [51-53] or the measurement of an exhaled 13 C-metabolite (13 CO₂) following the ingestion of a ¹³C-labelled substrate, which has been applied to the assessment of conditions such as gastrointestinal disorders, liver function or identification of Helicobacter pylori infection [52, 54, 55]. These tests have to be carried out under defined conditions with respect to the time between administration of the substrate and breath measurement [16]. The search for endogenous markers is the focus of much research and some breath compounds have already been linked to particular medical conditions, of which some examples are given in Table 1.3. The diagnostic potential may not only lie in the identification of a single marker for a particular disease state. The use of multiple markers has also been investigated, such as the 'breath methylated alkane contour' devised by Phillips et al. that is based on the overall profile formed by the abundance of alkanes and monomethylated alkanes measured in breath [56], which has been applied to the assessment of various conditions (see Section 1.3.2). It is not just breath VOCs that are being measured for non-invasive disease diagnosis; the analysis of VOCs in urine headspace [57-59], skin emissions [60-62] and exhaled breath condensate (EBC) [63-65] have also been investigated.

Condition	Compound	Reference
Lipid peroxidation	Alkanes, aldehydes	[50, 66]
Diabetes	Acetone, propan-2-ol, methyl nitrate	[47, 67-69]
Cancer	Alkanes, methylated alkanes, aromatic compounds, aldehydes	[36, 70-74]
Heart transplant rejection	Alkanes, methylated alkanes	[75, 76]
Lung transplant rejection	Carbonyl sulphide	[77]
Airway inflammation	Nitric oxide, halogenated amines	[78, 79]
Cholesterol-lowering therapy	Isoprene	[15, 45, 80]
Liver disease	Sulphur compounds, carboxylic acids, ammonia, ketones	[1, 2, 81-84]
Kidney disease	Dimethylamine, trimethylamine, ammonia	[5, 85]
Oral malodour	Sulphur compounds	[86-88]
Trimethylaminuria	Trimethylamine	[89]
Congestive heart failure	Acetone	[90]

Table 1.3 Some example applications for exhaled breath compounds.

The following sections provide an overview of some of the main groups of compounds that have been identified in breath.

1.3.2 Hydrocarbons

Numerous aliphatic and aromatic hydrocarbons have been measured in exhaled breath [6, 17, 40, 91]. Gut bacteria are one source of $C_1 - C_5$ straight-chain alkanes, which are produced by the anaerobic fermentation of carbohydrates [51]. The process of lipid peroxidation is responsible for producing hydrocarbons such as alkanes and alkenes [50]. Lipid peroxidation is the free radical attack on polyunsaturated fatty acids (PUFAs), which are found in cell membranes. Reactive oxygen species, including oxygen-containing free radicals, are produced during normal metabolism and are usually scavenged by antioxidants to prevent them from causing damage. When the production of reactive oxygen species exceeds the antioxidant defences oxidative stress can result, where the radicals start to attack biomolecules such as proteins, DNA and lipids. The most commonly studied lipid peroxidation products in exhaled breath are ethane and pentane (see for example [92-95]), which result from ω -3 and ω -6 PUFAs respectively [50]. Exhaled alkane levels have been reported to show an age dependency

where increased levels in older subjects were linked to age-related increases in oxidative stress [56, 94, 96], although the observation of high levels in a small group of younger subjects [97] suggests that this trend may be limited to adults. Oxidative stress is associated with many clinical conditions [50, 98], and examples where increased breath alkanes have been observed include lung disease [99-101], heart transplant rejection [75, 76], inflammatory bowel disease [102] and haemodialysis [103, 104]. The profile formed from the measurement of alkanes and methylated alkanes has been used to classify patients with cancer [70, 71], diabetes [105], and unstable angina [106].

1.3.3 Isoprene

Isoprene (2-methyl-1,3-butadiene) is an unsaturated hydrocarbon present in breath that is primarily thought to originate as a by-product of cholesterol synthesis [107-109]. This is supported by the observation of reduced isoprene levels following the administration of cholesterol-reducing drugs [15, 45, 80]. Breath isoprene concentrations have been reported to show an age dependency, displaying lower concentrations in the breath of children in comparison to adults [110]. This relates to the observation of low or nondetectable concentrations in the breath of babies [3, 111]. However, with the exception of one study that observed lower concentrations in young adults in comparison to older adults [112], breath isoprene concentrations are generally reported to show no correlation with age in adult subjects [94, 113]. Breath isoprene levels were reported to display a circadian rhythm peaking around 2 - 6 am [113], although this was later thought to link to states of sleep and wakefulness [110, 114]. Owing to its low solubility and high volatility, exhaled isoprene concentrations are sensitive to changes in heart rate and breathing rate [15], which has been used to explain the rapidly increasing concentrations observed following the start of exercise [15, 32, 115]. The previously described increase in exhaled isoprene levels during 'sleep' has since been described as an effect of the increase in heart rate upon being woken to provide a breath sample [15], and may similarly explain the link found between elevated isoprene levels and psychological stress [6]. Increased breath isoprene concentrations have been found in haemodialysis patients, which were further elevated after dialysis [116, 117]. Elevated isoprene concentrations have also been associated with liver disease [82] and acute myocardial infarction [118], while lower isoprene concentrations have been observed during respiratory exacerbation in cystic fibrosis [119] and chronic heart failure [120].

1.3.4 Alcohols

Methanol and ethanol are the most abundant endogenous alcohols reported in exhaled breath (Table 1.1). Short chain alcohols seem to have, at least in part, bacterial origins. C1 - C5 alcohols are produced by anaerobic fermentation of carbohydrates by gut bacteria [51]. Methanol can be produced from dietary sources such as the breakdown of pectin-containing fruits or the artificial sweetener aspartame [46, 121]. Alcohols can also be produced by the metabolism of alkanes by cytochrome P450 enzymes in the liver [50, 122]. While the majority of exhaled methanol is thought to be produced systemically, ethanol appears to be mostly generated in the oral cavity, and may be enhanced after the consumption of sugary foods [23, 35, 123]. It is well known that breath ethanol concentrations are elevated after the consumption of alcoholic drinks, forming the basis of the road-side breathalyzer test. Methanol concentrations are also increased after alcohol consumption, with the levels persisting in breath for longer owing to the inhibition of methanol metabolism by ethanol [124, 125]. Elevated ethanol concentrations have been linked to obesity and non-alcoholic liver disease [126, 127]. Propanol has been found to have both oral and systemic sources [23, 31]. Propan-2-ol is thought to be produced from the enzymatic reduction of acetone [47, 66] and may become elevated in diabetics [47]. Elevated propanol concentrations have been observed in cirrhosis patients [30], and has been tentatively linked with lung cancer [36]. The measurement of pentanol in breath has been recently reported, which was found to be present in low concentrations in the breath of children and young adults [25].

1.3.5 Ketones

Acetone (propanone) is one of the most abundant VOCs found in exhaled breath (Table 1.1). Acetone, along with acetoacetic acid and β -hydroxybutyric acid, are collectively known as the 'ketone bodies' which are formed from fatty acids in the liver [128]. Acetone is formed from the decarboxylation of acetoacetic acid [44]. Acetone levels are elevated during fasting or starvation [68, 129] when carbohydrates are scarce and the break down of fatty acids provides a source of energy, and also during high fat, low carbohydrate 'ketogenic' diets [130]. Acetone concentrations are elevated in uncontrolled diabetes owing to the insulin deficiency that prevents glucose being taken up from the blood, causing the body to use fatty acids as a source of energy instead [67, 68]. It is the elevated acetone concentrations that are responsible for the 'fruity' smell of diabetic breath. Acetone can also be formed from the enzymatic conversion of propan-2-ol [44, 47], and the ingestion of propan-2-ol solution has been followed by an increase in breath acetone [131]. Butan-2-one and pentan-2-one have been detected in the breath of healthy subjects [30, 31, 132, 133]. Elevated concentrations of acetone, butan-2-one and pentan-2-one have all been associated with liver disease [30, 83, 127], and higher acetone concentrations have also been observed in patients with congestive heart failure [90].

1.3.6 Aldehydes

 $C_1 - C_{10}$ aldehydes have been detected in breath of healthy subjects, of which acetaldehyde (ethanal) is the most commonly reported [23, 35, 132, 134, 135]. Acetaldehyde is the first metabolite of the enzymatic oxidation of ethanol [48], and the relatively low concentrations observed in breath are thought to originate mainly from the metabolism of endogenous ethanol [66]. As ethanol concentrations are elevated after the consumption of alcoholic drinks, acetaldehyde concentrations are similarly increased [136]. Acetaldehyde appears to have both oral and systemic sources [23], and it is thought that bacteria in the oral cavity are capable of producing acetaldehyde after higher levels were measured in the dead space air in comparison to alveolar air following the consumption of alcohol [137]. The simplest aldehyde, formaldehyde (methanal), has been measured in low concentrations in the breath of healthy individuals [23, 36], of which some is thought to be produced systemically [23]. Aldehydes can be produced during lipid peroxidation [61, 65, 66, 132], and their measurement in breath and other biological samples have been associated with various forms of cancer [36, 59, 72, 73, 138].

1.3.7 Nitrogen-containing compounds

Ammonia is an abundant inorganic compound that is found in breath, and is a product of protein metabolism [28]; the protein origin has been demonstrated with the observation of increased breath ammonia concentrations in volunteers after being fed a protein-rich meal [139]. Exhaled ammonia is thought to mostly originate from the oral cavity, with systemic ammonia concentrations found to be around an order of magnitude lower (~ 100 ppbv) in healthy subjects [23, 140]. Increased ammonia levels have been measured in breath following a mouthwash with urea solution suggesting that there is either enzymatic or bacterial activity in the mouth [123]. Ammonia is normally converted to urea by the liver, which is subsequently excreted by the kidneys [141]. Ammonia levels can rise when liver function is impaired, and elevated breath ammonia concentrations have been observed in conditions such as cirrhosis [84]. Elevated breath ammonia concentrations have also been observed in end-stage renal failure [85, 141]. H. *pylori* bacteria produce urease enzymes that break down urea into ammonia and carbon dioxide, and the elevation of these products can be used to make a positive diagnosis of infection [142]. This is usually achieved through the use of ¹³C-urea and the measurement of ¹³CO₂ [54], although the use of exhaled ammonia measurement after the ingestion of non-labelled urea has been investigated as an alternative [142, 143].

Low-level nitrile compounds have been detected in breath including hydrogen cyanide, which is mainly produced in the mouth and presumed to originate from oral bacteria [23], and acetonitrile, although this is largely as a result of smoking [43]. A number of nitrogen-containing compounds have been associated with disease states. Exhaled

hydrogen cyanide and hydrogen nitrate were found to be significantly elevated in patients with *H. pylori* infection [144]. Methyl nitrate, which is present in pptv levels, has been suggested to be an exhaled biomarker of hyperglycaemia as it was found to be increased in type I diabetics and correlated with blood glucose levels [69]. Trimethylamine, which is mainly derived from the bacterial action on dietary precursors in the intestine, is responsible for the strong 'fish' odour breath of individuals with the metabolic disorder trimethylaminuria, whereby a deficiency in the enzyme responsible for trimethylamine metabolism causes levels in the body to increase [89]. Trimethylamine and dimethylamine are thought to contribute to 'uraemic breath' as elevated levels have been observed in subjects with end-stage renal failure, which were subsequently found to decrease after dialysis [5].

There has been much interest in the measurement of exhaled nitric oxide since being identified as a component of breath. Nitric oxide originates from the cells of the airways, where it is synthesised from the amino acid L-arginine by nitric oxide synthase enzymes [79]. Concentrations of exhaled nitric oxide are increased in subjects with airway inflammation, such as those with asthma, which is thought to result from the expression of an inducible form of nitric oxide synthase enzyme during the inflammatory process [145, 146]. Nitric oxide is also present in the nasal cavity in much higher concentrations than that found in the airways [147], therefore presenting a potential source of contamination of breath samples that has to be excluded during sampling [148]. The measurement of exhaled nitric oxide is one of the few breath tests to have been developed for use in clinical practice. Halogenated amines have also been detected in breath and have been suggested as possible markers of airway inflammation [78].

1.3.8 Sulphur-containing compounds

Volatile sulphur compounds (VSCs) such as methanethiol, dimethyl sulphide, dimethyl disulphide and hydrogen sulphide appear to largely originate in the oral cavity [37, 39] and are thought to result from the action of anaerobic bacteria on sulphur-containing

amino acids [87], although dimethyl sulphide has also shown systemic production [37, 133]. VSCs in the body are thought to be produced from the metabolism of the sulphurcontaining amino acid methionine [2, 82, 149]. Some VSCs, mainly allyl sulphides, can be present in breath as a result of dietary intake [38]. While present in lower levels in healthy subjects [31, 87], methanethiol, dimethyl sulphide and hydrogen sulphide are major contributors to oral malodour and are often the focus of breath odour research [86-88]. Halitosis has both oral and extra-oral sources, the latter resulting from malodorous compounds in the blood which may be linked to systemic disease [86, 87, 150]. Thiols and dimethyl sulphide are thought to contribute to the 'musty' odour of fetor hepaticus in subjects with liver failure [2]. Several studies have reported high levels of dimethyl sulphide in the breath of patients with liver cirrhosis [81, 83, 150, 151], and one study also found carbonyl sulphide and carbon disulphide levels to be increased by liver disease [82]. Carbonyl sulphide was also found to be increased in subjects with acute lung transplant rejection [77].

1.4 Analysis of breath VOCs

From an analytical point of view, breath is a challenging sample because of the complex chemical composition, low analyte concentrations and high humidity. One of the reasons behind the increased interest in breath analysis over the last 40 years is the advances in analytical instrumentation. The ideal requirements of an instrument for the analysis of trace constituents in exhaled breath can be summarised as follows:

- Quantitative: for the determination of absolute trace gas concentrations
- Sensitive: needs high sensitivity with detection limits of low ppbv pptv
- Specific: the ability to measure a target analyte without interference from other components
- High temporal resolution: at least 1 s timescale for real-time analysis
- Unaffected by humidity

With the range of VOCs present in breath, a variety of different analytical techniques have been used to measure them.

Many early studies of the trace composition of exhaled breath used gas chromatography (GC) based methods [1-6], and GC coupled with mass spectrometry (GC/MS) is still the most commonly used technique (see for example [31, 133, 135, 152, 153]). Gas chromatography involves the separation of components in a mixture based upon the rates at which they travel in a carrier gas, the mobile phase, through a stationary phase contained in a column [154]. The interaction between the sample compounds and the stationary phase causes the different chemical compounds to elute from the column at different times, known as the retention time. GC/MS (usually electron ionisation) aids compound identification based on both the retention time and the characteristic fragmentation patterns displayed in the mass spectrum, which can be compared against those contained in spectral libraries. GC/MS has been used to identify a large number of VOCs found in breath [6, 17, 133, 152]. The main disadvantage of GC/MS for breath VOC measurement is that pre-concentration is required, and the timescale for preconcentration and separation of compounds on the column mean that real-time measurements are not possible. Pre-concentration is commonly achieved through the use of sorbent traps, coated fibres called solid phase microextraction (SPME) or cryotrapping [134, 152, 155-158], usually followed by thermal desorption into the GC. The presence of water vapour can cause problems for some pre-concentration methods and for chromatography columns [158-161]. Low molecular weight volatile compounds such as formaldehyde and 'sticky' compounds such as amines are difficult to analyse by GC [160], and on some GC columns pentane, regarded as a marker of lipid peroxidation, and isoprene, a major hydrocarbon in breath, co-elute [94, 162, 163]. GC × GC instruments have been designed to provide greater separation of breath constituents [164].

Numerous breath studies have been carried out by Smith and Španěl using selected ion flow tube mass spectrometry (SIFT-MS). The SIFT-MS technique is based on chemical ionisation reactions between either H_3O^+ , NO^+ or O_2^+ reagent ions and sample VOCs in

a flow tube [165]. VOC assignments are made based on *m/z* values, although problems can occur with overlapping peaks and isobaric species. Switching between the different reagent ions can assist in some situations [166] and SIFT-MS has been coupled with GC for separation and identification of isobaric species [167]. The SIFT-MS technique can perform real-time analysis at low ppbv levels without pre-concentration [165]. Longitudinal studies have been performed using SIFT-MS monitoring breath ammonia, acetone, propanol, isoprene, ethanol, acetaldehyde and methanol over 6 month periods [28, 32, 34, 35] to build up a greater understanding of these compounds in healthy subjects and allowing the determination of normal concentration distributions (see [29] for a review). SIFT-MS has also provided information on the sources of some common VOCs, classified in terms of systemic or oral origin [23, 37, 140], and has been applied to the investigation of VOC variation in certain diseases [85, 116, 143, 168].

Developed by Lindinger et al., proton transfer reaction mass spectrometry (PTR-MS) is another chemical ionisation technique utilising proton transfer reactions between H_3O^+ ions and sample VOCs in a drift tube (discussed in detail in Chapter 2). PTR-MS also has the ability for real-time analysis and has demonstrated its use in monitoring breath VOCs down to the pptv level without pre-concentration [131]. As in SIFT-MS, VOCs assignments are based on *m/z* values only and can suffer from overlapping peaks and isobaric species. GC has been coupled with PTR-MS for the unambiguous identification of VOCs [169]. Neither PTR-MS nor SIFT-MS can detect low mass alkanes in breath, such as ethane, which is commonly used as a marker of lipid peroxidation. PTR-MS has been applied to the examination of exhaled breath from healthy subjects [112, 131, 170-173], and demonstrated the monitoring of VOCs during processes such as exercise [15, 115] and sleep [174, 175]. PTR-MS has also been applied to a number of medical applications including the investigation of cancer biomarkers [36, 80], identification of infection [144], the monitoring of hypercholesterolemia therapy [15, 80] and the investigation of metabolic disorders [176].

Alternatives to mass spectrometry-based techniques have also been developed. Ion mobility spectrometry (IMS) utilises chemical ionisation reactions between reagent ions

and sample molecules and separates ions based on their drift time while under the influence of a weak electric field [177]. For complex mixtures such as breath the resolution is often insufficient, so sample pre-separation is required, which is usually achieved through the direct coupling of a multi-capillary chromatographic column (MCC) [177, 178]. The use of the MCC also helps to minimise the humidity effects on the product ions through separation on the column [178]. Identification in MCC-IMS is based on both the retention time on the column and the drift time in the IMS. An advantage of IMS instrumentation in comparison to other spectrometers is the relative low cost and operation at atmospheric pressure, which means that a vacuum system is not required thus allowing the development of small technology [178]. Breath collection can be performed on-line through the direct exhalation into a sample loop prior to the MCC, and the total time required for separation and analysis is around 10 minutes [177]. IMS offers detection limits in the ppbv – pptv range [177]. Owing to the chemical ionisation processes, low mass alkanes are not detectable by IMS [160]. The potential of IMS for breath analysis may not necessarily be in the identification of specific components, but in recognising differences between different breath spectra, which has been applied to identification of lung cancer patients [177] and sarcoidosis patients [179, 180].

A number of research groups have applied laser-based techniques to the analysis of exhaled breath, including the measurement of the lipid peroxidation products ethane [40, 95, 181-183] and ethene [181, 184, 185], as well as other trace gases including nitric oxide [186-191], carbon monoxide [192], carbonyl sulphide [193], acetaldehyde [194], ammonia [195] and acetone [196]. Many techniques are based on absorption spectroscopy, a good review of which is given in a recent publication [197]. Laser spectroscopy has high molecular specificity, high sensitivity and on-line, real-time capability, as illustrated for example with the measurement of ethane at pptv concentrations [95]. Laser methods usually measure only a single molecular species at a time, although the simultaneous measurement of NO and CO_2 has been demonstrated [187]. These techniques provide a means to monitor some of the VOCs in breath that are not detectable by other real-time methods such as PTR-MS and SIFT-MS.
While breath analysis research is likely to continue using specialised laboratory instruments, small, inexpensive and simple instruments are required for breath analysis to make the transition from the research setting to clinical practice, ideally in the form of benchtop instruments or even hand-held 'breathalyzers'. Specific sensors for the detection of single compounds or non-selective sensor arrays coupled with pattern recognition (sometimes called 'electronic noses') provide small-scale and cheaper alternatives. The use of electronic noses has been investigated for the identification of lung cancer [198, 199], asthma [200] and halitosis [201, 202]. When a marker or pattern of markers for a particular disease has been defined, sensors or sensor arrays can be specifically designed.

1.5 Summary

This chapter has provided a brief introduction to the field of breath research, giving an indication of the chemical complexity of exhaled breath. The analysis of the trace species in exhaled breath may identify links with disease and therefore provide a means for non-invasive medical diagnosis. This thesis aims to demonstrate that the technique of CIR-TOF-MS is a valuable tool for breath research, allowing the simultaneous monitoring of a range of breath VOCs with high sensitivity in real-time. The details of the CIR-MS technique and the assessment of its performance for breath research follows in Chapter 2.

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Chapter 2.

Chemical ionisation reaction time-of-flight mass spectrometry: instrumentation and performance

2.1 Introduction

The focus of this thesis is the application of CIR-TOF-MS to the analysis of exhaled breath. The Leicester instrument adopted the general 'CIR-MS' title after displaying the ability to utilise alternative CI reagents [1, 2], although PTR-MS using H_3O^+ reagent ions still remains the main ionisation method. The CIR-TOF-MS instrument has been well characterised, particularly with regard to atmospheric measurement [3, 4], although the application to breath analysis will bring new challenges. This chapter describes the CIR-TOF-MS instrument and operating principles, and assesses its performance under the conditions encountered during breath measurement.

2.2 Proton transfer reaction mass spectrometry

2.2.1 Instrumentation

For any sample to be analysed by mass spectrometry, the neutral molecules must first be ionised. The chemical ionisation (CI) technique was first introduced by Munson and Field in 1966 [5] as an alternative to the traditional electron ionisation (EI) method. In an EI source, the interaction between high energy electrons in the region of 70 eV and the gaseous analyte results in the ejection of an electron from the latter to form a radical cation [6]. In this process energy in excess of the ionisation energy of the molecule, which is typically around 10 eV for VOCs, is transferred. The excess energy often

results in extensive fragmentation of the molecular ion. Characteristic fragmentation patterns can be used to determine analyte identity, but identification becomes very complicated when analysing complex mixtures. In comparison, the CI method is based on gas phase ion-molecule reactions between the analyte and a primary reagent ion. CI is described as a 'soft' ionisation technique as it produces ions with little excess energy (a few eV at most [7]), resulting in little or no fragmentation. Often only a single analyte ion is produced, so the resulting mass spectra are relatively simple, making CI more suitable for the analysis of multi-component mixtures.

The type of ion-molecule reaction is dependent upon the chemical properties of both the CI reagent used and the individual analyte species, but generally ionisation will proceed via one of four reactions: proton transfer, charge transfer, anion abstraction or association [7]. Proton transfer reactions have become the most widely used, and form the basis of the PTR-MS technique. Typical reagent gases and the corresponding reagent ions for proton transfer include methane/ CH_5^+ , ammonia/ NH_4^+ and water vapour/ H_3O^+ [5, 8]. Exothermic proton transfer will occur for compounds with a proton affinity greater than that of the reagent gas, which has to be taken into account when selecting a suitable reagent. Table 2.1 provides proton affinity values for a selection of compounds. With respect to the analysis of air and breath, methane has a low proton affinity (543.5 kJ mol⁻¹ [9]) so the reactions with the major components of these samples (N₂, O₂, CO₂, H₂O) may dominate, whilst ammonia with a high proton affinity (853.6 kJ mol⁻¹ [9]) excludes many of the relevant VOCs. Water vapour, with a proton affinity of 691.0 kJ mol⁻¹ [9], is the most suitable reagent as the H_3O^+ ions will react with a large number of VOCs but not with the bulk components, and is almost exclusively used in PTR-MS.

PTR-MS, which combined CI with the flow-drift tube technique [10], was developed by Lindinger et al. and introduced in the 1990s [8, 11]. Prior to the development of PTR-MS, gas chromatographic techniques (GC-FID, GC/MS) were commonly used for VOC analysis [12]. Whilst offering high sensitivity and a means of identifying compounds, GC methods require off-line collection and pre-concentration, which along with the

time needed for chromatographic separation means limited temporal resolution, making it difficult to monitor mixtures with relatively fast changing concentrations [13]. In contrast, PTR-MS offers on-line, real-time measurements of trace gases without the need for pre-concentration.

All PTR-MS instruments feature the following components: (1) an ion source for the production of the reagent ions, (2) a drift tube that acts as the reaction chamber where proton transfer between the reagent ions and neutral analytes takes place, and (3) a mass analyser/detection system. The majority of PTR-MS instruments reported in the literature are commercially available Ionicon Analytik systems [14], most of which feature a hollow cathode discharge (HCD) ion source coupled to a quadrupole analyser (see [8] for example).

In a HCD ion source, the discharge process causes the electron ionisation of water vapour to produce ions such as O^+ , H^+ , H_2^+ , OH^+ and H_2O^+ , all of which react further with neutral water molecules to subsequently produce H_3O^+ ions, either in the discharge region itself or the small source drift region that follows [11]. Therefore, high concentrations of H_3O^+ ions are produced (> 99% purity [11]) without the need for preselection of ions before entering the drift tube, as opposed to SIFT-MS which requires a mass filer to select a single reagent. Some O_2^+ and NO⁺ impurity ions are formed as a result of back diffusion of air from the drift tube into the ion source, an unwanted occurrence since these ions can also react with VOCs [13]. HCD sources normally operate between 2 – 3 mbar, and H_3O^+ count rates of 10⁶ counts s⁻¹ are routinely achieved [15-17]. Radioactive ion sources (²⁴¹Am, ²¹⁰Po) have been employed as an alternative to discharge sources and can operate at higher pressures (up to 13 mbar) [18, 19]. Higher drift tube pressures can translate into higher instrument sensitivity [15, 18, 20]. Reagent ions from the ion source are drawn into the drift tube that consists of a series of ring electrodes and insulating spacers, and is normally 5 - 15 cm in length [21]. The sample is continuously introduced into the drift tube, with the air containing the trace analyte species acting as the buffer gas. The potential gradient over the drift tube directs the ions towards the exit and into the analyser.

As previously stated, the majority of PTR-MS instruments use quadrupole analysers, which act as mass filters that only allow the transmission of ions of a particular mass to the detector at any given time. This means that while dwelling on one mass value, ions in all other mass channels are lost. The duty cycle for a quadrupole analyser is inversely proportional to the number of mass channels monitored. For the analysis of complex samples, quadrupole instruments achieve high sensitivity over a complete mass range when sufficient sampling times are available. However, to achieve comparable sensitivity on short timescales quadrupoles are typically operated so that only a small number of preselected mass channels are monitored at the expense of losing information on other components. Detection at the pptv level has been reported for dwell times of 1 – 10 seconds [16, 22]. Another limitation of quadrupole analysers is the relatively low mass resolution [18].

Some instruments with alternative analysers have been developed in the form of iontrap (IT) or TOF systems, both of which offer the simultaneous measurement of ions. The first PTR-IT instrument was reported by Prazeller et al., which coupled the IT analyser to a standard hollow cathode ion source and drift tube [23]. Similar instruments have since been reported [24, 25]. The IT accumulates and stores ions simultaneously over an entire mass range, before scanning them out to the detector. The advantages over quadrupole analysers include a higher duty cycle, up to 95 - 99% compared to 1 - 99%10% with a quadrupole in a typical operating mode [23, 24], and the resolution of isobaric compounds with MS/MS capability, whereby collision-induced dissociation is performed on ions of a single mass after the ejection of all others from the trap [21]. The detection limits of IT systems are almost comparable to that of quadrupole instruments, and sub-ppbv levels have been achieved [25]. Since the first publication of the Leicester time-of-flight instrument [26], a few other PTR-TOF-MS systems have been reported. Ennis et al. coupled a hollow cathode source to a reflectron-TOF and achieved 1 ppbv detection limits for integration times of less than 60 seconds [27]. The same instrument also displayed a mass resolution that was sufficient to differentiate between nominal isobars. Tanimoto et al. designed a linear-TOF system that had limited mass resolution,

but achieved sub-ppbv detection limits for 1 minute integration times [19]. A limitation of TOF-systems is the duty cycle, which is typically less than 3% [21, 26].

SIFT-MS is a related technique that owing to the extensive studies of Smith and Španěl (reviewed in [28]) is regularly referenced throughout this thesis, so a brief description is given here. The SIFT technique was originally developed for the study ion-molecule reactions [29], but is now also used for VOC analysis [28]. SIFT-MS differs from PTR-MS in the use of a flow tube rather than a drift tube, whereby ions travel in a fast-flowing carrier gas rather than by the influence of an electric field. A mixture of H_3O^+ , NO^+ and O_2^+ ions are generated in a microwave discharge source, then with the use of a quadrupole mass filter a single reagent ion is selected for injection into the flow tube; ion pre-selection is another difference between the SIFT-MS and PTR-MS methods. Reagent ions enter the fast-flowing helium carrier gas (~ 1 mbar) in the flow tube (~ 0.3 – 1 m in length) [28]. The sample is introduced into the flow tube and the resulting analyte ions are detected downstream by a quadrupole mass spectrometer. The dilution of the sample in the helium flow means that the instrument sensitivity is typically lower than that observed in PTR-MS [13, 21].

2.2.2 Proton transfer reactions

Exothermic proton transfer reactions between H_3O^+ ions and sample VOCs occur for those compounds with a proton affinity greater than that of water, resulting in the production of protonated pseudo-molecular ions (Equation 2.1).

$$H_3O^+ + M \xrightarrow{k} MH^+ + H_2O$$
 (2.1)

M represents the neutral analyte compound, MH^+ its protonated product ion and k the proton transfer reaction rate coefficient. The reaction rate coefficient indicates the speed of the reaction, and exothermic proton transfer will proceed at or near the collision rate, i.e. proton transfer occurs on every collision [8]. Measured reaction rate coefficients and

calculated collision-limited rate coefficients (k_c , see [21] for a review of calculation methods) have been found to be in good agreement [8]. Proton transfer reaction rate coefficients are of the order of 10^{-9} cm³ molecules⁻¹ s⁻¹ (Table 2.1).

	Molecular Formula	Molecular Mass	Protonated Mass	Proton Affinity (kJ mol ⁻¹) ^a	$k_{\rm c}$ (10 ⁻⁹ cm ³ s ⁻¹) ^b	$(D)^{c}$
Oxygen	O ₂	32	33	421.0	-	-
Nitrogen	N_2	28	29	493.8	-	-
Carbon Dioxide	CO_2	44	45	540.5	-	-
Water	H ₂ O	18	19	691.0	-	-
Formaldehyde	CH ₂ O	30	31	712.9	2.92	2.33
Formic Acid	$\mathrm{CH}_2\mathrm{O}_2$	46	47	742.0	2.02	1.41
Methanol	CH ₄ O	32	33	754.3	2.33	1.70
Toluene	$\mathrm{C_7H_8}$	92	93	784.0	2.12	0.38
Benzene	C_6H_6	78	79	750.4	1.97	0.00
Pent-1-ene	C_5H_{10}	70	71	-	1.87	0.50
Acetaldehyde	C_2H_4O	44	45	768.5	3.36	2.75
Ethanol	C_2H_6O	46	47	776.4	2.26	1.69
Acetonitrile	C_2H_3N	41	42	779.2	4.74	3.92
Acetic Acid	$C_2H_4O_2$	60	61	783.7	2.27	1.74
Water Dimer	(H ₂ O) ₂	36	37	808.0 [§]	-	-
Acetone	C_3H_6O	58	59	812.0	3.00	2.88
Isoprene	C_5H_8	68	69	826.4	1.94	0.25
Butan-2-one	C_4H_8O	72	73	827.3	3.38	2.78
Dimethyl Sulphide	C_2H_6S	62	63	830.9	2.53	1.55
Hexan-2-one	$C_6H_{12}O$	100	101	-	4.00^{\dagger}	2.80^{\dagger}
Ammonia	NH ₃	17	18	853.6	2.60^{*}	1.47*

Table 2.1 Properties of a selection of VOCs and inorganic compounds.

^a From [9]

 ${}^{b}k_{c}$ is the calculated collision-limited proton transfer reaction rate coefficient, from [30] ${}^{c}\mu_{D}$ is the permanent dipole moment, from [31] ${}^{\$}$ From [32]

[†] From [33]

* From [34]

The reagent ions are present in large excess of the trace analyte molecules, therefore proton transfer reactions do not significantly deplete the H_3O^+ signal, which effectively remains constant. Under these pseudo-first order conditions, analyte concentrations can be estimated using Equation 2.2.

$$[M] = \frac{1}{kt} \frac{[MH^+]}{[H_3O^+]} \approx \frac{1}{kt} \frac{i(MH^+)}{i(H_3O)}$$
(2.2)

In the previous equation, $i(MH^+)$ is the protonated analyte ion signal, $i(H_3O^+)$ is the hydronium reagent ion signal, [M] is the analyte concentration in the sample and *t* is the reaction time, which is taken as the time that the ions spend in the reaction region. Therefore if *k* and *t* are known, the concentration of a specific analyte can be determined through the measurement of the MH^+/H_3O^+ signal ratio. However, the accuracy of calculated concentrations can be limited for reasons including the uncertainties associated with the rate constant, uncertainty in the reaction time, variation in ion transmission, differing mobilities of reagent and analyte ions, and the fact that the equation does not account for additional processes such as fragmentation [17, 21, 35]. Equation 2.2 can be modified to take some of these factors into account, however calibration with a specific gas standard is the preferred method for determining reliable analyte concentrations.

Owing to their low proton affinities (Table 2.1), the major components of air and breath do not undergo proton transfer with H_3O^+ , whereas a wide range of VOCs do. The main exception is low mass alkanes. Methane, ethane, propane and isobutane all have proton affinities lower than that of water (543.5, 596.3, 625.7 and 677.8 kJ mol⁻¹ respectively [9]), so proton transfer to these compounds is endothermic. Given that for a particular homologous series of organic compound, the proton affinities generally increase with carbon chain length, proton transfer to alkanes should eventually become exothermic, which has been estimated to occur near hexane [36]. Even though proton transfer becomes exothermic for larger alkanes, no direct proton transfer products have been observed, although it is possible that the protonated product ions undergo fragmentation

[36]. In terms of breath sampling, this means that the lipid peroxidation products ethane and pentane cannot be detected when using H_3O^+ as the CI reagent.

With the exception of ethene (proton affinity = 680.5 kJ mol⁻¹ [9]), proton transfer is exothermic for alkenes. The difference between alkanes and alkenes is the electron-rich double bond to which the electrophilic H_3O^+ can attack (Figure 2.1). Protonation will be regiospecific in the case of unsymmetric alkenes, with the proton bonding to the least substituted carbon to leave the charge on the more stable, higher substituted carbocation [37]. The mechanism for arenes is similar to that for alkenes, whereby the proton will bond to one of the double bonds in the benzene ring.



alkenes: R/R' = H, alkyl arenes: R = H, alkyl, R' = part of benzene ring

Figure 2.1 The mechanism of protonation for unsaturated VOCs.

Oxygenated VOCs (OVOCs) such as aldehydes, ketones and alcohols readily undergo proton transfer. For OVOCs, an electron lone pair located on the oxygen atom of the carbonyl or hydroxyl group provides the site for protonation (Figure 2.2). The detection of the simplest aldehyde, formaldehyde, can be difficult as the proton affinity is only slightly higher than that of water so the exothermicity of the reaction is small (21.9 kJ mol⁻¹). As a consequence, the reverse reaction is not negligible. Even though the rate coefficient of the reverse reaction is much lower than that of the forward reaction, the concentration of H₂O in the reactor is much greater than that of formaldehyde, so the overall rates become comparable [13, 38]. This means that proton transfer between H_3O^+ ions and formaldehyde is less efficient, especially with increased humidity.



Ketones: R/R' = alkyl Aldehydes: R = H, alkyl, R' = H



Alcohols: R = alkyl

Figure 2.2 The mechanism of protonation for some OVOCs.

Similarly to the OVOCs, sulphur-containing and nitrogen-containing compounds have electron lone pairs to which the proton can bond (Figure 2.3).



Amines: R = H, alkyl

Figure 2.3 The mechanism of protonation for some sulphur-containing and nitrogen-containing compounds.

As proton transfer is mostly non-dissociative each VOC will produce one product ion, which is measured as the molecular mass plus one. If fragmentation does occur it is usually via a simple mechanism such as the elimination of a molecule of water, which can commonly occur following protonation of alcohols (Figure 2.4) [39], and some larger aldehydes and carboxylic acids [33, 40].



Figure 2.4 The elimination of water from a protonated alcohol.

2.2.3 Cluster ion chemistry

As a result of unreacted neutral water vapour molecules in the drift tube, H_3O^+ ions can become hydrated producing water cluster ions, $H_3O^+(H_2O)_n$:

$$H_2O^+(H_2O)_{n-1} + H_2O + X \longleftrightarrow H_3O^+(H_2O)_n + X$$
(2.3)

where X is a third body. The extent of water clustering increases as the humidity of the sample increases, so becomes more significant in humid samples such as breath. The water cluster ions can complicate interpretation of mass spectra as they also react with the sample VOCs in the drift tube. Taking the $H_3O^+(H_2O)$ cluster ion as an example, if the proton affinity of a VOC is greater than that of the water dimer (calculated to be 808 \pm 6 kJ mol⁻¹ [32]), then direct proton transfer can occur (Equation 2.4) [15, 41].

$$M + H_3O^+(H_2O) \longrightarrow MH^+ + 2H_2O$$
(2.4)
In such cases, this reaction will usually proceed as efficiently as proton transfer from H_3O^+ (Equation 2.1) [13, 42]. For molecules that are sufficiently polar, reactions with water cluster ions can also occur via the alternative route of ligand switching (Equation 2.5) [13, 42].

$$M + H_3O^+(H_2O) \longrightarrow MH^+(H_2O) + H_2O$$
(2.5)

$$\longrightarrow \mathrm{MH}^{+} + 2\mathrm{H}_{2}\mathrm{O} \tag{2.6}$$

The proton in the cluster ion complex formed by the switching reaction will be associated to the molecule with the largest proton affinity [42]. For molecules with a large proton affinity, the reaction becomes sufficiently exothermic to disrupt the bonding of the water ligand causing it to dissociate (Equation 2.6) [42], leaving MH⁺ ions analogous to that obtained via the direct proton transfer route. Therefore ligand switching reactions provide a pathway for polar VOCs to react with the water cluster ions in situations where they cannot do so by direct proton transfer. For non-polar compounds with a low proton affinity that therefore are unable to react with the water cluster ions, the sensitivity will be reduced as the sample humidity increases [16, 41] since the increase in cluster ion formation reduces the amount of 'free' H_3O^+ ions available for reaction.

The $MH^+(H_2O)$ cluster ions formed in Equation 2.5 are normally more weakly bound than $H_3O^+(H_2O)$ cluster ions and can be dissociated in the drift tube to form MH^+ ions [41]. The electric field applied over the length of the drift tube can be used to reduce cluster ion formation. The parameter E/N, where E is the electric field and N is the gas number density, is often used to describe drift tube conditions. Operating at high E/Nvalues, by either increasing the electric field or reducing the drift tube pressure and therefore lowering N, increases the collision energy between the cluster ions and buffer gas molecules, which can lead to dissociation. However, increasing the E/N ratio can also lead to fragmentation of the protonated analyte ions, resulting in a reduction of the protonated ion signal and also complicating spectral interpretation. It is therefore important to find an optimum E/N setting to minimise cluster formation but without causing severe fragmentation of the protonated analyte ions. Most PTR-MS instruments usually operate at E/N values between 100 – 140 Townsends (Td) [21], where 1 Td is equal to 10^{-17} V cm².

2.2.4 Drift tube calculations

As mentioned above, the E/N ratio is used to describe the conditions inside the drift tube. The electric field (in units of V cm⁻¹) is defined as the potential difference (Δ V) over a given distance (L), as shown in Equation 2.7.

$$E = \frac{\Delta V}{L} \tag{2.7}$$

The gas number density is the number of molecules in a given volume (V), so is given in units of cm⁻³, and can be calculated using the ideal gas law (Equation 2.8).

$$N = \frac{nN_{\rm A}}{V} = \frac{PN_{\rm A}}{RT}$$
(2.8)

In the previous equation, P is the drift tube pressure (mbar), N_A is Avogadro's number $(6.022 \times 10^{23} \text{ mol}^{-1})$, T is the drift tube temperature (K) and R is the universal gas constant (8.314 \times 10⁴ cm³ mbar K⁻¹ mol⁻¹, converted from SI units to experimental units). The E/N ratio can therefore be calculated using Equation 2.7 and Equation 2.8, and has overall units of V cm², although values are commonly reported in terms of Townsends.

In an electric field, positive ions will travel with a velocity in the field direction:

$$v_{\rm d} = \mu E \tag{2.9}$$

where v_d is the drift velocity (cm s⁻¹) and μ is the mobility (cm² V⁻¹ s⁻¹). In terms of the reduced mobility (μ_0) at standard temperature and pressure (STP: 1013.25 mbar, 273.16 K) the relationship becomes:

$$\mu_0 = \mu \frac{P}{P_0} \frac{T_0}{T} = \mu \frac{N}{N_0}$$
(2.10)

P, *T* and *N* represent the pressure, temperature and gas number density in the drift tube and P_0 , T_0 and N_0 represent the pressure, temperature and gas number density at STP conditions. Calculated using Equation 2.8, N_0 is equal to 2.687 × 10¹⁹ cm⁻³. Reduced mobility measurements for H₃O⁺ and H₃O⁺(H₂O) ions in nitrogen have been determined to be 2.76 and 2.28 cm² V⁻¹ s⁻¹ respectively [43]. Substituting Equation 2.10 into Equation 2.9 gives:

$$v_{\rm d} = \mu_0 N_0 \left(\frac{E}{N}\right) \tag{2.11}$$

Equation 2.11 shows that the ion drift velocity is proportional to the E/N ratio. The reaction time will therefore show an inverse relationship to E/N, and can be estimated using:

$$t = \frac{L}{v_{\rm d}} = \frac{L}{\mu_0 N_0} \left(\frac{E}{N}\right)^{-1}$$
(2.12)

The average centre-of-mass kinetic energy (KE_{cm}) for a collision between an ion-neutral pair in the drift tube, such as between a H_3O^+ ion and an analyte molecule, can be calculated using Equation 2.13 [15, 44].

$$KE_{cm} = \frac{\left(m_{ion} + m_{buffer}\right)m_{neutral}}{2\left(m_{ion} + m_{neutral}\right)}v_{d}^{2} + \frac{3}{2}k_{B}T$$
(2.13)

In Equation 2.13, m_{ion} , m_{buffer} and m_{neutral} are the masses of the ions, buffer gas molecules and neutral molecules respectively, k_{B} is the Boltzmann constant (1.380 × 10⁻¹⁹ cm² kg s⁻² K⁻¹, converted from SI to experimental units) and *T* is the buffer gas temperature. When the buffer gas is air, the average mass of 28.8 u is used [1]. For collisions between ions and buffer gas molecules, m_{neutral} can be substituted for m_{buffer} [32]. Equation 2.13 shows that by increasing the *E/N* value and therefore increasing the ion drift velocity, the energy for an ion-neutral collision is increased, hence the use of higher *E/N* values to reduce cluster ion formation.

2.2.5 Time-of-flight mass spectrometry

The following is a brief overview of the operating principles of time-of-flight mass spectrometry, the mass spectrometric technique used throughout this thesis. The basis of time-of-flight analysers is that a discrete packet of ions of different m/z ratios with the same initial kinetic energy will take different times to transverse a field-free region called a flight tube. The m/z ratio is determined by measuring the time it takes the ions to pass through the field free region to the detector.

In the acceleration region, ions acquire the same kinetic energy. An ion of mass m and charge q accelerated by a potential V will have a kinetic energy defined by Equation 2.14 and Equation 2.15.

$$KE = qV = zeV \tag{2.14}$$

$$KE = \frac{1}{2}mv^2 \tag{2.15}$$

In Equation 2.14, z is the charge number and e is the elementary charge. It is then possible to equate these two terms and determine the drift velocity as shown in Equation 2.16.

$$v_{\rm d} = \sqrt{\frac{2zeV}{m}}$$
(2.16)

As the ions have the same kinetic energy but different mass, the ions will have different velocities and reach the detector at different times. Equation 2.16 shows that drift velocity is inversely proportional to mass, so lighter ions will have a greater velocity and reach the detector first. Over a distance L, the ions will be separated based on the ion flight time, t.

$$t = \frac{L}{v_{\rm d}} = L \cdot \sqrt{\frac{1}{2eV}} \cdot \sqrt{\frac{m}{z}}$$
(2.17)

Equation 2.17 shows how the measurement of ion flight times can be used to calculate the mass-to-charge (m/z) ratio. The ability to distinguish between ions of close mass is known as the resolution. For TOF-MS, the resolution (R) is defined by Equation 2.18 [6].

$$R = \frac{m}{\Delta m} = \frac{t}{2\Delta t} \tag{2.18}$$

In this equation, *m* and *t* are the mass and flight time of an ion, and Δm and Δt are the full width-half maximum (FWHM) of a peak in the resulting spectrum when presented on either a mass or time scale.

Prior to the point of extraction, ions will have some initial kinetic energy and spatial distributions, so that after injection into the flight tube ions of the same mass will have slightly different energies, and therefore slightly different flight times, which will result in broader peaks in the mass spectrum [6, 45]. Mass resolution can be improved by using a reflectron-TOF analyser, also described as an 'ion mirror', whereby an electric field is used to deflect the ions back through the flight tube to an off-axis detector [6]. Ions of a higher kinetic energy penetrate the reflectron more deeply than the lower

energy ions, therefore spending more time entering and leaving the reflectron such that the ions arrive at the detector at the same time. The use of a reflectron also increases ion flight length and therefore flight time, which improves separation of ions of similar mass and further improves resolution.

2.3 The Leicester CIR-TOF-MS instrument

2.3.1 Overview

Full details of the development and characterisation of the CIR-TOF-MS instrument can be found in the theses of R. S. Blake [3] and K. P. Wyche [4]. The instrument consists of a radioactive ion source and drift tube that were built in-house, coupled to a commercial orthogonal reflectron time-of-flight mass spectrometer (Kore Technology Limited, Ely, UK) (Figure 2.5 and Figure 2.6). The main features of the CIR-MS instrument are described below.



Figure 2.5 The Leicester CIR-TOF-MS instrument (dimensions $2.0 \times 0.8 \times 1.8$ m).



Figure 2.6 A schematic diagram of the CIR-TOF-MS instrument.

2.3.2 Gas inlet system

The gas inlet system supplies the ion source/drift tube with a constant flow of water vapour and sample gas. The gas flow rates are controlled by mass flow controllers (MFCs) (Tylan FC260). To minimise memory effects, all gas lines are made of perfluoroalkoxy (PFA) polymer tubing (Swagelok, Manchester, UK), and all fittings of the gas inlet system are made of either PFA (Galtek integral ferrule fittings, Entegris) or stainless steel (Swagelok). Gas lines between the MFCs and the ion source/drift tube are maintained at 40°C through the use of a heating wire. This prevents condensation in the lines when analysing a humid sample.

Water vapour is generated by bubbling high-purity nitrogen (grade N6.0, BOC Special Gases) through a glass vessel containing high-purity deionised water (15 M Ω). The nitrogen flow through the vessel is regulated by a needle valve.

During breath analysis, the breath sampling apparatus is simply connected to the gas inlet unit before the sample MFC. Details regarding the sampling apparatus are described in Chapter 3.

2.3.3 Ion source and drift tube

The radioactive ion source and drift tube (see Figure 2.7) are based on a design by Hanson et al. [18]. The ion source consists of a radioactive strip of ²⁴¹Am (NRD, Grand Island, NY, USA) mounted inside a stainless steel ring and housed in a stainless steel surround. The radioactive strip emits α -particles with an energy around 5 MeV. A continuous flow of water vapour enters the top of the ion source at a set flow rate of 30 sccm (sccm: standard cubic centimetres per minute). Water molecules are ionised by the α -particles to form H₃O⁺ ions, which are assumed to proceed via the mechanism shown in the following equations [46].

$$H_2 O \xrightarrow{\alpha} H_2 O^{\bullet +}$$
(2.19)

$$H_2O^{\bullet+} + H_2O \longrightarrow H_3O^+ + HO^{\bullet}$$
(2.20)

The drift tube, situated directly below the ion source, is approximately 11 cm in length and consists of a series of 6 stainless steel ring electrodes (0.2 cm depth) separated by 2 cm insulating Semitron spacers. Viton O-rings sit in a groove in the upper and lower surface of the spacers to provide a seal. A thin spacer (Tufnol, 0.17 cm) separates the last electrode from the base flange. The small region before the exit of the drift tube is referred to as the collision dissociation cell (CDC) and is 0.8 cm in length.

The sample is continuously introduced at a flow rate of 220 sccm into the upstream end of the drift tube, through the wall of the spacer located between E2 and E3. The combined sample and vapour flows result in a drift tube pressure of 6 mbar measured by either a Baratron (MKS) or Pirani (Leybold) pressure gauge. The downstream end of the drift tube is evacuated using a rotary vane pump (5 m³ h⁻¹, Edwards RV5). If lower

sample and vapour flows are required, the amount of gas removed by the rotary pump can be restricted by partially closing an in-line tap, thus maintaining the drift tube pressure. The drift tube temperature is kept constant using a heating wire, which is coiled around the outside and maintains a temperature of 40°C.



Figure 2.7 A diagram of the ion source/drift tube arrangement of the CIR-TOF-MS instrument. E1 – E6 represents electrodes 1 – 6 and R1 – R6 represents resistors 1 – 6, of which R1 – R5 are fixed at 1 M Ω and R6 is variable between 0 – 1 M Ω . SI, VI, PG and PO refer to the sample and vapour inlets, pressure gauge and pump outlet respectively. The main drift cell region is located between E1 and E6 and the collision cell between E6 and ground (G); the two regions can be operated at different *E/N* values. (Diagram courtesy of C. Whyte).

The electrodes are connected to a resistor chain consisting of five 1 M Ω resistors and one variable resistor (maximum 1 M Ω , usually set to 860600 Ω) over the CDC. The top electrode (E1) is held at a high positive potential of 1320 V, which decreases with each subsequent electrode, creating a voltage gradient to draw the reagent ions from the ion source into the drift tube. The main section of the drift tube experiences a constant electric field of 113 V cm⁻¹, whilst the chosen setting of the variable resistor over the CDC results in a higher electric field of 239 V cm⁻¹, which helps to collisionally dissociate cluster ions prior to detection. Using the operating conditions described, the number density within the drift tube has been calculated to be 1.388×10^{17} cm⁻³. The drift tube voltage and variable resistor setting given above provide the desired *E/N* value of 80/170 Td, where the first value refers to that of the main drift cell and the second value refers to that of the CDC. The reasons behind this choice of *E/N* value are discussed in Sections 2.4.3 and 2.4.4.

The sample ions exit the drift tube via a 200 μ m aperture. The size of the aperture dictates the flow of gas from the drift tube into the mass spectrometer; a smaller aperture here would allow higher pressures in the drift tube whilst maintaining acceptable pressures downstream in the analyser, but would reduce the ion transmission [3]. The 200 μ m aperture provides a compromise between the ion transmission and the drift tube pressure.

2.3.4 Mass analyser and detection

After passing through the aperture, ions enter the transfer chamber containing the Einzel lens elements for focussing the ion beam. The chamber is evacuated by two turbomolecular pumps (70 L s⁻¹, Varian V70) each backed by a rotary pump. The ion beam then passes into a secondary chamber containing the pulsed extraction system for injecting small packets of ions into the orthogonally positioned reflectron time-of-flight analyser. The chamber is evacuated by a large turbomolecular pump (255 L s⁻¹, BOC Edwards EXT 255) backed by a rotary pump, positioned at the base of the chamber. The system is differentially pumped throughout such that when a relatively high pressure exists in the drift tube, the low pressure required in the analyser is still achieved. The pressure inside the TOF-MS is in the region of 9×10^{-7} Torr (approximately 1.2×10^{-6} mbar), as measured by a pressure gauge (cold cathode gauge, MKS Series 943) situated in the secondary chamber.

Inside the flight tube of the TOF-MS, sample ions are separated according to their mass and detected by a micro-channel plate (MCP) detector. The output from the MCP is sent via a pre-amplifier to a time-to-digital converter (TDC). The TDC is responsible for triggering the pulsed extractor and recording ion arrival times at the detector.

2.3.5 Data collection and processing

Data collection is controlled through the supplied GRAMS/AI software (Thermo Scientific). GRAMS/AI converts the time data from the TDC into mass-to-charge ratios (m/z) using Equation 2.21, displaying the raw spectra as a plot of recorded signal in counts against m/z.

$$m/z = \left(\frac{t - t_0}{C_{\rm b}}\right)^2 \tag{2.21}$$

In the previous equation, t is the arrival time of the ion, and t_0 and C_b are conversion parameters determined by the software after calibration to two peaks of known mass. Approximate values for t_0 and C_b are 0.39 and 5.58 respectively under normal experimental settings.

The time taken to acquire a single scan is about 80 μ s for a mass range up to 200 u, so around 10⁴ scans s⁻¹ can be achieved. Several scans are integrated to obtain meaningful data through the improvement of the signal-to-noise (S/N) ratio. One second integration times are used for on-line breath measurement, while most other experiments typically use one minute integration times. The outputted mass spectra represent the accumulation of scans over the chosen integration time. Experimental parameters such as mass range (usually 12 – 200 u), experiment length (integration time) and the number of experiments are set in the collect options of GRAMS/AI. Since the masses of most VOCs do not exceed 200 u, this value was typically used as the upper mass limit for the experiments throughout this thesis. For the data to be processed and analysed outside of the GRAMS/AI software, two separate programs are used, MaxiSum and MaxiGroup [3]. Firstly the MaxiSum program transforms the raw mass spectral data through a summing process, effectively integrating the area under each peak over a defined window on either side of each nominal mass value (\pm 0.3 u). This produces an integrated signal for every mass channel. The MaxiGroup program then outputs the data as an Excel-readable file and has the optional function of summing the data to effectively model larger integration times.

2.3.6 Normalisation

Using the convention described by Warneke et al. [16], data are normalised to 10^6 reagent ion counts per second, whereby 'reagent ions' are classed as both H_3O^+ and $H_3O^+(H_2O)$, using the following equation.

$$i(\mathrm{MH}^{+})_{\mathrm{ncps}} = i(\mathrm{MH}^{+})_{\mathrm{cps}} \times \left(\frac{10^{6}}{i(\mathrm{H}_{3}\mathrm{O}^{+})_{\mathrm{cps}} + i(\mathrm{H}_{3}\mathrm{O}^{+}(\mathrm{H}_{2}\mathrm{O}))_{\mathrm{cps}}}\right)$$
 (2.22)

In Equation 2.22, $i(MH^+)_{ncps}$ is the normalised protonated analyte signal and $i(MH^+)_{cps}$, $i(H_3O^+)_{cps}$ and $i(H_3O^+(H_2O))_{cps}$ are the raw signals of the protonated analyte, H_3O^+ ion and $H_3O^+(H_2O)$ ion respectively. The normalisation process tries to account for any changes in reagent ion signal over time.

2.3.7 Gas standards generator

Throughout this thesis the CIR-MS instrument was calibrated using a gas standards generator that allowed the generation of multi-component calibration gas mixtures in the laboratory using permeation tubes, which produced precise gas concentrations whilst being maintained at a given temperature. Calibration gas mixtures were generated using a KIN-TEK 491M modular gas standards generator (supplied by Eco Scientific,

Stroud, UK), consisting of 491M-B base module coupled to a 491M-PM auxiliary module and a 491M-HG humidification module. The base module consisted of a single glass permeation oven housed inside an accurately controlled heating block (\pm 0.1°C), and the dilution gas controls. Permeation tubes were placed inside the oven that was set to the temperature at which the tubes were certified. Nitrogen was used as the carrier gas, a small flow (0.1 L min⁻¹) of which was passed through the oven to collect the permeate before recombining with a larger dilution gas flow. The auxiliary module, situated downstream of the base module, provided an additional permeation oven that could be set to a different temperature, therefore allowing the inclusion of more compounds in the generated mixture. The dilution gas flow was varied to give different calibration gas concentrations, and had an operating range of 0.2 to 9 L min⁻¹. The lower limit came from the minimum flow required over the two ovens, although the minimum experimental dilution flow also needed to be greater than the sample inlet flow to the CIR-MS instrument.

Concentrations were calculated using Equation 2.23.

$$C = \frac{\left(E \times K_0\right)}{\left(F_{\rm T} \times 1000\right)} \tag{2.23}$$

In the previous equation, *C* is the concentration in ppmv, *E* is the emission rate in ng min⁻¹, F_T is the total dilution gas flow in L min⁻¹, and K_0 is a compound specific constant which converts the emission rate from units of ng min⁻¹ to nL min⁻¹. This constant was calculated using Equation 2.24, where *M* is the molecular weight of the compound.

$$K_0 = \frac{22.4}{M}$$
(2.24)

The humidification module sat downstream of both the base and auxiliary modules. Water vapour was added without the trace components of the mixture coming into contact with water, so there was no loss of soluble compounds. A portion of the dry dilution gas was passed through a saturator, which consisted of a highly permeable tube immersed in water. Water permeated through the membrane into the gas flow to create a saturated mixture, which was then added to the flow from the base/auxiliary modules to form the final calibration gas. The level of humidity was determined by the ratio of the dry to the saturated flow.

Relative humidity is a temperature dependent value, and as the temperature inside the humidity module differed from the temperature that the mixture was delivered into, i.e. ambient laboratory temperature, the relative humidity reading on the module had to be corrected (Equation 2.25).

$$RH = RH_{S} \times CF = RH_{S} \times \left(\frac{P_{S}}{P_{D}}\right)$$
(2.25)

In Equation 2.25, RH is the relative humidity of the calibration gas (i.e. the mixture entering the CIR-MS instrument), RH_S is the relative humidity measured by the sensor inside the humidity module, and CF refers to the correction factor, which was determined by dividing the vapour pressure at the sensor temperature (P_S) by the vapour pressure at the delivery temperature (P_D). In experimental terms, a target RH_S value was usually calculated for a required RH to make sure that the calibration was carried out at the correct humidity.

It was possible to heat the sample line between the humidifier output and the mass spectrometer inlet so to raise the delivery temperature and equal that measured at the sensor, in which case the RH_s reading would have represented the true RH. In practice this made it difficult to achieve high humidity values as the RH_s limit of the humidity module was in the region of 85 - 90%, so the only way of obtaining a saturated mixture was to deliver the mixture into cooler conditions. This point is illustrated in Table 2.2. When sensor and delivery temperatures are equal the CF is 1 (Table 2.2 (a)), so to obtain a saturated mixture the humidifier has to reach a RH_s of 100%, which in practice

is not possible. The CF increases with increasing sensor temperature and decreasing delivery temperature, so a saturated calibration gas could be achieved with RH_S values as low as 50% (Table 2.2 (b)). The sensor temperature could not be controlled, but generally increased the longer the instrument was running.

Table 2.2 (a) Some example correction factors calculated using Equation 2.25. The $P_{\rm S}$ and $P_{\rm D}$ values used to calculated the correction factors are available from literature sources e.g. [31]. (b) An example of how the correction factor affects the RH_S value, for a true RH of 100%.

(a)	Sensor Temperature						(b)	Sensor Temperature				
Room Temperature	°C	30	32	34	36		oom Temperature	°C	30	32	34	36
	24	1.423	1.594	1.784	1.993			24	70.3	62.7	56.1	50.2
	26	1.263	1.415	1.583	1.769			26	79.2	70.7	63.2	56.5
	28	1.123	1.258	1.408	1.573			28	89.1	79.5	71.0	63.6
	30	1.000	1.121	1.254	1.401		Я	30	100.0	89.2	79.7	71.4

It was often found that after setting the required RH_S , measurements would show a slow but gradual decline over the duration of the experiment. In addition to this, sudden 'spikes' in humidity were sometimes observed, which may have been an effect relating to the sensor rather than a true increase in humidity, but meant that for a period of time the RH_S readings were uncertain. To assist data collection, measurements were considered valid if the RH_S was kept within a \pm 3% range of the target true RH; data recorded outside of this range were excluded. It was somewhat easier when requiring 100% RH as any value above the equivalent RH_S would achieve saturation.

For humid samples, the calculation had to be modified to take into account the extra dilution caused by the water vapour, as shown in Equation 2.26, where $C_{\rm H}$ is the concentration after the correction for humidity.

$$C_{\rm H} = \left(1 - \left(\frac{\rm RH_{\rm s} \times P_{\rm s}}{100 \times P_{\rm D}}\right)\right) \times C \tag{2.26}$$

Alternatively, if a specific concentration value was required, the necessary flow could be calculated using Equation 2.27, where F_D is the dry dilution flow set on the base module.

$$F_{\rm D} = \left(1 - \left(\frac{\mathrm{RH}_{\mathrm{S}} \times P_{\mathrm{S}}}{100 \times P_{\mathrm{D}}}\right)\right) \times F_{\mathrm{T}}$$
(2.27)

At 0% RH, $F_{\rm D}$ and $F_{\rm T}$ are equal.

A number of permeation tubes were used throughout this thesis, as shown in Table 2.3. All permeation tubes were certified gravimetrically, whereby the devices were weighed periodically whilst being maintained at a constant temperature, until a steady weight loss per unit time was achieved (within 2 - 5% accuracy).

Table 2.3 Permeation tube details. All permeation tubes were disposable tubular devices, from either VICI (VICI Metronics, Poulsbo, WA, USA) or KIN-TEK (Supplied through Eco-Scientific, Stroud, UK).

Permeation Tube	Tube Type	Supplied by	Emission Rate (ng min ⁻¹)	Certification Temperature (°C)	
Ammonia	Low Emission	VICI	545	30	
Acetonitrile	Standard	VICI	167	30	
Acetaldehyde	Standard [*]	KIN-TEK	86	30	
Acetone	High Emission	VICI	541	30	
Dimethyl sulphide	Standard	VICI	17.8	30	
Pent-1-ene	High Emission	KIN-TEK	357	30	
Acetic Acid	High Emission	VICI	626	40	
Isoprene	Standard	KIN-TEK	33	40	
Methanol	Standard	KIN-TEK	62	45	
Ethanol	High Emission	VICI	1354	70	
Hexan-2-one	High Emission	KIN-TEK	811	80	

* Extended life permeation tube

2.4 CIR-MS instrument characterisation for breath measurement

2.4.1 CIR-MS mass spectrum and mass resolution

Figure 2.8 shows an example of a raw CIR-MS mass spectrum, displaying the m/z against the signal intensity, in comparison to that obtained after data processing, i.e. after peak integration.



Figure 2.8 Comparison of a raw (top) and a processed (bottom) CIR-MS mass spectrum of breath.

The dominant peak at m/z 19 represents the H₃O⁺ reagent ion signal, and the secondary peak at m/z 37 represents the H₃O⁺(H₂O) cluster ion signal. As CIR-MS/PTR-MS instruments produce only singly charged ions, the m/z scale can be treated as a mass scale. In the CIR-MS system, NO⁺ (m/z 30) and O₂⁺ (m/z 32) impurity ions are observed at low intensities of less than 0.5% and less than 0.01% respectively, relative to the H₃O⁺ signal. Raw integrated H₃O⁺ count rates of 2 – 4 × 10³ counts s⁻¹ (cps) are typically acquired while scanning a 12 – 200 u mass range.

The mass resolution $(m/\Delta m)$ of the CIR-MS instrument under standard operating conditions is displayed as a function of mass in Figure 2.9, and was determined to be in excess of 1000 over the majority of the mass range. This is an order of magnitude greater than that reported for a quadrupole PTR-MS instrument [18] and a linear-PTR-TOF system [19], both of which quoted $m/\Delta m \sim 100$, although it is thought that the resolution would need to be greater than 2000 before it becomes possible to distinguish between nominally isobaric species [21]. Resolution sufficient to determine between the nominal isobars ethanol and methanoic acid has been demonstrated for a reflectron-PTR-TOF system [27].



Figure 2.9 The CIR-MS mass resolution as a function of m/z.

The CIR-MS instrument has previously demonstrated the use of alternative CI reagents to distinguish between isobaric species based on the different ionisation pathways for different functional groups, e.g. aldehyde and ketone isobars [2]. This work was carried out using dry samples with residual water vapour being suppressed using a cold trap to minimise the possibility of H_3O^+ formation, which could interfere with the subsequent analysis. Therefore, the application of alternative CI reagents to determine isobars in humid samples such as exhaled breath would be more difficult. Exploratory breath measurements made using NO⁺ found that a reasonable reagent ion signal could be generated and maintained, but the presence of water in the system meant there was significant interference from H_3O^+ and its ionisation products, so further work would be required to resolve this problem and investigate its use in breath analysis.

2.4.2 Reagent ion distribution in the drift tube

The generation of $H_3O^+(H_2O)_n$ cluster ions increases with the humidity of the sample being analysed, therefore making the effects of ion clustering particularly relevant during the analysis of exhaled breath. Increasing the *E/N* of the CDC can be utilised to reduce cluster ion formation by increasing the collision energy with the buffer gas molecules, with the aim of dissociating the weakly bound MH⁺(H₂O) ions back to their unhydrated form, although the H₃O⁺(H₂O)_n cluster ion abundance can be similarly affected.

Figure 2.10 shows the relative intensities of the H_3O^+ , $H_3O^+(H_2O)$ and $H_3O^+(H_2O)_2$ ions measured as a function of CDC *E/N* under both dry and humid sampling conditions. The results indicated that most reagent ions would be present as either H_3O^+ or $H_3O^+(H_2O)$, with the larger $H_3O^+(H_2O)_2$ cluster ions only becoming noticeable at low CDC *E/N* or at increased sample humidity. Even under dry sample conditions, some $H_3O^+(H_2O)$ cluster ions were present as a result of water vapour from the ion source reaching the drift tube [16]. Whilst effective at reducing cluster ion populations and therefore simplifying the resultant mass spectra, the use of the CDC masks the true ion distributions in the drift tube, underestimating the degree of cluster ion formation. Figure 2.10 shows that the $H_3O^+(H_2O)$ cluster ions dominate at the lowest CDC *E/N* of 120 Td, only 40 Td greater than the typical reactor cell *E/N*, with the H_3O^+ and $H_3O^+(H_2O)_2$ ions being present in smaller amounts, although under humid sampling conditions the amount of H_3O^+ ions is minimal. This indicated that cluster ion chemistry would dominate in the reaction region where lower *E/N* values are typically used.



Figure 2.10 The variation in the H_3O^+ , $H_3O^+(H_2O)$ and $H_3O^+(H_2O)_2$ ion distribution as a function of collision cell E/N, with the main drift cell E/N maintained at 80 Td. The data are presented as the percent of the total ion signal, i.e. the sum of H_3O^+ , $H_3O^+(H_2O)$ and $H_3O^+(H_2O)_2$ raw signals. The circles/solid lines represent the measurements made whilst analysing a dry sample gas, and the triangles/dotted lines represent the measurements made whilst analysing a humid (100% RH) sample gas.

The change in the measured $H_3O^+(H_2O)$ abundance with sample humidity at a specific *E/N* setting can exploited as an internal reference for estimating the humidity of the sample. The trend between the $H_3O^+/H_3O^+(H_2O)$ ratio, or *m/z* (19/37), and the relative humidity of the sample is displayed in Figure 2.11. The higher the sample humidity, the lower the ratio will be as a result of the increased $H_3O^+(H_2O)$ ion signal. By extrapolation, *m/z* (19/37) for a completely dry sample should equal 26, while a saturated sample would have a value of 3.



Figure 2.11 The change in the $H_3O^+/H_3O^+(H_2O)$ ratio, m/z (19/37), as a function of sample humidity at an E/N ratio of 80/170 Td. The data are fitted with an exponential decay trendline. At a sample relative humidity of 0%, the m/z (19/37) has been calculated to be 26.

2.4.3 Instrument sensitivity: sample humidity and drift tube E/N

Quantitative analysis in mass spectrometry aims to find the relationship between the signal intensity and analyte concentration. This is achieved through the calibration of the instrument response to a series of known concentrations. If a plot of the analyte concentration against the measured signal shows good linear response, the instrument sensitivity can be determined from the gradient of a linear fit to the data. Therefore, the experimentally determined compound-specific sensitivity value ($S_{\rm M}$) is defined as:

$$S_{\rm M} = \frac{i(\rm MH^+)}{C_{\rm M}}$$
(2.28)

where $i(MH^+)$ is the measured signal in ncps and C_M is the concentration in ppbv. The sensitivity is therefore in units of ncps ppbv⁻¹. Humidity-dependent sensitivities have

been reported for some VOCs [15, 16, 41], owing to the changes in ion chemistry associated with the increasingly abundant water cluster ions. This may have important implications for the detection of some compounds in breath.

As described in Section 2.3.3, the drift tube essentially consists of two parts; the main reactor cell where the chemical ionisation reactions occur, and the CDC before the drift tube exit, which can operate at an independent E/N value. The E/N values chosen over these two sections need to be balanced to maximise the protonated ion signal, and hence the sensitivity of the target species. To first assess the effect of the reactor cell conditions on sensitivity, a series of calibration experiments were performed for a group of compounds that consisted of ammonia, acetaldehyde, acetone, pent-1-ene and hexan-2-one, using the permeation tubes detailed in Table 2.3 and the gas standards generator described in Section 2.3.7. These compounds offered a selection of chemical functional groups with differing proton affinities (768.5 – 853.6 kJ mol⁻¹) and dipole moments (0.50 – 2.88 D). The calibrations were carried out at relative humidities between 0 – 100% (in steps of 20% RH) and main drift cell E/N values between 80 – 140 Td (in steps of 20 Td), whilst keeping the CDC constant at 170 Td.

Figure 2.12 displays sensitivity contour plots for ammonia, acetone and hexan-2-one as a function of E/N and relative humidity. The sensitivities of these three compounds appeared to show little dependence on the humidity of the sample, although they were affected by the change in E/N. The independence of the sensitivity with humidity could have been explained by the high proton affinities and dipole moments of these compounds. The proton affinities of ammonia and acetone are 853.6 kJ mol⁻¹ and 812.0 kJ mol⁻¹ respectively. The proton affinity of hexan-2-one has not yet been determined, but given that proton affinities increase with carbon chain length, it was assumed that the value for hexan-2-one would be greater than that for acetone, and the value of 843.2 kJ mol⁻¹ quoted for hexan-3-one [21] provides a reasonable estimation. Therefore, these three compounds can react via direct proton transfer with both H₃O⁺ and H₃O⁺(H₂O) ions. Additionally, the large dipole moments of ammonia, hexan-2-one and acetone (1.47, 2.80 and 2.88 D respectively) mean that ligand switching reactions with the

 $H_3O^+(H_2O)_n$ cluster ions can also occur [42]. Given the large proton affinities of these compounds, ligand switching with $H_3O^+(H_2O)$ cluster ions is likely to be dissociative, so would also result in the production of MH^+ ions. For ligand switching with larger water cluster ions (n = 2, 3), acetone has been found to mainly produce $MH^+(H_2O)$ ions [42], in which case the weakly bound water ligand could have been dissociated in the CDC. Therefore, as the humidity was increased and the reagent ion distribution changed, the ability to form MH^+ product ions remained largely unaffected. However, the sensitivities were found to decrease with increasing *E/N*. Given that this corresponded to an increase in the collision energy, the initial response might have been to suggest that the protonated analyte ions were being fragmented, although this would have been unlikely to occur for a small molecule such as ammonia. This observation may have been better explained by the increase in the drift velocity, and therefore a reduction in the reaction time [4].



Figure 2.12 Sensitivity as a function of main drift cell E/N and relative humidity for ammonia, acetone and hexan-2-one respectively.

Figure 2.13 shows a similar sensitivity contour plot for acetaldehyde. Acetaldehyde has a lower proton affinity (768.5 kJ mol⁻¹) than the previous compounds, such that direct proton transfer cannot occur between acetaldehyde and $H_3O^+(H_2O)$ ions. However, the large dipole moment (2.75 D) means that acetaldehyde is still able to react with

 $H_3O^+(H_2O)_n$ cluster ions via ligand switching. Given the proton affinity of acetaldehyde, the exothermicity of the ligand switching reaction is likely to be insufficient to cause dissociation and would therefore result in the formation of $MH^+(H_2O)_n$ ions [42]. The greatest sensitivity for protonated acetaldehyde was observed at low humidity and low E/N conditions. At low E/N values the reagent ions would be largely present in cluster form, so the observed MH⁺ ions were probably formed from the dissociation of $MH^{+}(H_2O)$ ions in the CDC. This would have also explained the low sensitivity for the $MH^+(H_2O)$ ions in this region (Figure 2.13). The sensitivity towards the MH^+ ion was found to decrease as the sample humidity was increased, suggesting that the reagent ion distribution was being shifted towards the formation of larger $H_3O^+(H_2O)_n$ ions, and therefore the production of larger $MH^+(H_2O)_n$ ions. These larger $MH^+(H_2O)_n$ ions were probably dissociated in the CDC to MH⁺(H₂O) ions, hence the observed increase in the $MH^+(H_2O)$ sensitivity at high humidity. At higher E/N values the increased availability of H_3O^+ ions for proton transfer may have been expected to increase the MH^+ sensitivity, however it appeared that this was counterbalanced by fragmentation and reaction time effects since a reduction in sensitivity was observed. Acetaldehyde can form acyl fragment ions (CH₃CO⁺, m/z 43), although this is not the only species to fragment to this mass channel (see below).



Figure 2.13 Sensitivity as a function of main drift cell E/N and relative humidity for acetaldehyde and the acetaldehyde-water cluster ion respectively.

Figure 2.14 shows the sensitivity contour plot for pent-1-ene. No proton affinity is available for pent-1-ene, but given that the proton affinity for hex-1-ene is $805.2 \text{ kJ mol}^{-1}$ [9], and that proton affinities decrease with carbon chain length, pent-1-ene cannot react by proton transfer with H₃O⁺(H₂O) cluster ions. Pent-1-ene also has a low dipole moment (0.5 D) and is also unable to undergo ligand switching, so there is no means for this molecule to react with water cluster ions. Therefore, the greatest sensitivity would have been expected to occur at conditions where cluster ion formation was minimal, i.e. low humidity and high *E/N*. While the area of highest sensitivity was indeed found at low humidity, high *E/N* values were found to have a negative effect on sensitivity. The reduction in pent-1-ene sensitivity above 100 Td correlated with the increased formation of alkyl fragment ions (C₃H₇⁺, *m/z* 43) and also of allyl fragment ions (C₃H₅⁺, *m/z* 41) at *E/N* values greater than 120 Td. As sample humidity increased, pent-1-ene sensitivity decreased in response to the reduced availability of 'free' H₃O⁺ ions.



Figure 2.14 Sensitivity as a function of main drift cell E/N and relative humidity for pent-1-ene and fragments of pent-1-ene respectively.

For the measurement of species in exhaled breath, given the high relative humidity, optimal sensitivity may have been expected when operating at higher E/N values, since this would be associated with a reduction in the amount of cluster ion formation. However, the results showed that a lower reactor cell E/N correlated with the greatest

sensitivity for most of the test compounds, especially the OVOCs. With the exception of the hydrocarbon lipid peroxidation products, of which ethane and pentane cannot be detected by CIR-MS, many of the commonly reported breath VOCs are polar molecules that would generally be expected to display similar sensitivity patterns to that demonstrated in Figure 2.12 and Figure 2.13. Therefore, a reactor cell E/N value of 80 Td was judged to be the most suitable for breath measurements.

2.4.4 Instrument sensitivity: CDC E/N

The CDC offers a region that can operate at an E/N higher than that of the main drift cell, which can assist the dissociation of cluster ions. Conversion of weakly bound MH⁺(H₂O) cluster ions to their unhydrated form benefits from an increased protonated analyte signal and simplification of the mass spectra, although too high a collision energy in the CDC can also have a negative effect by inducing fragmentation. In the previous sensitivity investigation, the CDC E/N remained constant at 170 Td whilst the effect of changing the reactor cell E/N was monitored. In addition to determining the optimum E/N value for the reaction chamber, the E/N for the CDC required similar investigation. Using the method described previously, calibration experiments were carried out for two hydrocarbons, isoprene and pent-1-ene, and two OVOCs, acetaldehyde and acetone. The calibrations were performed under dry conditions only, varying the CDC E/N between 110 – 190 Td (in 20 Td steps), whilst keeping the main reactor cell constant at 100 Td.

Figure 2.15 displays the sensitivity as a function of the CDC E/N for the two hydrocarbon compounds. Both isoprene and pent-1-ene showed the greatest sensitivity at E/N values below 150 Td. Increasing the E/N beyond 150 Td started to reduce the sensitivities as a result of the fragmentation of the protonated analyte ions. The fragments at m/z 41 and m/z 43 in this particular case were thought to arise mainly from pent-1-ene, although isoprene could have also contributed to m/z 41, as could acetaldehyde to m/z 43 in the form of an acyl fragment ion rather than an alkyl fragment ion. The opposite trend was found for the OVOCs, where the greatest sensitivity was

observed at E/N values above 150 Td (Figure 2.15). The ability of the OVOCs to undergo ligand switching reactions results in the formation of MH⁺(H₂O) ions, which can be dissociated to the core MH⁺ ions if the E/N, and therefore collision energy, in the CDC is sufficient. In moving to lower E/N values, the degree of cluster dissociation was reduced and a larger proportion of the analyte was contained as the hydrated form. As such, the sensitivity towards the protonated analyte was reduced.



Figure 2.15 Sensitivity as a function of CDC E/N for the hydrocarbons isoprene and pent-1-ene (upper plot), and the OVOCs acetaldehyde and acetone (lower plot). Legend assignments: m/z 45 acetaldehyde MH⁺ ion, m/z 63 acetaldehyde MH⁺(H₂O) cluster ion, m/z 59 acetone MH⁺ ion, m/z 77 acetone MH⁺(H₂O) cluster ion, m/z 69 isoprene MH⁺ ion, m/z 71 pent-1-ene MH⁺ ion, m/z 43 and m/z 41 fragment ions (F⁺).

Based on the compounds that were tested, the most suitable CDC *E/N* appeared to lie between 150 - 170 Td. *E/N* values above this range caused extensive fragmentation of the hydrocarbon ions, while lower *E/N* values caused the OVOCs to become hydrated. These experiments were carried out under dry conditions, so for the analysis of exhaled breath a significant increase in the abundance of $H_3O^+(H_2O)$ ions, and therefore MH⁺(H₂O) ions, would be expected. As a result, the upper limit of 170 Td was determined to be the optimum setting for the CDC. The combination of a low reactor *E/N* for optimum ionisation conditions with a relatively high CDC *E/N* to dissociate cluster ions was thought to provide the most suitable conditions for the analysis of VOCs in exhaled breath.

2.4.5 Correction of increased permeation rates

As detailed in Section 2.3.7, the calibration of the CIR-MS instrument was carried out using permeation tubes and a laboratory gas standards generator. During some calibrations, the temperature of the permeation oven inside the gas standards generator was found to be higher than the required oven temperature of 30°C that was needed for most of the permeation tubes. The combined effects of a reasonably high laboratory temperature (at times up to 26°C) and the heat generated by the gas standards generator, especially when the humidifier module was in use, were the most likely cause. If a humidified gas mixture was required, the effect could be minimized by keeping the auxiliary module off, therefore leaving a gap between the oven in the base module and the humidifier module. The oven temperature sometimes reached 34°C, which could have significantly increased the emission rate of the permeation tubes leading to an underestimation of the calculated concentrations. On some occasions calibration under elevated oven temperatures was unavoidable, so it was therefore necessary to investigate what affect this had on the data and to try to devise a correction.

In an attempt to find a suitable correction for elevated oven temperatures, a number of methods were tested. The first method involved correcting the signal recorded by the instrument. A series of measurements were carried out over a range of temperatures,

both above and below the certification temperature of the permeation tubes. The percent signal change as a function of temperature was plotted (Figure 2.16), which then provided a correction factor that could be applied to data by scaling down the signal accordingly.



Figure 2.16 The signal change (%) observed for a number of compounds at varying permeation oven temperatures (0% RH, 80/170 Td). For simplicity, the graph shows only the exponential growth trend lines which were fitted to the original data.

The second method attempted to correct the concentration by applying a general rule that the emission rate of the permeation tube increases by 10% for every 1°C increase in oven temperature [47]. Similarly, the third method involved a more precise calculation for the emission rate at a higher oven temperature (Equation 2.29) [47].

$$\log_{10} E_1 = \log_{10} E_0 + \alpha (T_1 - T_0)$$
(2.29)

In the previous equation, E_0 is the certified emission rate at temperature T_0 , E_1 is the new emission rate at temperature T_1 , and α is a device specific temperature coefficient, which should be available from the device manufacturers. VICI quote α values of 0.030 for high emission tubes and 0.034 for standard emission tubes [48]. These values were not provided for the KIN-TEK tubes, so the previous values were similarly applied to tubes of the same emission type, with the understanding that these values may not have been accurate. The new emission rates calculated using both methods are displayed in Table 2.4. New concentrations were calculated using Equation 2.23, substituting the certified emission rate with that estimated for a higher oven temperature. These methods of concentration correction estimated a 32 - 46% increase in the emission rates, and therefore in the resulting concentrations, for 4°C rise in oven temperature.

Table 2.4 Table of permeation tube emission rates (ng min⁻¹) estimated for oven temperatures above the certification temperature, where E_I refers to the certified emission rate at 30°C, E_2 refers to the emission rate calculated using the general rule of a 10% increase in *E* for every 1°C increase in oven temperature, and E_3 refers to the emission rate calculated using Equation 2.29. For some permeation tubes (marked *) α values for Equation 2.29 were not provided. Therefore for ammonia, a low emission permeation tube, a value of 0.034 was used to estimate the emission rate, and for acetaldehyde and pent-1-ene, assuming that the standard and high emission tubes from VICI and KIN-TEK were similar, emission rates were calculated using α values of 0.034 and 0.030 respectively.

Compound	E_1		E_2				E_3			
Compound	30°C	31°C	32°C	33°C	34°C	31°C	32°C	33°C	34°C	
Ammonia [*]	545	600	659	725	798	589	637	689	745	
Acetonitrile	167	184	202	222	245	181	195	211	228	
Acetaldehyde [*]	86	95	104	114	126	93	101	109	118	
Acetone	541	595	655	720	792	580	621	666	713	
Dimethyl sulphide	17.8	19.6	21.5	23.7	26.1	19.2	20.8	22.5	24.3	
Pent-1-ene*	357	393	432	475	523	383	410	439	471	

In order to determine the most suitable correction method, two calibration experiments (0% RH) were carried out using a set of 30°C certified permeation tubes. On an occasion when the temperatures were low enough to do so, the first calibration was carried out with the permeation oven set to the certified temperature for the tubes. For the second experiment, the previous calibration was repeated with the oven temperature raised to 34°C to simulate the maximum elevated temperature observed. Each of the

three correction methods outlined above were then applied to the data collected at 34°C, and the results compared to those achieved at 30°C (Table 2.5). The results were assessed based on the difference between the sensitivity values obtained from the calibration curves.

Table 2.5 Comparison of sensitivity values (*S*, in ncps ppbv⁻¹) determined from calibrations performed at the certified temperature of the permeation tubes (30°C) and also at an elevated oven temperature (34°C). The different methods of correction were applied to the data obtained at 34°C and the resulting sensitivity values compared to those values obtained at 30°C. $\Delta S'$ represents the percent difference between the 30°C sensitivity value and the uncorrected 34°C sensitivity value, and ΔS represents the percent difference between the 30°C sensitivity value and the corrected 34°C sensitivity value. **Method 1** refers to the correction by scaling of the measured signal, **Method 2** refers to the general emission rate correction of a 10% increase in *E* for every 1°C increase in oven temperature and **Method 3** refers to the emission rate correction calculated using Equation 2.29. $R^2 > 0.99$ was achieved for all calibration curves.

Compound	2000	34°C Uncorrected		34°C Corrected						
Compound	30 C			Method 1		Method 2		Method 3		
	S	S	$\Delta S'$	S	ΔS	S	ΔS	S	ΔS	
Ammonia	27.2	35.0	29%	29.2	7%	23.9	-12%	25.6	-6%	
Acetonitrile	105.3	134.4	28%	105.0	0%	90.0	-14%	96.4	-7%	
Acetaldehyde	54.5	70.8	30%	59.5	9%	48.4	-11%	51.8	-5%	
Acetone	114.7	151.3	32%	129.3	13%	103.3	-10%	114.7	0%	
Dimethyl sulphide	93.3	133.6	43%	96.2	3%	88.6	-7%	94.9	0%	
Pent-1-ene	8.3	11.5	38%	9.0	7%	7.7	-12%	8.6	-2%	
Average ΔS			33%		7%		-11%		-3%	

Without correction for the elevated oven temperature, there was on average a 33% increase in the sensitivity values. The signal correction provided a good estimation of the certified sensitivity with an average difference of 7%, although the correction may have been specific to the conditions under which the measurements were made, so additional measurements may have been required if correcting data obtained at a different humidity or E/N value. The rule based on a 10% increase in the emission rate per 1°C increase in oven temperature gave on average an 11% difference and generally underestimated the sensitivity. The more accurate calculation provided the closest result with an average difference of 3%, even with the uncertainty in the α value used in Equation 2.29 for ammonia, acetaldehyde and pent-1-ene. Method 3 was judged to be the most suitable for the correction of calibration data given that the same calculation

could be used for any instrumental conditions, and out of the two calculation methods it provided the closest estimation to the certified sensitivity especially for the compounds of most interest to breath research (i.e. acetone, dimethyl sulphide).

It should be noted that some of the permeation tubes that were used during this experiment were past the certification date, which meant that the emission rates could not be guaranteed. Acetaldehyde was found to decompose to acetic acid, which became increasingly visible in the recorded mass spectra. Therefore, some of the sensitivity values quoted during this experiment were for use as a relative measure only.

2.4.6 CIR-MS sensitivity summary and limit of detection

Monitoring trace VOCs in exhaled breath in real-time requires high instrument sensitivity and low detection limits on at least a one second timescale. Following the establishment of optimum settings, further calibration experiments were performed to assess the CIR-MS sensitivity for a broader range of compounds. With the exception of those compounds previously analysed (Section 2.4.3), calibrations were carried out for all of the permeation tubes listed in Table 2.3, at both 0% and 100% relative humidity, using the gas standards generator.

Figure 2.17 presents example calibration data from which sensitivity values are obtained. The calibration curves demonstrated the good linear response of the CIR-MS instrument over the concentration range tested. While these results demonstrate the linearity over relatively low ppbv concentrations, previous work has demonstrated good linear response over a 5 – 50 ppmv range [26]. Figure 2.17 compares two acetonitrile calibrations that were performed at both one minute and one second integration times. The results show that after normalisation the determined sensitivity values were equivalent, however the variability in the one minute data was much lower owing to signal-to-noise (S/N) improvement that results from the accumulation of more scans; for *n* measurements the S/N improves by a factor of \sqrt{n} [49].



Figure 2.17 Comparison of acetonitrile calibrations (0% RH) recorded at one minute integration times (top) and one second integration times (bottom).

The limit of detection (LOD) is described as the concentration that gives an instrument signal that is significantly different from the 'blank' or 'background' signal [50]. The background levels of the CIR-MS instrument were estimated from the measurements made while passing a high-purity nitrogen sample through the system. From this data, the average background signal and the standard deviation of that signal (σ_B) were determined, and the limit of detection (in ppbv) was then calculated using Equation 2.30 [51].

$$LOD_{M} = \frac{2\sigma_{B}}{S_{M}}$$
(2.30)

The factor $2\sigma_{\rm B}$ represents the chosen S/N level. This equation shows that the detection limit can be improved through decreasing the variability of the instrument background, i.e. by increasing the integration time.

Table 2.6 A summary of the CIR-MS sensitivities (S, in ncps $ppbv^{-1}$) and limits of detection (LOD, in ppbv). The calibration experiments were performed over the concentration ranges (ppbv) shown, and the measurements were made at 1 minute integration times and an E/N of 80/170 Td. $R^2 > 0.99$ was achieved for all calibration curves unless otherwise stated. The limits of detection were calculated using Equation 2.30, with the subscripts denoting the integration time in seconds.

Compound	0% Relative	Humidit	у		100% Relative Humidity				
Compound	Range	S	LOD ₆₀	LOD_1	Range	S	LOD ₆₀	LOD_1	
Methanol	15 - 43	50.8	2	11	17 - 43	40.9	1	9	
Pent-1-ene	14 - 229	7.2	8	52	29 - 229	3.4	21	159	
Acetaldehyde	6-88	143.9	1	4	11 - 88	87.5	1	5	
Ethanol	329 - 1648	$0.5^{\$}$	280	1975	329 - 942	0.5 [§]	311	2059	
Acetonitrile	15 - 228	117.1	0.4	1	19 - 165	95.4^{\dagger}	0.2	1	
Acetic Acid	39 - 584	64.6	2	14	39 - 334	27.0^{*}	4	23	
Acetone	26 - 418	105.4	2	19	52-418	98.0	3	15	
Isoprene	2 - 27	18.9	2	13	3 - 14	7.1 [§]	3	26	
Dimethyl Sulphide	1 - 17	80.3^{\dagger}	0.4	2	1 - 10	74.4^{\dagger}	0.2	2	
Hexan-2-one	29 - 445	176.3	0.2	2	57 - 455	169.5	0.2	1	
Ammonia	90 - 1436	46.5	6	49	180 - 1436	45.9	5	42	

 ${}^{\$}_{*} R^{2} = 0.95$ ${}^{\$}_{*} R^{2} = 0.81$

[†] Calibration data were corrected using Method 3 as described in Section 2.4.5.

Table 2.6 compiles the sensitivity values that were obtained during the earlier sensitivity investigation (Section 2.4.3) and those carried out since the E/N optimisation. Using these sensitivity values, the detection limits were calculated using Equation 2.30. Focussing on breath analysis conditions (100% relative humidity), the sensitivities measured by the CIR-MS instrument ranged from 0.5 - 169.5 ncps ppbv⁻¹, while the detection limits at one second integration times ranged from 1 ppbv - 2 ppmv, although the detection limits for the majority of the compounds were found to be less than 50 ppbv. As shown in Table 2.6, the CIR-MS instrument showed poor sensitivity, and therefore limit of detection, for ethanol. This has also been reported for other PTR-MS instruments [52, 53] and is thought to result from the fragmentation of protonated ethanol to m/z 19. The contribution to this mass channel cannot normally be monitored because of the overlap with the H₃O⁺ reagent ion signal, however significant fragmentation was demonstrated with the use of deuterium-labelled ethanol and the measurement of H₂DO⁺ at m/z 20 [52]. This will be an issue for the analysis of ethanol in breath as endogenous concentrations are typically a few hundred ppbv, so are therefore below the limit of detection on a one second timescale. However, CIR-MS showed good performance with respect to other breath compounds, for example, acetone and isoprene showed one second detection limits of 15 ppbv and 26 ppbv respectively, while the concentrations of these compounds in breath are typically in excess of 100 ppbv.

The effect that sample water vapour concentration has on the measured ion signals has been well reported [15, 16, 41] and demonstrated within this chapter, but further complications could arise from the effects of the other bulk components of breath. In a recent publication, Keck et al. investigated the effect of the carbon dioxide content of a sample with respect to the measured ion signals [54]. It was reported that a 5% increase in carbon dioxide generated 'softer' conditions within the drift tube, increasing both cluster ion and VOC signals, especially for fragmenting compounds such as ethanol and propanol. This was thought to result from the reduction of the mobility of the ions within the drift tube because of the change in buffer gas composition. As it is important to simulate a sample as accurately as possible during the calibration procedure, the effect of increased carbon dioxide concentration should be assessed in the CIR-MS system, and if a significant effect is observed, future calibration mixtures should include 5% carbon dioxide in addition to being saturated.

2.5 Summary

The CIR-TOF-MS technique has been examined with respect to its application and suitability to breath research. The main benefit of TOF-MS over quadrupole instruments is the simultaneous monitoring of all mass channels in a given range, therefore making it an ideal system for analysing complex mixtures of trace VOCs in exhaled breath in real-time, providing that high sensitivity can be achieved within short integration times. Investigation of the effects of sample humidity on the ion chemistry occurring in the drift tube has allowed the determination of optimum settings to maximise the sensitivity towards some of the common species found in breath. Following instrument optimisation, the CIR-MS performance was assessed and was found to have good sensitivity for a number of VOCs, such that the one second limits of detection at the low ppbv level were demonstrated. Therefore, the CIR-TOF-MS instrument should prove to be a capable means to actively monitor exhaled breath in real-time.
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Chapter 3. Development of a suitable breath sampling apparatus and on-line collection procedure

3.1 Introduction to breath sampling

Ever since the early days of breath analysis, researchers have realised that the way that breath is sampled is a crucial issue [1, 2]. The current status of breath sampling and the matters that need to be addressed have been summarised in recent publications [3-5]. This chapter discusses the development of a suitable sampling apparatus for the collection and transport of exhaled breath between patient and instrument, along with the similar development of the sampling protocol for the on-line collection of breath to test the real-time ability of the CIR-MS instrument for breath analysis.

The design of a breath sampling apparatus will vary according to how the breath will be sampled, but generally the following points are taken into account [6, 7]. The apparatus must be comfortable and safe for the patient, which means the use of disposable components such as mouthpieces and bacterial filters to prevent contamination from one patient to the next. There should be virtually no resistance to expiration, therefore any tubing or other collection components should be wide-bore, so that it is possible for most people to provide a breath sample. The apparatus should maintain sample integrity, i.e. not allow loss of analytes or contamination of the sample. This means that care should be taken with the choice of materials used to construct the collection apparatus, especially with regard to plastics and rubber that may emit volatiles that would contaminate the breath sample during collection. The body of the apparatus and tubing should be heated to prevent condensation of the breath on the internal walls,

which is important since water soluble VOCs could be lost by partitioning into the aqueous phase.

Breath sampling can be carried out on-line or off-line. On-line collection requires a subject to breathe into apparatus that is connected directly to the analytical instrument, whereas off-line collection requires the breath to first be collected in some form of container. Off-line sampling is useful in situations where the instrument cannot be located at the site of breath collection. In these circumstances, samples are usually collected in Tedlar bags, stainless steel canisters or sorbent traps (discussed further in Chapter 5), although there is concern over contamination and sample loss [8, 9], which makes on-line sampling more favourable. Breath is not a homogeneous sample as the composition of the dead space air differs from that of the alveolar breath. Different fractions may therefore need to be examined depending on the focus of the analysis, as concentrations of VOCs generated in the airways will be higher in the dead space air, whereas VOCs originating from the blood will be highest in the alveolar air [10, 11]. Exhaled breath can be collected as a mixed expiratory sample whereby the whole breath is collected, i.e. both the dead space and alveolar air, or as an end-expiratory sample in which only the alveolar air is collected. The collection of mixed expiratory breath is easier, but most researchers focus on collecting only the alveolar breath as it is not diluted by the dead space air. On-line, real-time analysis has the ability to sample whole breath, but temporally separate the alveolar fraction.

Where real-time analysis is not possible, researchers have come up with a number of methods to collect only the end-exhaled or alveolar portion of breath. Haldane and Priestly designed a piece of apparatus that is now referred to as the Haldane-Priestly tube, whereby the different portions of breath are separated spatially [12]. As a subject exhales into a long tube, the dead space air will be located furthest downstream, while the end-exhaled breath is contained in the upstream end from where the sample is taken. Other sampling systems have since been based on this principle, such as the breath collection apparatus used by Phillips et al. that consists of a similar tubular reservoir into which the exhaled breath enters with the dead space downstream, while the alveolar

sample is removed via a port near the subject's mouth [6]. Alternatively, Schubert et al. designed a CO_2 -controlled sampling system for the collection of alveolar air [13, 14]. CO_2 concentrations are low in inspired air (approximately 0.04%), but rapidly increase in exhaled breath (approximately 5%). Sample collection is therefore initiated only when CO_2 levels, which are measured using a fast responding analyser, reach a level representative of alveolar air.

Breath collection usually requires a subject to provide either a single exhalation or repeated tidal exhalations over a defined amount of time. For single exhalations, the breath must be representative of all subsequent breaths. During tidal breath sampling, it has been found that when a subject is asked to breath normally through a collection apparatus, hyperventilation can occur, which can cause changes in the VOC distribution across the alveolar-capillary membrane [4]. Induced hyperventilation for 20 seconds has been shown to decrease the concentration of ethanol in the breath of a volunteer after alcohol ingestion compared to the control breathing manoeuvre, although concentrations returned on resuming normal breathing ([2] and reference therein). Similar results were found after 2 minutes of induced hyperventilation for end-tidal isoprene concentrations, although end-tidal acetone concentrations were unaffected; the lower gas solubility which affects exchange over the alveolar membrane was thought to account for this observation [15].

Owing to the wide variety of analytical instruments available, which often have different requirements and capabilities (in terms of what compounds they can detect, the instrument sensitivity and detection limits, and whether sampling can be achieved in real-time), research groups tend to follow their own sampling protocols and comparison of data between groups is therefore not always possible. It has been recommended that standard protocols should be generated for single breath collection, end-tidal collection and constant tidal collection [4]. Standardisation of the breath sampling method is one of the important issues that must be resolved for breath analysis to have more widespread use outside of research laboratories. A standardised sample is crucial for quantitative analysis. Without standardisation of the breath collection technique, the

proportion of alveolar air and dead-space air can vary from breath sample to breath sample [1], and reproducible and comparable VOC concentrations will not be obtained. As mentioned previously, it is important that guidelines are generated that all researchers can then follow. With these guidelines in place, researchers can then work towards building up a basic knowledge about breath markers and their origin, and generate normal concentration values in healthy subjects as a function of age, gender, ethnicity and body mass index so abnormal concentrations can then be identified [4].

There is not yet any agreement between researchers on the issue of background correction to take into account atmospheric air VOCs. VOCs in air can vary widely, depending on factors such as location or time of day. Ambient VOCs have been observed to significantly distort the interpretation of breath analysis results [16]. Phillips et al. use the 'alveolar gradient' method whereby room air levels are subtracted from breath levels [17]. A positive gradient means that the VOC abundance is higher in exhaled breath than in inspired air thus suggesting an endogenous origin, where a negative gradient indicates that the VOC results from an exogenous source. Another method requires the patient to breathe hydrocarbon-free pure air for a couple of minutes before analysis in an attempt to 'wash out' the lungs [18], but it has been found that even high-purity breathing air has up to 1 ppmv total hydrocarbon content [19]. Both methods assume that a person rapidly reaches steady state with their environment, but this may not be the case. While the lungs can be washed-out within a few minutes, lipid soluble compounds stored in tissues need a more extensive wash-out period; unmetabolised anaesthetics have been detected in breath up to six weeks after surgery [4].

The nitric oxide breath test is a good illustration of how issues in breath sampling were overcome to become one of the few breath tests ready for use in clinical practice. Nitric oxide originates from cells of the airways and is considered as a marker of airway inflammation, so the measurement of nitric oxide in exhaled breath can be used for the assessment of conditions such as asthma [20]. Exhaled nitric oxide concentrations showed an inverse relationship with flow rate [21], and nitric oxide was also found to be

present in the nasal cavity and sinuses in higher concentrations than that found in the airways [22], therefore presenting a potential source of contamination of exhaled breath samples. To control the variability in the reported nitric oxide concentrations, the American Thoracic Society and European Respiratory Society generated guidelines for the measurement of exhaled nitric oxide [23]. The standardized single breath technique required an inhalation to total lung capacity and an exhalation at a constant flow rate of 50 mL s⁻¹ (controlled by visual/audio feedback) against resistance to exclude nasal contamination by means of soft palate closure. Both desk-top and hand-held instruments have since been designed to collect breath samples in accordance with the recommended guidelines [24, 25].

3.2 Apparatus development

3.2.1 Early designs

The first step in the development of the CIR-MS instrument for on-line, real-time breath analysis was to design a suitable piece of apparatus for capturing and transporting exhaled breath. Before the design phase commenced, an opportunity for a clinical trial arose involving a group of young cystic fibrosis patients (see Section 3.3.2 for study details). The apparatus was therefore originally designed with this trial in mind. The original intention was to collect continuous tidal exhalations over a set period of time as this was thought to be most representative of normal breathing. The apparatus design would directly couple to the CIR-MS instrument to take advantage of the real-time capability, therefore removing the need to separate the dead space and alveolar breath.

Early attempts at breath sampling used simple disposable cardboard mouthpieces attached to ¹/₈ inch PFA tubing that was routinely used with the CIR-MS system. The first design was very basic, consisting of a mouthpiece (supplied by Leicester Royal Infirmary) and a custom Teflon adapter to which the PFA sample line was connected (Figure 3.1 (a)). This design was then developed to include a bacterial filter (supplied by Leicester Royal Infirmary) that was required for infection control, and an

inlet/overflow to allow room air to be drawn into the CIR-MS instrument during an inhalation and excess breath to be vented during an exhalation (Figure 3.1 (b)). It soon became clear that the small diameter tubing created too much resistance to breathe against.



Figure 3.1 The early designs of the breath sampling apparatus: (a) a basic design consisting of a cardboard mouthpiece, and (b) a similar design incorporating a bacterial filter and an inlet for continuous air flow into the instrument. The arrows indicate the direction of the sample flow to the CIR-MS instrument.

Whilst maintaining the mouthpiece and filter arrangement, future designs needed to be constructed of similar diameter components in order to make sampling easier and more comfortable. The bacterial filter was a necessary addition to the apparatus as infection control in cystic fibrosis patients is crucial, but this also meant that only the front end of the apparatus needed to be disposable. The alternative would have been to use an entirely disposable apparatus, or a careful cleaning procedure would have been required in between patients, which would have been time consuming. The use of a disposable bacterial filter reduced the cost of consumables, was more time-effective, and allowed for more flexibility in the apparatus design as it did not have to be made with disposability in mind.

3.2.2 Low resistance designs

Further designs were created using components from commercially available hospital breathing circuits. As these components were intended for use in ventilation and anaesthetic breathing systems, they were designed for patient comfort and had large internal diameters for low resistance. All components were easily replaceable, relatively cheap, and came in standard sizes (15 or 22 mm, male and female fittings).

In changing to the larger diameter components, there was then the issue of how to connect the apparatus to the ¹/₈ inch sample inlet of the CIR-MS instrument. Any form of in-line reducer would have caused the original problem of resistance. The solution was to attach a ¹/₈ inch sampling line such that it branched off the wide-bore section of the apparatus, which would draw a portion of breath from the main flow. This design meant that the sampling apparatus could be directly connected to the instrument and met the requirements in terms of patient comfort.

Trying to capture an entire breath was difficult owing to the large breath flow in comparison to sample inlet flow, and the variation in the flow rate over the course of a breath cycle, which caused the sample inlet MFC to fluctuate whilst trying to maintain the set inlet flow. Significant interruption to the inlet flow would have affected the drift tube pressure and ultimately the E/N value, so it was important that the inlet flow remained steady. The use of an in-line collection bag to act as a breath reservoir from which a constant sample could be drawn would have avoided this problem, but this would have allowed the mixing of the dead space air and the alveolar breath. By sampling a portion from the main flow, the MFC was found to remain constant (± 2 sccm), and the required inlet flow was maintained during an inhalation by instead sampling ambient air.

The first design to be constructed using wide bore components is shown in Figure 3.2, which was also the first design to be used with patients during a clinical study. The disposable component included a plastic mouthpiece and an adult electrostatic bacterial/viral filter (from Flexicare Medical Limited, Mountain Ash, Mid Glamorgan, UK), which were easily removed and replaced in between patients. The shape of the new mouthpiece, with a flattened end, was more comfortable to use than the previous cylindrically shaped cardboard mouthpiece.



Figure 3.2 The first breath sampling apparatus design to be constructed from hospital breathing system components, including a digital volume transducer for breath flow rate measurements. The arrows indicate the direction of the sample flow to the CIR-MS instrument.

This design also featured a Digital Volume Transducer (DVT; VMM-400 Ventilation Measurement Module from Interface Associates, Laguna Niguel, CA, USA) to record volume and flow rate measurements (see Section 3.2.4). The 15 mm outer diameter of the cartridge of the DVT meant that it could be connected directly into the apparatus.

Various connectors (all from Intersurgical, Wokingham, Berkshire, UK) were used throughout the apparatus to join the components together, including a T-piece connector with two non-return valves that allowed a subject to breathe in room air during an inhalation, but directed the breath through the rest of the apparatus on exhalation. A section of tubing (22 mm diameter, 150 mm in length, from Flexicare) joined the T-piece to a custom Teflon connector, to which the PFA sampling line was attached. A portion of the exhaled breath was drawn through the sampling line by the mass spectrometer and the remainder was vented through an exit valve. An additional inlet for ambient air (not visible in Figure 3.2) ensured that a constant flow of gas was supplied to the instrument during an inhalation stage when no breath was passing through the apparatus, and also allowed ambient background measurements to be made. The dead volume of the apparatus was approximately 170 mL.

The sample line between the apparatus and the instrument was heated to 40°C, and a heated blanket (made by Infroheat Limited, Wolverhampton, UK) surrounded the entire sampling apparatus, which was also heated to 40°C. Heating the apparatus and sampling line avoided condensation, and therefore prevented the loss of soluble VOCs.

The mouthpiece could be substituted for a face mask if required. Facemasks may be needed in situations where subjects are not able to use mouthpieces. An advantage of using standard sized components was that the change could be made without affecting the rest of the sampling apparatus. The decision to primarily use mouthpieces was based on the ease of maintaining a seal between the patient and the apparatus for the duration of the experiment. This was more difficult to achieve when using a facemask, especially for prolonged periods, as the patient was required to hold the mask in position against the face.

3.2.3 Further modifications

The first design that was constructed using the wide bore components was also the largest design in terms of size and dead volume, i.e. the amount of air contained in the

apparatus that had to be displaced by the breath sample. In an attempt to reduce the size, the design was re-evaluated and the large section of tubing was replaced with a small connector, which produced the apparatus shown in Figure 3.3 (a).



Figure 3.3 (a) A reduced size breath sampling apparatus, and (b) a more recent design. The arrows indicate the direction of the sample flow to the CIR-MS instrument.

The design was then further reduced to the version shown in Figure 3.3(b) by removing the Teflon connector and exit valve, and instead the PFA sample line was attached directly to the connector situated below the T-piece. Ambient air, which in previous

designs was drawn in through a separate PFA line, was instead drawn in through the open end of the apparatus and was displaced by breath during an exhalation. All other apparatus features remained as described previously. With the exception of all tidal breath measurements, this design was used for all on-line breath sampling described throughout this thesis. The dead volume of the new design was approximately 100 mL.

One problem that resulted from reducing the size of the apparatus was that the heated blanket, which was originally based on the larger apparatus design, started to become more impractical. During later work in this thesis, the heated blanket had to be substituted for a heating tape that was directly wrapped around the apparatus. The heating tape was secured in place with cable ties and surrounded in foil. This method worked in terms of overall effect, but the removal of the disposable parts of the apparatus became much more difficult as the heating tape had to be undone each time and then refitted with the new parts in place. Since the apparatus was still going through development, the heated blanket was not replaced, but it would be highly recommended once the design is finalised since the convenience would be especially useful during clinical trials where a regular changing of mouthpieces/filters is likely.

3.2.4 The Digital Volume Transducer

The DVT consisted of a small turbine cartridge (dead volume 6.7 mL) containing an ultra light weight impeller blade and an infrared beam. Air flow passing through the cartridge caused the blade to rotate, interrupting the beam, which was detected and translated into flow direction and volume measurements. Consecutive breaths could be measured as the readings were zeroed when a change in flow direction was detected. The small turbine had a flow range of $0.05 - 3 \text{ L s}^{-1}$.

The DVT was used in conjunction with the Ventilation Measurement Module (VMM), which processed and displayed the signals from the DVT. The VMM could be used as a stand-alone unit, which showed limited data on a digital display, but provided more useful information if connected to a computer. The accompanying software displayed

real-time breath curves and windows containing the most recent inhalation/exhalation volumes and minute ventilation/respiratory frequency (10 breath moving average) (Figure 3.4). Through this software the data could be saved to file, although these files contained volume information only. The VMM outputted readings every 0.01 seconds, so a time scale could be added to the recorded volume data and from this the flow rate, minute ventilation and respiratory frequency could be deduced.



Figure 3.4 A screen-capture of the DVT software.

In the output file, positive numbers indicated an inspired volume and negative numbers indicated an expired volume. This data was imported into Excel where, for purposes of this work, the signs of the volume data were switched, i.e. the exhalation volume became positive and vice versa, so that when plotted against the data recorded by the CIR-MS instrument correlations could be made between the point of exhalation and the VOC signal increases. The resulting transducer data can be plotted as shown in Figure 3.5, displaying inhaled and exhaled volumes against time. The DVT provided a record of the breathing pattern for any given experiment and allowed the quantification of

breath measurements. In addition to the inhaled and exhaled volumes, the overall flow rate (mL s⁻¹) for each breath could be determined, as well as the minute ventilation (L min⁻¹) and respiratory frequency (breaths min⁻¹).



Figure 3.5 Example DVT measurements displaying one minute of tidal breathing. The overall flow rate $(mL s^{-1})$ can be calculated for each breath using the total exhaled volume (*V*) and the exhalation time (*t*). Exhaled minute ventilation (L min⁻¹) can be worked out by summing all of the exhaled volumes in a one minute period, and the respiratory frequency (breaths min⁻¹) refers to the number of inhalation/exhalation cycles in one minute.

3.2.5 Apparatus testing

Throughout the apparatus development process, care was needed with the choice of materials used. Some types of plastic tubing and facemasks were found to emit volatiles; m/z 59 and m/z 99 were commonly observed and were tentatively assigned to acetone and cyclohexanone respectively. These signals may have resulted from solvent use during the production process. The release of cyclohexanone from plastic medical components has been previously reported [26, 27]. Even though background measurements were always recorded before a breath sample was collected, sources of contamination should be minimised.

A bacterial filter was a necessary part of the breath sampling apparatus to prevent cross infection from one patient to the next. Experiments were carried out to assess whether the bacterial filter or any other part of the sampling apparatus would trap breath VOCs. To quantify any changes that may have been observed, known concentrations of VOCs were passed through the breath apparatus using the gas standards generator (Section 2.3.7), and reference measurements were obtained by passing the same mixture through standard PFA gas line. Using acetone as an example, Figure 3.6 confirms that there was no VOC loss during passage through the sampling apparatus over the concentration range tested under both dry and humid conditions. Ammonia, methanol, acetonitrile, acetaldehyde and pent-1-ene were also included in the standard mixture, and for which the same result was observed.



Figure 3.6 Comparison of acetone measurements made after passing a standard gas mixture (0% and 100% relative humidity) through both the breath sampling apparatus and standard PFA gas line as a reference. Measurements were made at 1 minute integration times and each marker represents a 5-point average, all with a standard deviation < 2%. $R^2 > 0.99$ for all linear fits.

The same experimental arrangement was also used to test for emissions from the apparatus by passing high-purity nitrogen through both the standard PFA gas line and the breath sampling apparatus. By comparison, no detectable volatiles were found to be emitted from the apparatus.

The bacterial filter was also tested for loss of humidity, which was important for quantification purposes. Exhaled breath is saturated, and given that the apparatus was heated to approximately body temperature, the humidity should have remained the same through to the sample inlet. With this in mind, calibrations were carried out at 100% relative humidity. If the filter reduced the humidity of the sample, then quantification would have been inaccurate for humidity-dependent VOCs. Using a humidified nitrogen flow generated by the KIN-TEK humidification module, humidity measurements were made (using a dew point hygrometer, General Eastern Hygro M4) both with and without the filter in-line. Inspection of Figure 3.7 shows that no significant difference was observed.



Figure 3.7 Comparison of the relative humidity readings recorded with and without an in-line bacterial filter. No significant difference was observed, assessed using a one-tailed Student's t-test (OriginPro) whereby p > 0.05 (average: without filter 84.5% RH ± 1.4%, with filter 85.5% RH ± 1.4%).

3.3 Protocol development

3.3.1 Tidal breath collection

In addition to the breath sampling apparatus, a sampling protocol had to be similarly developed. As previously stated, the original intention was to collect continuous tidal exhalations over a defined period of time as this was thought to be most representative of normal breathing.

The initial testing of both the apparatus and the collection method was performed on members of the research group (1 male/4 females, 23 - 29 years old). The procedure involved tidal breathing over a 5 minute period. This allowed enough time for the patients to relax and get used to the procedure, as well as providing a good sized data set for each sample. Subjects were asked to inhale and exhale through the apparatus shown in Figure 3.2. Nose-clips were not used for reasons of patient comfort, however the DVT recordings would have shown if the procedure had not been performed correctly (e.g. if the subject had not breathed only via the mouth). No problems were found in breathing through the apparatus for 5 minutes. No attempt was made to control diet prior to breath analysis, although a minimum of 2 hours was normally left between the consumption of any food or drink (other than water) and analysis. Ambient air measurements were similarly collected over a 5 minute period before breath collection. All measurements were made at 1 second integration times, and over a mass range of 12 -200 u.

The tidal breath collection method was used prior to the E/N optimisation that was discussed in Section 2.4.3 and Section 2.4.4, so all measurements were performed at a higher E/N of 130/190 Td. The drift tube of the CIR-MS instrument also had a slightly different arrangement to that described in Section 2.3.3, in terms of the coupling of the drift tube to the transfer chamber; full details of which can be found in [28]. In brief, the entire drift tube sat higher on the base flange with the exit aperture housed on an extra plate that was situated below the last electrode (separated by a thin PTFE film), rather

than on the underside of the base flange. This design allowed for an intermediate pumping chamber between the drift tube exit and the transfer optics, which meant that the drift tube could be operated at a slightly higher pressure of 7.6 mbar. Sample and water vapour inlet flows were also higher at 275 sccm and 50 sccm respectively.

Figure 3.8 shows example CIR-MS and DVT measurements that were recorded during a tidal breath collection. In order to assist the determination of endogenous and exogenous compounds, inspiratory air levels have to be taken into account. The ambient air background levels were always measured for a period of time prior to breath sampling and were used as a reference, but were not subtracted from the breath measurements so to maintain the true observed intensities. One of the most noticeable differences between the CIR-MS measurements of ambient air and breath was the abundance of the $H_3O^+(H_2O)$ cluster ions. At the time of sampling, the relative humidity of the ambient air was measured to be around 30%, whereas breath has a relative humidity of 100%, so an increase in the water cluster abundance would be expected. Presented as the m/z (19/37) ratio, the ratio was lower in the breath data because of the higher $H_3O^+(H_2O)$ ion signal relative to the H_3O^+ signal. This provided a means to assess the quality of a breath sample in that the m/z (19/37) ratio would need to be below a certain value if an alveolar (saturated) sample had been provided. The signals that relate to endogenous compounds would be expected to be present in greater levels in exhaled breath than in the inhaled air, since this indicates of a source of production within the body. However, it should be noted that this generalisation does not always apply, for example, the exhaled VOCs that result from smoking are exogenous in origin, but will typically display higher concentrations in breath than in the ambient air at the site of sampling. Figure 3.8 displays two examples of signals that are thought to represent compounds of endogenous origin. The most dominant signal in the breath of all of the test subjects was observed at m/z 59, which showed a clear elevation over the levels measured in the ambient air. Some signals, such as that observed at m/z 69, showed some degree of overlap between the exhaled and the ambient measurements, although the average levels in breath were still found to be higher.



Figure 3.8 Example tidal breath data showing (in descending order): DVT measurements, the H_3O^+ to $H_3O^+(H_2O)$ ratio as an indication of sample humidity, and the exhaled m/z 59 and m/z 69 levels, both in comparison to the ambient air levels. The first 10 seconds of data were discarded to allow for the flushing of the dead volume of apparatus. The dotted lines represent the air and breath averages taken over the full sample period.



Figure 3.9 The effect of data summing on the signal variability, using m/z 69 as an example. The blue circles represent ambient air data points and the red circles represent breath data points. The measurements that were originally collected at 1 second integration times could be summed to reduce the variability and increase signal separation.

Even though the experiments were performed in real-time, the individual breaths could not be clearly resolved in the resulting data. Referring back to the m/z 59 data shown in Figure 3.8, the measurements did not show a return to ambient levels at any point throughout the experiment, which would have been expected in between exhalations. This suggested that during an inhalation, rather than drawing a sample of ambient air through the air inlet, the instrument was also drawing some of the previous exhaled air isolated in the lower half of the apparatus by the valve system. As breath-by-breath resolution was not achieved, the data did not need to be presented on the original 1 second timescale, and summing multiple signals using the MaxiGroup program (Section 2.3.5) benefitted from reducing the variability of the signals. Using m/z 69 as an example, the effect of summing the data is shown in Figure 3.9. The amount of variability in both the air and the breath data sets was reduced as the data were summed into larger groups, providing better separation and aiding identification of weaker breath signals from the ambient air background. Summing the data into 10 second groups was chosen to be the most suitable for the analysis of tidal breath data, as this provided a reasonable compromise between signal separation and the number of measurement points over the course of the experiment, so any temporal changes in the levels could still be monitored.

The data from the test subjects were re-processed after summing into 10 second groups, and following this a number of signals were identified where the levels in exhaled breath were greater than that in the ambient air. These signals are displayed in Table 3.1. Since m/z values are not unique indicators of compound identity, the VOC assignments are tentative. The previously mentioned signals at m/z 59 and m/z 69 were assigned to acetone and isoprene respectively. Acetone, one of the most abundant breath VOCs, showed much variation even in this small group of healthy adults. The signal at m/z 33, which was assigned to methanol, showed a strong correlation with m/z 51 (r = 0.995), which presumably represented a methanol-water cluster ion. The signal at m/z 63 was thought to correspond to protonated dimethyl sulphide. These VOCs were common to all of the test subjects, suggesting that these compounds originated from core biological processes. A number of other signals were observed that were only

present in some of the test subjects. The signal at m/z 18 was assigned to ammonia, for which levels in the breath of most of the test subjects were comparable to that measured in the ambient air. The higher levels in the breath of two of the subjects may have reflected differences in diet [29]. The identity of the signal observed at m/z 81, which was only observed in the breath of one individual, was more uncertain. While it may have represented a dimethyl sulphide-water cluster ion, there was poor correlation with the dimethyl sulphide signal at m/z 63 (r = 0.035). A signal at m/z 137 was also observed in the breath of the same individual, which could have represented limonene, a compound that has been previously identified in exhaled breath and again may be a reflection of diet [30]. Limonene is also known to produce a fragment at m/z 81 [31].

Table 3.1 The breath signals measured in a group of five adults (A1 - A5) for a tidal breath collection, shown in comparison to ambient air measurements. The values shown represent a 5-minute average (10 s grouped data) with the relative standard deviation (%) given in the brackets.

	Signal (ncps)							
m/z	Air	A1	A2	A3	A4	A5		
(19/37)*	45 (±7%)	12 (±4%)	13 (±3%)	13 (±4%)	14 (±4%)	13 (±5%)		
18	2450 (±16%)	2611 (±13%)	2639 (±14%)	3033 (±12%)	4278 (±20%)	4940 (±13%)		
33	4038 (±9%)	29527 (±14%)	19985 (±13%)	24688 (±11%)	12200 (±10%)	10077 (±8%)		
51	61 (±95%)	1035 (±25%)	688 (±27%)	813 (±28%)	431 (±29%)	358 (±42%)		
59	4753 (±12%)	27929 (±7%)	13966 (±5%)	15260 (±7%)	37604 (±12%)	21396 (±5%)		
63	72 (±138%)	1268 (±25%)	954 (±28%)	822 (±32%)	729 (±37%)	849 (±25%)		
69	166 (±56%)	2139 (±23%)	1060 (±36%)	1400 (±33%)	1120 (±28%)	1548 (±21%)		
81	74 (±75%)	68 (±94%)	62 (±82%)	343 (±50%)	63 (±83%)	98 (±64%)		
137	42 (±110%)	24 (±113%)	45 (±92%)	188 (±56%)	36 (±150%)	54 (±88%)		

* unitless

The DVT measurements for the test subjects showed that tidal volumes ranged from 400 mL – 900 mL, minute ventilations between 6 – 14 L min⁻¹ and respiratory frequencies between 9 – 22 breaths min⁻¹, with one volunteer displaying a very high tidal volume and minute ventilation of 2 L and 45 L min⁻¹ respectively. Tidal volumes are typically in the region of 500 mL for healthy adults and minute ventilations between 6 – 8 L min⁻¹ [32], so hyperventilation in some subjects was a possibility. Hyperventilation, caused by either rapid and/or deeper breathing, has been previously

found to occur during tidal breath collection, which was confirmed through the measurement of reduced end-tidal CO_2 concentrations [15, 19]. Breathing through a mouthpiece has been shown to affect breathing patterns by increasing tidal volume and minute ventilation [33, 34], making it difficult to breathe 'normally' through a piece of apparatus. The use of facemasks has been suggested to have less of an effect [35].

3.3.2 Testing of the tidal collection method: The cystic fibrosis trial

3.3.2.1 Introduction

Cystic fibrosis is the most common life-limiting inherited condition that affects over 8000 people in the UK [36]. Cystic fibrosis is caused by a mutation in a single gene that is responsible for the production of a protein which channels chloride ions out of mucus-secreting cells. In cystic fibrosis patients, an abnormal form of this protein disrupts chloride ion transport and movement of water out of the cells, so mucus becomes thick and sticky. Cystic fibrosis affects many organs in the body, although mainly the lungs and pancreas, where the thick mucus clogs the airways and obstructs the glands responsible for secreting digestive enzymes into the intestines. In the airways the sticky mucus provides an ideal environment for bacterial growth meaning cystic fibrosis sufferers are constantly at risk of bacterial chest infections. The lungs eventually become permanently colonised by bacteria leading to chronic infection, which is the main cause of morbidity and mortality in cystic fibrosis patients [37].

It is common for bacteria such as *Staphylococcus aureus* and *Hemophilus influenzae* to infect the lungs of cystic fibrosis patients, although *Pseudomonas aeruginosa* will eventually dominate [37, 38]. Chronic *P. aeruginosa* infection is near impossible to eradicate and is associated with a poor prognosis for the cystic fibrosis patient [37]. If a patient is suspected of having an infection, the current procedure is to prescribe a broad spectrum antibiotic covering them for a number of common bacteria while a cough swab is taken for culture. When the culture reveals which bacteria are present, the treatment can be modified accordingly. Bacterial identification often involves time-

consuming laboratory techniques, so quicker methods of diagnosis are currently being investigated. With early detection and antibiotic treatment, chronic *P. aeruginosa* colonisation can be delayed [39].

Numerous VOCs have been found to be emitted from *P. aeruginosa* and other bacterial cultures in vitro [40-46], which is discussed further in Chapter 6. If these bacteria similarly produce VOCs in vivo that are detectable in the breath of infected individuals, then a breath test may provide a quicker diagnosis of bacterial infection. Only a few studies have focused on the analysis of breath from cystic fibrosis patients. One study found significantly higher pentane levels and significantly lower dimethyl sulphide levels in the breath of cystic fibrosis patients in comparison to the healthy control group, of which exhaled pentane was higher in patients with pulmonary exacerbation suggesting increased oxidative stress [47]. Another study that focussed on breath sulphides identified carbonyl sulphide as being significantly higher in the breath of cystic fibrosis patients in comparison to the healthy control group, although no significant difference in dimethyl sulphide levels were observed [48], contradicting the findings of the previous study. It has also been reported that breath isoprene levels can be reduced in cystic fibrosis patients with acute respiratory exacerbation [49], although this was again contradictory to another study that found no difference between exacerbated and stable cystic fibrosis patients or between cystic fibrosis and healthy subjects [47]. Both propan-2-ol and hydrogen cyanide have also been identified in the breath of cystic fibrosis patients infected with *P. aeruginosa* [50, 51].

This section provides the details of a trial study performed to examine VOCs in the exhaled breath of children with cystic fibrosis. The aim was to investigate differences between the breath of cystic fibrosis and healthy children, and the differences within cystic fibrosis subjects that may arise as result of pulmonary infection.

3.3.2.2 Patients and methods

Breath samples were collected from a small group of cystic fibrosis patients who were attending a clinic at Leicester Royal Infirmary. A total of 10 cystic fibrosis children (5 males/5 females, mean age 14) took part, with 4 healthy children (3 males/1 female, mean age 9) forming the control group (Table 3.2). All subjects were brought to the laboratory at the University of Leicester for analysis. All cystic fibrosis subjects displayed chest symptoms at the time of the trial, and were taking or had recently completed antibiotic treatment. Some subjects were additionally taking steroid-based anti-inflammatory medication and/or were receiving DNase treatment to reduce mucus viscosity. None of the subjects in the control group showed signs of illness at the time of the trial and were free of any chronic lung condition. In the cystic fibrosis group, sputum or cough swabs for culture were not collected at the time of breath collection, but the relevant details were obtained from patient medical records. Written consent was provided by the legal guardians of the test subjects and approval for the trial was obtained from the Leicestershire Ethics Committee.

Table 3.2 Demographics of the cystic fibrosis group (CF1 – CF10) and the healthy control group (N1 – N4). The microbiology results for the cystic fibrosis subjects were obtained from patient medical records and were based on sputum or cough swab cultures taken in the 10 days prior to breath sampling. Some patients showed no microbiological growth (-).

Patient ID	Gender	Age	Microbiology Results	Patient ID	Gender	Age
CF1	F	15	Staphylococcus aureus	N1	М	9
CF2	М	16	-	N2	М	7
CF3	М	14	-	N3	F	10
CF4	М	15	Aspergillus fumigatus	N4	М	11
CF5	F	14	Aspergillus fumigatus*			
CF6	F	14	Mixed flora			
CF7	F	10	-			
CF8	F	12	-			
CF9	М	13	Staphylococcus aureus			
CF10	М	13	Staphylococcus aureus			

* No swabs were taken within 10 days of breath sampling, but a swab from 14 days previous tested negative, while a swab taken 16 days after tested positive for *Aspergillus fumigatus*. Therefore it was possible that the patient may have been infected at the time of breath analysis.

In terms of infection control, all surfaces within the collection area were wiped with a bleach solution before the subject arrived to give the breath sample. The heated blanket was cleaned using an alcohol wipe, away from the CIR-MS instrument so that the interference of volatiles from the wipe was kept to a minimum. A new mouthpiece and bacterial filter were attached to the apparatus (Figure 3.2) on arrival of the patient.

Each patient was sat down for a few minutes to relax before sampling, during which time the experimental procedure was explained and the patient details were taken, and 5 minutes of ambient air background measurements were recorded. The patient was then seated at the breath collection apparatus and the height of the apparatus adjusted to a comfortable position, but so that their head was tilted slightly back. Breath collection involved tidal breathing, inhalation and exhalation via the mouth for 5 minutes. Puzzles were positioned in front of the children in an attempt to take the focus away from breathing through the apparatus and to help pass the time. Directly after analysis, the mouthpiece and bacterial filter were removed and disposed of. All breath samples were collected during the afternoon, and no food had been consumed in the previous 2 - 3 hours. Both ambient air and breath measurements were recorded at 1 second integration times over a mass range of 12 - 200 u.

Prior to analysis, all data were summed into 10 second groups. For statistical analysis of the data, the significance between the different patient groups was assessed using a two sample Student's t-test (OriginPro) where a p-value < 0.05 was considered statistically significant. Principal component analysis was performed using PLS Toolbox (Eigenvector Research Inc., Wenatchee, WA, USA), which operated in MATLAB.

3.3.2.3 Results

Both the healthy and the cystic fibrosis children found no discomfort in performing 5 minutes of tidal breathing through the sampling apparatus. The microbiological results (Table 3.2) revealed that none of the cystic fibrosis patients were infected with *P*. *aeruginosa* at the time of breath sampling, and *S. aureus* and *A. fumigatus* were instead

found to be the most common infecting organisms. Although the original focus was on the identification of *P. aeruginosa* infection, the data set still provided a means to look for general differences between healthy and cystic fibrosis children, and also between cystic fibrosis children with and without infection.



Figure 3.10 DVT measurements for 5 subjects taking part in the cystic fibrosis trial, displaying a 1 minute section of a 5 minute tidal breath collection. The results highlight the degree of variability in tidal breathing from person to person, and also the usefulness of having a record of breathing pattern.

The DVT was a valuable part of the sampling apparatus as it provided a record of the breathing pattern during any given experiment. Comparison of the exhalation profiles

from different volunteers highlighted the degree of variability that can occur when a subject is asked to breathe tidally through the sampling apparatus (Figure 3.10). In the 60 second periods displayed in Figure 3.10, significant inter-individual variability was observed in terms of the respiratory frequency and tidal volumes. A certain degree of intra-individual variability in breathing pattern was also observed, which was more visible in some subjects than in others. It was difficult to tell at the time of sampling whether a patient had complied with the collection procedure. Figure 3.10 (top graph) suggests that some subjects may have had difficulty in following the directions as requested, which was only realised at the data processing stage whereby it was too late to repeat the breath collection. In this particular case, only the first minute breath collection was affected, suggesting that for this individual there may have been a period of adjustment to breathing through the apparatus, and the remaining collection time provided a valid sample. In another patient, the tidal breaths throughout the 5 minute experimental period were found to be less than 150 mL, leading to an uncertainty in whether any alveolar air had been sampled. This was also reflected in the CIR-MS data in which the m/z (19/37) ratio was higher than all other breath samples (m/z (19/37) ~ 20; compare with the values presented in Table 3.1), indicating that the sample was not reliable. The data for this patient was therefore excluded from the analysis.

Figure 3.11 shows the average levels of each of the signals observed in the breath of the trial subjects, displaying the results in groups of cystic fibrosis patients with infection, cystic fibrosis patients without infection and healthy controls, all compared to the ambient air levels. The commonly observed breath signals at m/z 33, m/z 59, m/z 63 and m/z 69 (methanol, acetone, dimethyl sulphide and isoprene respectively) generally showed no trend across the groups. Exhaled acetone and dimethyl sulphide levels in the current trial were found to be comparable to that observed in the breath of the healthy adults (Table 3.1). However, the exhaled methanol levels observed in the trial subjects were considerably lower than that observed in the group of healthy adults. This may have been a reflection of the variability in exhaled methanol levels, given that two of the adult volunteers who contributed to the data in Table 3.1 also provided breath samples on the following day and on that occasion displayed lower exhaled methanol levels,

equivalent to those found in the trial subjects. Ambient methanol levels on one particular day during the trial were abnormally high, higher than that found in the breath of the two patient samples collected on that day. It was uncertain whether the high background levels had influenced the breath measurements since the exhaled levels fell within the normal range observed during this study. With the exception of one cystic fibrosis patient, exhaled isoprene levels in the trial subjects were generally lower than that observed in the healthy adults (Table 3.1), which may relate to age difference between the two groups as it has been reported that younger children have lower breath isoprene concentrations than adults [52]. There have been contradictory reports regarding dimethyl sulphide and isoprene concentrations in the breath of cystic fibrosis patients (Section 3.3.2.1). As previously stated, for both of these compounds the present results indicated that there was no significant difference between the cystic fibrosis and healthy control groups (mean dimethyl sulphide: CF = 965 ncps vs. N = 831 ncps, p = 0.28, and mean isoprene: CF = 393 ncps (excluding outlier) vs. N = 493 ncps, p = 0.13), although conclusive results were difficult to obtain from such a small sample size.

Despite there being no patients involved in the current study with *P. aeruginosa* infection, the propan-2-ol and hydrogen cyanide content of breath samples from cystic fibrosis subjects were examined for completeness. Throughout the trial the signals in the corresponding mass channels never exceeded ambient levels. One cystic fibrosis patient with infection at the time of analysis displayed elevated levels of m/z 81 and m/z 137 in their breath. These signals were previously observed in one of the healthy adults (Table 3.1) and were both attributed to limonene. Given that these signals were not elevated in the breath of any of the other infected cystic fibrosis patients, and that they were previously observed in healthy subjects, it was unlikely that they had any relevance to infection status.



Figure 3.11 The average levels measured for a selection of m/z values in the breath of cystic fibrosis patients with infection (red circles), cystic fibrosis patients without infection (green circles) and normal healthy children (purple circles), in comparison to ambient air levels (grey boxes). Each data point represents the average signal and error bars display the standard deviation.

The most interesting observation related to the signals at m/z 71 and m/z 89. The identities of these signals were again uncertain, and may have represented two different breath compounds showing similar trends (r = 0.947) or one single compound and its corresponding fragment or water cluster ion, for example a C₅ alcohol and its dehydration fragment. Levels of these two masses appeared to be higher in the breath of the infected cystic fibrosis patients in comparison to both the non-infected cystic fibrosis group (mean: CF_i = 159 ncps vs. CF_{ni} = 65 ncps, p < 0.05), and the healthy controls (mean: N = 54 ncps, p < 0.05). One non-infected cystic fibrosis patient also
showed higher levels of these masses that were comparable to that observed in the infected group, although the incorrect assignment of infection status was possible. Classification of infection was based on recent culture results obtained from patient medical records. However, it was possible that changes could have occurred in the time between the collection of that sample and breath analysis, especially as all of the patients were receiving some form of antibiotic treatment. Ideally for confident assignments to be made, samples for culture would have been collected on the same day as the breath analysis, which was only possible for one cystic fibrosis subject during the study. Alternatively, the signals at m/z 71 and m/z 89 may have related to airway condition rather than microbiological status, as airway inflammation is another important component of cystic fibrosis disease [53, 54], or even oxidative stress [55]. This would have required a more detailed clinical assessment for this to be investigated, the information for which was not available for this study.

Instead of inspection of the individual signals, the examination of the overall breath VOC profile may provide a more useful way of visualising the results. Therefore, multivariate analysis was used to explore whether the patterns formed by multiple signals were characteristic of breath samples belonging to a particular group of subjects. Principal component analysis is commonly used to reduce the dimensionality of a data set whilst maintaining most of the original variation to assist the identification of underlying trends. A principal component analysis was performed on the data collected during the current trial using a selection of 11 m/z values and the resulting biplot is shown in Figure 3.12, which presents the scores and loadings for the first principal component, describing the greatest variation in the data, against the third principal component as this provided the best separation of the groups. The biplot showed that the breath samples of the healthy subjects and the non-infected cystic fibrosis subjects were grouped together and were therefore not distinguishable from each other. However, this collective group was separated from the breath samples of the infected cystic fibrosis subjects, with the only exception being the sample of the one non-infected cystic fibrosis patient discussed previously whose breath profile was closer to that of the infected patients. Ideally, the extension of the trial to a larger number of individuals

would be required to properly investigate these observations, as would the collection of swabs for microbiological culture directly following exhaled breath analysis so that confident assignments of infection status can be made, which will allow for a more informed assessment of whether this factor relates to the patterns observed within breath. While this preliminary trial was limited by the small number of participants, the results suggested that pulmonary infection or other factors such as inflammation or oxidative stress may cause changes in the exhaled breath profile of cystic fibrosis patients, and the coupling of CIR-MS data to a statistical technique such as principal component analysis illustrated the potential of using of multiple variables to 'fingerprint' clinical conditions.



Figure 3.12 A principal component analysis biplot showing the grouping of exhaled breath samples from cystic fibrosis children with infection, cystic fibrosis children without infection and healthy children, in comparison to ambient air samples. The plot displays the first principal component against the third principal component. The principal component analysis was performed using 11 m/z values and the data were pre-processed by autoscaling.

3.3.3 Single breath collection

All of the breath sampling until this point had involved the collection of tidal breaths over a defined length of time. Testing of the protocol with a group of healthy adults and a group of children during the cystic fibrosis study highlighted areas where improvements could have been made. Asking the subjects to breathe tidally through the apparatus resulted in much variability in the breathing pattern and the possibility of hyperventilation, which may affect the distribution of molecules across the alveolar membrane over time. The prevention of hyperventilation has been reported by asking a subject to breathe at a defined rate of 10 breaths per minute and a tidal volume based upon a subject's height and weight [19]. In an attempt to control the number of breaths and also to reduce the sampling time, which would be beneficial when trying to collect large numbers of samples, a new method involving the collection of a single full exhalation was investigated.

The new procedure consisted of a full inhalation followed by a vital capacity exhalation. The method still required the inhalation to be taken through the apparatus, a requirement of the DVT to ensure the recording was reset, although this did also provide inhalation measurements. The procedure could be completed within 1 minute, with the breath itself typically taking less than 10 seconds, but the additional recording time allowed for ambient measurements on either side of the exhalation to be made. The apparatus had also undergone development since the cystic fibrosis trial, with a reduction in the overall volume and the removal of the exit valve (Section 3.2.3). The latter modification allowed more accurate ambient air measurements to be made in between exhalations following the problems that were found with the previous design. Previously, two separate samples (one air, one breath) had been required to the aid determination of endogenous compounds, but this assumed that no changes in ambient conditions had occurred in between the two samples being collected. The new procedure allowed both sets of measurements to be made during the same experiment, displaying ambient levels pre-exhalation as well as the return post-exhalation.

For single breath collection, it is important that the sample is representative. Since the new collection procedure was much quicker to perform, it allowed repeat measurements to be made so that the reproducibility of the samples could be assessed. Even though the overall time for breath measurement was shorter, a single vital capacity exhalation provided a larger portion of alveolar breath in comparison to a single tidal exhalation (Figure 3.13).



Figure 3.13 Comparison of a tidal and a vital capacity exhalation measured using the DVT. The exhalation volume, exhalation time and flow rate for the first tidal breath and the vital capacity breath in this example are as follows: tidal breath – 1422 mL, 3.51 s, 405 mL s⁻¹, full breath – 3455 mL, 4.17 s, 828 mL s⁻¹.

In the CIR-MS data obtained using the new breath collection procedure, it was found that plotting the measured signals for a particular m/z channel against time produced an exhalation profile for those VOCs that were present in breath in greater levels than the ambient air. This is illustrated in Figure 3.14, using acetone as an example. A rapid rise was observed relative to the initial ambient levels corresponding to the exhaled breath passing the sample inlet, which subsequently levelled off before returning to ambient levels post-exhalation, thus producing the characteristic shape of the exhalation profile. A good correlation was observed between the DVT and the CIR-MS measurements for the point of exhalation and the increase in the VOC signal. The m/z (19/37) ratio was used to estimate the relative humidity of the sample (Section 2.4.2), which suggested an increase from 28% relative humidity in ambient air to the expected 100% relative humidity for an end-exhaled breath. It is worth noting that the ratios quoted here differ from those quoted during the tidal breath collection owing to the use of different E/N values, which affects the reagent ion distribution. When the single vital capacity exhalation method was tested on two healthy adults, in addition to m/z 59, exhalation profiles were observed at m/z 33, m/z 51, m/z 63 and m/z 69 (Table 3.3). These profiles corresponded to the same 'core' VOCs found during the tidal breath experiments (Table 3.1).



Figure 3.14 Example CIR-MS and DVT measurements obtained for a single vital capacity exhalation.

	Signal (ncps)			
m/z	Air	A1	A2	
(19/37)*	11 (±6%)	3 (±5%)	3 (±2%)	
33	4413 (±19%)	9202 (±6%)	8130 (±9%)	
51	86 (±116%)	1013 (±3%)	648 (±26%)	
59	5265 (±15%)	38150 (±2%)	60515 (±5%)	
63	56 (±143%)	1385 (±18%)	1260 (±2%)	
69	223 (±73%)	2057 (±13%)	3602 (±18%)	

Table 3.3 The breath signals measured in two healthy adults (A1 and A2) for a single vital capacity breath collection, shown in comparison to ambient air measurements. The values shown represent a 3 breath average (peak height values) with the relative standard deviation (%) given in the brackets.

* unitless

It is worth noting that the ability to observe exhalation profiles was not based on the change in breath collection method alone, but in combination with instrument changes that had occurred since the end of the cystic fibrosis study. This saw the removal of the intermediate pumping chamber in order to bring the drift tube exit closer to the transfer optics to increase ion transmission (see [28] for details). These changes brought about the arrangement that is described in Section 2.3.3. After these modifications, the H₃O⁺ count rates had doubled from 2×10^3 counts s⁻¹ to 4×10^3 counts s⁻¹, which may have related to increased sensitivity [56]. In addition to the instrument changes, the *E/N* optimisation (Sections 2.4.3 and 2.4.4) identified the drift tube settings that maximised instrument sensitivity towards compounds of interest to breath analysis (80/170 Td).

In comparison to the tidal breath collection, the single exhalation method was easier to monitor to ensure that the procedure was performed correctly. In terms of monitoring the breath collection at the time of sampling, the inhalation-exhalation curves displayed by the DVT software were easy to view for a single exhalation. While this was also possible to do during tidal sampling, monitoring every breath over the 5 minute collection period was not very practical. Also, since the apparatus modifications that accompanied the change from tidal to single breath sampling allowed for the more accurate measurement of ambient levels following an exhalation, a number of signals (usually m/z 37 and m/z 59) showed visible changes on-screen in the raw CIR-MS mass

spectra, corresponding to the time of exhalation. Monitoring these signals during breath sampling could similarly be used as an indication of whether a good sample had been provided. If there was any concern about the quality of the breath sample, the process was easy and quick to repeat.

3.3.4 Testing of the single breath method: The breath alcohol experiment

3.3.4.1 Introduction

After the consumption of alcoholic drinks, elevated levels of ethanol can be found in breath, which is representative of the increased blood alcohol concentrations. With this knowledge, roadside breathalyser tests have been developed and are routinely used by police officers. The legal limit for breath ethanol is 180 ppmv [57], which is much greater than endogenous levels that are at most a few hundred ppbv. The metabolism of ethanol by the body is well understood. After alcohol ingestion, some ethanol is absorbed through the stomach, but most will be absorbed in the duodenum [58]. Ethanol is then carried in the blood to the liver where the majority is metabolised by enzymatic oxidation (Figure 3.15), the major pathway being the conversion of ethanol to acetaldehyde by the enzyme alcohol dehydrogenase, and the subsequent metabolism of acetaldehyde to acetic acid by the enzyme aldehyde dehydrogenase [58]. Acetic acid is eventually converted to carbon dioxide and water in the Krebs cycle [58].



Figure 3.15 The metabolism of ethanol.

A simple breath alcohol experiment was performed to test the single breath collection method on multiple volunteers in a situation where rapid, repeat measurements were required, and to demonstrate that via the analysis of breath CIR-MS was capable of monitoring processes occurring in the body using a simple, well-defined example. This trial also provided the first quantitative CIR-MS breath measurements.

3.3.4.2 Methods

Using the apparatus shown in Figure 3.3 (b), two healthy volunteers (1 male, age 30, BMI 24.1 and 1 female, age 25, BMI 20.3) were asked to provide a series of single vital capacity breaths. Five breath measurements were recorded over a 30 minute period prior to any alcohol consumption so that baseline levels could be obtained. The volunteers then consumed 50 mL of an alcoholic drink (undiluted 40% vodka, equivalent to 2 units) over a 10 minute period. The breath test was repeated immediately after finishing the drink, and then at regular intervals over the following 3 hours. No food had been consumed in the 2 hours prior to the experiment, and no other food or drink was consumed during the course of the experiment. Breath measurements were made at 1 second integration times over a mass range of 12 - 200 u, and at an *E/N* value of 80/170 Td. The measured signals were converted to concentrations using experimentally determined sensitivity values (Section 2.4.6).

3.3.4.3 Results

As the measurements resulting from a tidal breath collection did not show distinct exhalation profiles, the data were analysed by simply taking an average over the entire sampling period. However, the same method of analysis could not be applied to the measurements obtained using the single breath collection. Whilst a number of signals were found to produce exhalation profiles, not all produced smooth profiles such as that shown for acetone in Figure 3.14, in which case the end-exhaled values could not be accurately determined by using the height of the exhalation peak. Therefore, in order to determine the best way of analysing this data, two different methods were tested.



Figure 3.16 The method used for obtaining an 'end-exhaled average' based on the acetone exhalation profile.



Figure 3.17 Comparison of the data collected on the original 1 second timescale to the same data after being summed into 2 second groups, using acetone (m/z 59) and dimethyl sulphide (m/z 63) as examples. The arrows mark the 'peak height' values for the grouped data.

Given that acetone is consistently observed in breath and that the measurements typically produced good exhalation profiles, it was possible to use the acetone profile to identify the end-exhaled region of each breath, as shown in Figure 3.16. Assuming that the end-exhaled region of all exhalation profiles fell within the same time period, the first method involved taking an 'end-exhaled average' over this time window for all mass channels. This method benefitted from being relatively quick to perform since only one exhalation profile needed to be inspected, yet end-exhaled values in all mass channels could be obtained through a simple averaging process. The second method involved grouping the data, similarly to that performed on the tidal breath data except that the data were instead summed into 2 second groups. This was the maximum possible group size that still provided a sufficient time resolution to allow the mapping of an exhalation. Figure 3.17 compares exhalation profiles plotted as the original 1 second data and the 2 second grouped data. For acetone, a smooth profile was observed even on a 1 second timescale and grouping of the data produced a similar result, however for weaker profiles such as that observed for dimethyl sulphide, a significant improvement was seen. In most cases, grouping of the data allowed values to be determined by taking the 'peak height' of each exhalation profile. This method would have been more accurate than the previous since each mass channel had to be inspected individually, however the process was more time-consuming.

The breath measurements made before alcohol consumption represented baseline endogenous levels. Baseline measurements of m/z 47, m/z 45 and m/z 61, the mass channels that would have corresponded to ethanol, acetaldehyde and acetic acid respectively, did not display any form of exhalation profile in either subject even after the data were grouped owing to the very low concentrations usually present in breath [21]. Therefore, when analysing the data using the peak height method, since no exhalation profiles were observed, the values at the point in time corresponding to the acetone maximum were used. After alcohol consumption, exhalation profiles were immediately observed at m/z 47 and m/z 45, but it took a further 25 – 30 minutes before confident profiles were seen at m/z 61. Figure 3.18 displays the breath alcohol results for the two subjects, focussing on the relationship between ethanol and its metabolites,

acetaldehyde and acetic acid. The plots display both the end-exhaled average and the peak height data for comparison, and show that a reasonably good agreement between both methods was observed.



Figure 3.18 Breath alcohol measurements displaying the relationship between ethanol and its metabolites acetaldehyde and acetic acid (top plot = subject A1/male, bottom plot = subject A2/female). The data points display the values obtained using the peak height method, with the solid line linking adjacent points (m/z 47) or representing a 2-point moving average (m/z 45 and m/z 61). Similarly the dotted lines represent data obtained using the end-exhaled average method, however for simplicity the data points are not shown.

Post-consumption, an immediate increase in m/z 47 levels was observed, which initially represented the high ethanol concentrations still remaining in the mouth. The initial mouth levels were higher in the male subject than in the female subject, even though the same amount of alcohol had been consumed and the analysis had taken place at the same time with respect to finishing the drink. The higher mouth ethanol concentration may have been a reflection of the slightly lower exhalation flow rates produced by this individual, providing a longer contact time between the ethanol in the mouth and the exhaled air. Ethanol in the mouth is thought to dissipate within approximately 10 minutes of the completion of drinking [58]. A 'dip' in the ethanol levels was observed after 10 minutes, this event being more pronounced in one volunteer than the other. Levels then peaked again after 15 – 20 minutes before declining back towards, but not reaching, baseline levels. Smith et al. observed a similar trend in breath ethanol concentrations after drinking [59]. This secondary rise and fall marked ethanol entering the bloodstream and its subsequent metabolism. The ethanol peak corresponded to 182 \pm 11 ppmv in the male subject and 149 \pm 7 ppmv in the female subject.

Levels of m/z 45 were initially increased after alcohol ingestion in comparison to the baseline levels. These levels further increased until reaching a reasonably constant level, at which they remained for approximately 1.5 hours. Acetaldehyde is the first metabolite of ethanol, and the constant levels of m/z 45 probably reflect a steady rate of acetaldehyde production and metabolism. Acetaldehyde concentrations over this period were slightly higher in the female subject (77 ± 11 ppbv) in comparison to the male subject (52 ± 9 ppbv), which may have explained the lower ethanol concentrations observed in this individual, in that more ethanol had been metabolised. Acetaldehyde in large amounts is toxic to the body, so is further metabolised to acetic acid [58]. An increase in m/z 61 was observed, which may have represented the formation of acetic acid, although other isobaric species can occur in this mass channel [60].

Exhalation profiles were observed at m/z 29 and m/z 65 (Figure 3.19). Over the course of the experiment, m/z 65 followed a similar trend to m/z 47, indicating that ethanol-water cluster ions were being formed as a result of the high ethanol concentrations. The

same trend was also observed at m/z 29, showing that ethanol was also fragmenting through the loss of water (Figure 2.4). When ethanol concentrations were above 20 ppmv, the fragment and cluster ion signals were approximately 6% and 2% of the m/z 47 signal respectively. Owing to the higher acetaldehyde concentration in breath resulting from the metabolism of the ingested ethanol, it was possible that acetaldehydewater cluster ions may have interfered with the measurement of dimethyl sulphide at m/z 63. Normally, exhaled acetaldehyde concentrations are very low, in which case the signal observed at m/z 63 is thought to result only from the compound tentatively assigned to dimethyl sulphide, although under conditions where acetaldehyde levels are elevated it is possible that acetaldehyde-water cluster ions may also contribute. Comparison of the baseline and the post-consumption levels of m/z 63 suggested that no noticeable amount of acetaldehyde-water cluster ions were being formed, as levels remained constant throughout the experiment.



Figure 3.19 An example of the exhalation profiles observed for ethanol and its corresponding fragment and ethanol-water cluster ions.

Exhalation profiles were also observed at m/z 75 and m/z 93, both of which showed the same trend as m/z 47 throughout the experiment. As these masses did not relate to any of the products of ethanol metabolism, the identities were more uncertain, but on the basis of their masses they may have represented a C₄ alcohol and its corresponding

alcohol-water cluster ion; isobutanol (2-methylpropan-1-ol) has been identified as a congener that is present in vodka [61]. These masses have not been reported in previous breath alcohol studies [59, 62], although this may have been because these studies used quadrupole instruments that are often operated so that only pre-selected mass channels are monitored. This highlighted one of the benefits of TOF-MS and the ability to monitor all mass channels in a given range simultaneously such that unexpected results are not missed.

Figure 3.20 displays the exhaled methanol concentrations over the course of the breath alcohol experiment. Methanol levels initially dropped after alcohol ingestion, which was then followed by a continuous rise with the levels eventually exceeding the baseline measurements. Ambient methanol levels at the time of the experiment were higher than the endogenous levels measured in the breath of both subjects, such that a decline was observed relative to ambient levels during the exhalation phase. Only the measurements made during the last hour of the experiment showed exhaled levels that were greater than the ambient levels, i.e. producing normal exhalation profiles. As a result, the exhaled methanol measurements may have been somewhat unreliable during the earlier part of the experiment, although the same trend was also observed in a previous breath alcohol experiment during which the background methanol levels were sufficiently low to obtain exhalation profiles throughout the entire measurement period. Lindinger at el. reported the observation of increased methanol concentrations in breath after alcohol consumption [62]. Baseline levels represent a balance between endogenous production and metabolic loss, and the metabolic loss of methanol is known to be inhibited when the body contains elevated levels of ethanol, therefore causing methanol levels in the body to rise. Lindinger at el. observed a rate of increase of 150 ppbv hour⁻¹ from baseline levels of 300 - 350 ppby [62]. The enzymes responsible for the metabolism of ethanol also metabolise other short-chain alcohols, including methanol to its aldehyde counterpart formaldehyde [63]. As the enzymes have a greater affinity for ethanol [64], methanol metabolism is inhibited and therefore accumulates in the body when ethanol levels are elevated. Lindinger et al. did not observe an initial drop in breath methanol concentrations, and Smith et al. did not monitor exhaled methanol during their

measurements, but did report a dip in exhaled ammonia concentrations after ethanol ingestion and suggested that increased portal blood flow led to an enhanced removal of ammonia, which is broken down in the liver [59]. The reason for the drop from baseline levels was uncertain, but the subsequent trend was consistent with the inhibition of methanol metabolism and rate of increase was in good agreement with the rate reported by Lindinger et al.



Figure 3.20 Exhaled methanol concentrations over the duration of the breath alcohol experiment. Only the results for subject A2 are displayed, although similar results were also obtained for subject A1.

Baseline breath acetone levels, while much higher in the male subject $(1297 \pm 79 \text{ ppbv})$ in comparison to female subject $(389 \pm 52 \text{ ppbv})$, fell within the normal concentration range previously observed for healthy adults [65]. Acetone concentrations were found to display approximately a 250 ppbv increase in both volunteers from the average baseline levels to the average levels over the last 40 minutes of the experiment (3 hours later). This trend was also observed in similar breath alcohol experiment and was explained by the metabolism of fats as hunger set in [59]. Neither of the volunteers had eaten in the 2 hours prior to the first breath measurement and the entire experiment lasted over 3 hours, so hunger was the most probable cause. As previously mentioned, exhaled

dimethyl sulphide levels remained reasonably constant throughout the monitoring period in both subjects (16 ± 4 ppbv and 15 ± 4 ppbv). Breath isoprene concentrations were more variable, but this did not seem to be influenced by alcohol ingestion. In the female subject, isoprene concentrations throughout the experiment were 260 ± 96 ppbv, while in the male subject the concentrations were slightly higher at 498 ± 137 ppbv and increased to 989 ± 80 ppbv over the last hour. While the initial values for this subject fell within the concentration range previously reported for breath isoprene [52, 66], the later measurements were rather high. Isoprene is known to be sensitive to changes in breathing rate and heart rate [67], so it may have been possible that this increase was related to the subject becoming aware that the experiment was nearing an end.



Figure 3.21 A summary of the exhalation volumes and flow rates measured for each of the breaths provided by the two volunteers over the duration of the breath alcohol experiment. ^{*}Irregular exhalations (subject A1 only).

No attempt was made to control the exhalation volume or flow rate during breath collection. Figure 3.21 summarises the exhaled volumes and flow rates measured by the DVT for each of the breaths provided by the two volunteers over the duration of the breath alcohol experiment. Problems with DVT output midway through the experiment

meant that a number of files did not record. For the 26 files that were recorded, the female subject produced fairly consistent results (volume 3242 mL \pm 4%, flow rate 1064 mL s⁻¹ \pm 8%) and while the male subject displayed a few irregular results, the majority of the measurements showed reasonable consistency (volume 4008 mL \pm 6%, flow rate 874 mL s⁻¹ \pm 12%, outliers excluded). The CIR-MS data corresponding to these irregular exhalations showed no visible deviation in the measured VOC signal, suggesting that even with a lower exhaled volume an end-exhaled sample was still being achieved.

VOCs of airway origin tend to show concentrations that are dependent on exhalation flow rate owing to the time available for diffusion from the airway wall into the exhaled air [68, 69], and VOCs originating from the alveolar interface have been reported to shown dependencies on the duration of exhalation [70]. A standardised and reproducible sample is important for quantitative breath analysis. Some researchers [3, 71] have suggested aiming for the ATS guidelines that were generated for the standardised measurement of exhaled nitric oxide, which requires breathing at a set flow rate against resistance (Section 3.1). For the application to VOC measurement, breath collection devices have used restrictors to limit the breath flow and increase the internal pressure of the apparatus in order to achieve the standardised nitric oxide procedure [71]. However, nitric oxide originates from the airways while many other compounds originate from the blood, and without knowing whether resistance to exhalation affects the distribution of VOCs across the alveolar membrane, this technique should probably be avoided.

Exhaled flow control could be achieved through visual feedback to the subject supplying the breath sample. One possibility, as devised by the manufacturers of NObreath[®] [72], is to incorporate a flow guide into the apparatus. This simple but effective idea utilises an upright clear tube perpendicular to the flow direction, which contains a float that will be maintained at a marked height inside the tube when the correct breath flow is achieved. Another solution, based on the apparatus described in this thesis, is to modify the DVT software to display a target flow on screen over which

the real-time experimental breath flow can be superimposed, therefore acting as a visual guide to control the exhalation flow rate. Target exhaled volumes could be similarly displayed to create a complete standard exhalation curve. Target volumes would be subject dependent to account for gender, height and age effects on lung volume. The target volume could be based on a set percentage of the predicted vital capacity for a subject, values which are already available owing to their use in spirometry [73]. A 'library' of pre-determined target curves for the different combinations could be generated and a 'quality control' process employed, whereby any experimental curves outside a certain percent of the target breath flow and/or exhaled volume is discarded.

3.4 Summary

A breath sampling device has been designed to couple to the CIR-MS instrument to allow the on-line, real-time analysis of breath. The apparatus was designed to have a low resistance to allow subjects of varying age and health to provide a suitable sample, and was constructed from standard-sized components that did not emit or lead to loss of VOCs, therefore maintaining the integrity of breath samples on passage through the apparatus. The DVT proved to be a useful addition to the apparatus in terms of quantifying exhalation volumes and flow rates, and the record of breathing pattern provided a means to assess the quality of a given sample.

The initial analysis of tidal exhaled breath identified a set of common VOCs in a group of healthy adults, and highlighted two signals in the breath of infected cystic fibrosis children that may have been related to infection status, although further investigation would be required since this finding could have related to other factors such as inflammation or oxidative stress, and given that these signals were observed in only a small number of individuals, it needs to be determined whether this trend is observed in larger groups. Both the initial testing with the group of healthy adults and the subjects who participated in the cystic fibrosis trial showed that there were several problems associated with tidal breath collection, such as the degree of variability in breathing pattern and the risk of hyperventilation. Tidal breath collection also had the issue of collection times on a minute timescale to ensure that a sufficient number of measurements were made and required the collection of separate ambient air samples, which further increased the total collection time.

The sampling protocol was changed to adopt a single vital capacity exhalation procedure. This offered the advantage of capturing both ambient and exhaled levels in a single experiment, which significantly reduced the sampling time and therefore allowed a higher sampling frequency. In combination with instrument improvements and apparatus changes, the single breath method resulted in the ability to view real-time exhalation profiles for those signals that displayed greater levels in breath than in the ambient air. The ability of the CIR-MS technique to monitor processes within the body through the measurement of exhaled breath was demonstrated with the use a simple breath alcohol experiment. This test also highlighted the value of TOF-MS over the more commonly used quadrupole instruments, in that the simultaneous measurement of a given mass range meant that unexpected results were not missed, and provided the first quantitative CIR-MS breath measurements. With regard to future direction, suggestions for a controlled on-line collection method using the current apparatus have been made.

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Chapter 4. On-line, real-time breath analysis of a group of asthma patients

4.1 Introduction

Chronic airway inflammation underlies a number of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis [1]. Assessment of inflammation is important in the clinical management of pulmonary disease as it may allow the monitoring of disease progression or response to antiinflammatory treatment [2]. Inflammation can be assessed non-invasively through the use of sputum analysis, usually induced by the inhalation of hypertonic saline, which allows for the quantification of inflammatory cells, although the process itself causes a short-term inflammatory response making it unsuitable for day-to-day measurements [1]. Exhaled nitric oxide is recognized as a marker of airway inflammation [3], and a non-invasive breath test has been developed that allows rapid and repeatable measurements. Exhaled nitric oxide correlates with the degree of eosinophilic inflammation [4], as is often found in asthma [5], and eosinophilic inflammation is also associated with a positive response to steroid treatment [6], so the use exhaled nitric oxide may be of use for treatment monitoring in asthma. However, asthma is not always associated with eosinophilic inflammation [7], and both COPD and cystic fibrosis are predominantly associated with neutrophilic inflammation [8, 9]. Individuals with COPD and cystic fibrosis typically show normal to low exhaled nitric oxide concentrations, with the exception of unstable COPD in which levels can be increased [1, 8]. Therefore the identification of additional exhaled markers of inflammation would be useful, especially markers that may correlate with neutrophilic inflammation.

Nitric oxide may not be the only informative chemical in the breath of individuals with inflammation. Chloramines and bromamines have been detected in breath and are thought to be produced during inflammation, derived from hypochlorous and hypobromous acids, which are produced by neutrophils and eosinophils [10]. Several inflammatory cells, such as eosinophils and neutrophils, release increased amounts of reactive oxygen species [11]. The lipid peroxidation product ethane has been found to be elevated in the exhaled breath of asthmatics, which also correlated with exhaled nitric oxide measurements [12]. Elevated ethane was similarly found to be elevated in the breath of COPD patients [13]. In both studies the exhaled ethane concentrations were found to be lower in steroid-treated subjects [12, 13]. If lipid peroxidation is increased in individuals with asthma or COPD, other classes of lipid peroxidation products such as aldehydes [14, 15] may also be present; aldehydes have been detected in the exhaled breath condensate of COPD patients [16]. Dragonieri et al. used an electronic nose coupled with pattern recognition to discriminate between the exhaled breath of asthmatics and healthy controls, showing that there is a difference in the exhaled VOC profile between these groups, although no differences were found between mild and severe asthmatics [17].

Following the development of suitable sampling apparatus and a protocol for on-line breath collection, the next stage was to begin investigating exhaled breath composition in a larger group of subjects and the variation in diseased states. This chapter focuses on the tasks involved in the relocation of the CIR-MS instrument to a clinical setting and its application to a study investigating VOCs in the exhaled breath of asthmatics and the search for potential markers of inflammation.

4.2 Experimental methods

4.2.1 Relocation to Glenfield Hospital

The CIR-MS instrument and breath sampling set-up were housed in a small room in the Respiratory Physiology Department at Glenfield Hospital, June – July 2006. In

comparison to routine use in the laboratory, there were a number of additional measures associated with the relocation and use of the instrument in a hospital. As with all medical equipment, the CIR-MS instrument and experimental arrangement had to comply with the electrical safety requirements of the Medical Equipment Service based at the hospital; in brief the measures that had to be applied before the instrument could be used with patients are outlined below, and are also summarised in Figure 4.1.

- The instrument had to be physically shielded from the patient to limit the number of components that the patients were exposed to.
- All equipment used by or located near to the patient had to be electrically isolated from the mains supply.
- All conductive parts accessible to the patient had to pass electrical safety criteria for medical devices (i.e. they must not exceed a touch current of $100 \ \mu$ A).

With the CIR-MS instrument and pumping system situated behind a partition, the patient was only exposed to the equipment associated with breath sampling and the controls for the mass spectrometer. This included the trolley holding the breath sampling apparatus, the DVT equipment and laptop for controlling the DVT, and the wireless computer controls for operating the CIR-MS instrument (Figure 4.2). The computer monitor for viewing the CIR-MS software was fixed to the partition above the trolley. The DVT equipment and laptop were powered through an isolating transformer attached to the patient trolley. The heated blanket was powered from the main CIR-MS instrument supply via a transformer in the controller to deliver an extra low voltage (24 V).



Figure 4.1 A diagram of the experimental arrangement used at the hospital with details of the electrical safety requirements (diagram provided by the Medical Equipment Service).



Figure 4.2 The CIR-MS instrument and sampling apparatus as it was laid out at the hospital.

4.2.2 Instrument arrangement

The CIR-MS instrument operated under a slightly different arrangement to that described in Section 2.3.3. A second ²⁴¹Am strip was added in an attempt to increase H_3O^+ reagent ion yield, which was hoped to translate into higher sensitivity. The double source arrangement was accommodated in a taller stainless steel surround positioned in between the top plate of the ion source and the top electrode of the drift tube. No changes were made to the drift tube or the TOF-MS. Sample and water vapour inlet flows were maintained at 220 sccm and 30 sccm respectively. The drift tube was operated at a pressure of 6 mbar and a voltage of 1324 V, resulting in an *E/N* value of 80/174 Td. All breath and ambient air measurements were made at 1 second integration times, initially over a 60 second period, but this was shortened to 30 seconds further into the study in order to reduce the time required per patient. All experiments were measured over a mass range of 12 - 154 u.

4.2.3 Instrument calibration

As the CIR-MS instrument was operated with a different ion source arrangement, a series of calibration experiments were performed to determine the sensitivity values for a selection of compounds under these settings, which would also allow the assessment of sensitivity improvement in comparison to the standard arrangement. With the double ²⁴¹Am ion source fitted, methanol, acetonitrile, acetone, dimethyl sulphide and isoprene calibrations were carried out using the gas standards generator (Section 2.3.7) at 100% relative humidity (1 minute integration times, 12 - 200 u).

4.2.4 Breath collection

On arrival the patients were seated and the breath sampling procedure was explained. The patients were asked to fill out a questionnaire requesting information on the following:

- patient study ID number/study group
- date and time of analysis
- age
- height and weight, for calculation of body mass index (BMI, in kg m^{-2})
- smoking status: current, ex- or non-smoker
- other medical conditions/medication at the time of analysis
- recent food/drink consumption (within 2 hours of analysis)
- recent alcohol consumption (within 12 hours of analysis)

Each patient was asked to provide a single inhalation/vital capacity exhalation through the mouthpiece of the sampling apparatus shown in Figure 3.3 (b). The apparatus was maintained at 40°C using the heated blanket, and the sampling line between the breath apparatus and CIR-MS sample inlet was also heated to 40°C using a heating tape. The sample line had to be longer than usual (~ 1 m) in order to pass underneath the partition. Breath volume and flow rate data were recorded using the DVT, with each inhalation/exhalation cycle displayed on the screen to aid the assessment of the validity of a sample. The collection process was repeated until 3 valid breaths were obtained per patient. The first collection was sometimes considered as a practice if the procedure had not been performed correctly, usually meaning that a subject had not inhaled through the apparatus or had provided more than one exhalation during a single breath collection. All patients completed the entire process in 3 - 4 breaths.

Where possible lung function and FE_{NO} measurements were performed by the research nurses, or taken from a recent entry in the patient medical records. Lung function was measured using a spirometer (Vitalograph) to determine FEV_1 and FVC values, where FEV_1 refers to the forced expiratory volume in one second and FVC is the forced vital capacity. Fractional exhaled nitric oxide (FE_{NO} , 50 mL s⁻¹) measurements were made using a handheld device (NIOX MINO, Aerocrine).

At the start of each day ambient air measurements were recorded to determine background VOC levels, and measurements were made repeatedly throughout the day.

4.2.5 Patient details

A total of 68 subjects participated in the study, who were categorised as one of three groups: asthma, COPD or healthy control. Patient demographics are summarised in Table 4.1. The asthma and COPD groups consisted of hospitalised patients, outpatients attending appointments, or asthmatics members of the hospital staff. Healthy controls consisted of members of the hospital staff and volunteers from the university who travelled out to the hospital to take part in the trial. All healthy subjects were free of chronic respiratory disease. Some of the asthma and COPD group were also diabetic, and a number of subjects were current or ex-smokers.

Table 4.1 Demographics of the asthma, COPD and healthy control groups (age, BMI, FEV₁ and FEV₁/FVC presented as mean and range).

Group n	10	M:F	Age	BMI (kg m ⁻²)	FEV_1	FEV ₁ /FVC
	n				(% predicted)*	(%)*
Asthma	35	17:18	49 (19 – 75)	28.9 (21.5 - 47.9)	73% (36 – 119%)	70% (44 - 88%)
COPD	5	3:2	76 (61 – 87)	28.9 (24.3 - 31.4)	44% (34 – 51%)	50% (32 - 68%)
Control	28	14:14	35 (16 - 58)	23.9 (18.5 - 34.8)	98% (69 – 113%)	83% (62 - 99%)
Total	68	34:34	45 (16 - 87)	26.9 (18.5 – 47.9)	79% (34 – 119%)	73% (32 – 99%)

* For those where spirometry data was available (asthma n = 34, COPD n = 3, control n = 16)

The study was approved by the Leicestershire, Northamptonshire and Rutland Ethics Committee and all subjects provided written informed consent.

4.3 Results

4.3.1 Double ²⁴¹Am ion source calibration

The calibration results under the double ²⁴¹Am ion source arrangement are presented in Table 4.2. The addition of a second americium source increased raw hydronium counts from 4×10^3 cps to $6 - 7 \times 10^3$ cps over a 150/200 u mass range. Comparison of the raw

sensitivities, so not to be obscured by the normalisation process, obtained for the double source arrangement and the standard single source arrangement (Table 4.2) suggested that there was some improvement with the use of an additional source. With the exception of methanol, all other VOCs showed an increased sensitivity with the double source arrangement.

Table 4.2 A summary of the sensitivities obtained using the double ²⁴¹Am ion source arrangement at 100% relative humidity and 80/174 Td. $R^2 > 0.90$ were obtained for all linear fits. The last two columns present a comparison of the raw sensitivities obtained using the double source arrangement (S_{double}) against those obtained using the standard single source arrangement (S_{single}). The raw data were collected at 1 minute integration times, so the raw sensitivities are presented in units of counts per minute per ppbv⁻¹ (cpm ppbv⁻¹).

VOC	Range (ppbv)	S (ncps ppbv ⁻¹)	$S_{\text{double}}(\text{cpm ppbv}^{-1})$	$S_{\text{single}} \left(\text{cpm ppbv}^{-1} \right)^*$
Methanol	11 – 53	19.7	10.0	11.3
Acetonitrile	23 - 114	87.7	39.8	22.5
Acetone	80 - 320	95.4	32.1	23.7
Dimethyl sulphide	2 - 8	73.5	32.3	19.4
Isoprene	3 - 14	10.0	4.8	1.8

* Raw sensitivities were obtained from the calibration data corresponding to that presented in Table 2.6.

4.3.2 Exhaled breath measurements

The single breath collection protocol was suitable for the rapid analysis of large numbers of subjects, with a triplicate breath collection typically completed within 5 - 10 minutes. A total of 68 subjects were recruited for the study, totalling 204 breaths for analysis. The data set also included subgroups such as diabetics and smokers, which provided the first opportunity to investigate how such conditions may affect the breath VOC profile. The breath data were analysed using the end-exhaled average method described in Section 3.3.4.3, since this method provided the quickest means to analyse the large amount of data generated during this study. For one subject, one out of the three breaths displayed substantially lower levels in all of the measured breath signals. Unfortunately the DVT did not record during this experiment, so there was no record of the exhaled volume to asses whether a full exhalation had been provided. However, a
m/z (19/37) value of 6 indicated that an end-exhaled sample had not been provided, therefore this sample was excluded, leaving two valid breaths for this subject and a total of 203 breaths for analysis.



Figure 4.3 Exhaled volume and flow rate distributions (n = 174).

As mentioned above, the DVT did not always register an exhalation, and in such cases the CIR-MS measurements for these breaths had no corresponding exhalation volume and flow rate data. Only 2 out of the 68 subjects had no DVT records from the triplicate breath collection. All other subjects had at least partial (18 subjects), if not complete sets of volume and flow rate measurements. The data that were collected provided an indication of the range in the exhalation volume and flow rate that result from an uncontrolled single vital capacity exhalation, as displayed in Figure 4.3.

Figure 4.4 displays the FE_{NO} concentrations for those subjects who took the test. FE_{NO} concentrations were determined for twelve subjects within the control group. A FE_{NO} value of 33 ppbv has been suggested as an upper limit for healthy adults [6], and all subjects had concentrations equal to or less than this value. The only exception was one 'healthy' subject who produced a very high FE_{NO} concentration of 187 ppbv, but it should be noted that this person showed symptoms of a respiratory infection within the days following the test, which is known to cause increased exhaled nitric oxide concentrations [18]. Only 3 COPD patients provided FE_{NO} measurements, one of which had slightly higher concentrations (42 ppbv) than the control group. Reported FE_{NO} concentrations in subjects with COPD are variable, partly because of the influence of smoking which is known to reduce FE_{NO} levels [6, 19]. One COPD subject showed no detectable levels of nitric oxide, although this person was the only current smoker within this group. As would be expected, FE_{NO} concentrations showed the greatest variability within the asthma group.



Figure 4.4 Comparison of FE_{NO} concentrations in the asthma group (n = 27), COPD group (n = 3) and control group (n = 12).

All subjects displayed exhalation profiles at m/z 33, m/z 51, m/z 59, m/z 63 and m/z 69, which were assigned to methanol and its water cluster ion, acetone, dimethyl sulphide and isoprene respectively. The end-exhaled average signals and ambient air measurements are summarised in Table 4.3. As will be discussed in Section 4.3.3.1, the exhaled VOC measurements were not normally distributed, so the data are described by the median and quartiles.

			Signal (ncps)		
m/z	Median	Lower quartile	Upper quartile	Minimum	Maximum
Air					
33	780	683	917	456	2378
51	34	25	47	12	109
59	6223	5168	7847	3854	16607
63	41	32	50	12	171
69	264	222	305	148	1086
Breath					
33	7083	5012	9943	2666	30651
51	392	245	574	61	1499
59	53653	38524	72467	23003	404933
63	774	618	993	300	1767
69	1964	1556	2685	708	4725

Table 4.3 Descriptive statistics for the measured breath VOC signals (n = 203), showing only those signals that were observed in all subjects and their corresponding levels in ambient air.

Some subjects with high acetone levels (> 1.5×10^5 ncps) showed exhalation profiles at m/z 77 (> 3×10^2 ncps), which presumably corresponded to an acetone-water cluster ion. Some subjects displayed a signal at m/z 42 (Figure 4.5), which was assigned to acetonitrile, a compound commonly observed in the breath of smokers [20]. This was the first time that acetonitrile had been measured in breath using CIR-MS. Exhalation profiles at m/z 18 were observed in one control subject (Figure 4.6), which was assigned to ammonia. Ammonia is a normal breath compound for which exhalation profiles are not routinely observed during CIR-MS analysis, as the levels in breath do not normally

exceed the levels measured in ambient air. The elevated levels in this individual may have been a reflection of diet [21].



Figure 4.5 An example of an acetonitrile exhalation profile from a smoker compared to that of two non-smokers.



Figure 4.6 An example of an ammonia exhalation profile from one healthy subject, with the acetone profile also displayed for comparison.

In addition to the previously mentioned compounds, a signal at m/z 89 was observed in the breath of a few individuals (Figure 4.7). Exhalation profiles in this mass channel were observed for a number of asthma patients, one COPD patient and a few control subjects. The levels measured in the breath of these subjects ranged from 1000 – 2000 ncps, and were typically below 500 ncps in ambient air. The shape of some of the m/z89 exhalation profiles were unusual in that when compared against their corresponding acetone profiles, they did not immediately return to pre-exhalation levels at the end of the exhalation, as was usually observed for exhalation profiles in other mass channels. The reason for this was uncertain; perhaps the compound remained in the sampling line between the apparatus and the instrument for longer, especially as the sampling line had to be made longer for the current study in order to pass underneath the partition between the patient and the instrument. These effects might not have normally been noticeable under the standard arrangement used in the laboratory.



Figure 4.7 An example of two m/z 89 exhalation profiles from different asthmatics, with the acetone profiles also displayed for comparison.

This signal had been previously observed in the breath of cystic fibrosis patients (Section 3.3.2.3), but not in any healthy subjects until the current study. During the cystic fibrosis trial, it was suggested that the signal at m/z 89 may have related to the presence of infection, although its occurrence in the breath of a patient who was thought to be free of infection at the time of sampling introduced the idea that it may have

instead related to inflammation or oxidative stress. Assuming that the signals observed at m/z 89 resulted from the same compound, its occurrence in the breath of some of the asthma and COPD patients during the current study could have supported the inflammation or oxidative stress hypothesis, although the presence in the breath of some healthy subjects complicated the issue. Two of the asthma patients and one of the healthy controls who displayed m/z 89 exhalation profiles provided FE_{NO} measurements of 56 ppbv, 12 ppbv and 24 ppbv respectively. The lowest of these FE_{NO} values corresponded to the highest intensity m/z 89 signal, which may have suggested that there was little correlation with eosinophilic inflammation, although this was difficult to assess based on the comparison of only three samples. If it was the case that this signal related to a compound of airway origin, then the comparison of the measured signal intensities may be complicated by differences in exhalation flow rates.

During the cystic fibrosis study, a signal at m/z 71 was also observed, which showed a strong correlation with m/z 89. This suggested that this pair of signals may have resulted from a single compound, such as a protonated species and its corresponding fragment or water cluster ion. For those subjects who displayed m/z 89 exhalation profiles, only one individual also displayed exhalation profiles at m/z 71. This subject did however have the highest levels of m/z 89. Based on the mass, the signal at m/z 89 could have represented a C₅ alcohol. This would also have been consistent with the fragmentation to m/z 71 through the loss of a water molecule, which is a common fragmentation pathway for alcohols. Low concentrations of pentanol have been measured in exhaled breath [22]. Pentanol can be produced from the metabolism of pentane [23], which itself is a product of lipid peroxidation, a condition that has been associated with both asthma and COPD [12, 13]. However, gut bacteria are another source of low molecular weight alcohols [24]. Alternatively, allyl methyl sulphide, which is nominally isobaric with pentanol, can also appear in breath as a result of diet [25].

One asthmatic and one COPD patient also displayed exhalation profiles at m/z 73. Again on the basis of mass, this signal may have resulted from a C₄ aldehyde or ketone. The low intensity made it difficult to determine whether these were true exhalation profiles, although good correlation with the acetone profile was observed. As a result of the low intensity, it may have been possible that this compound was present in the breath of more subjects, but that the results were overlooked. Unfortunately comparison of these results to FE_{NO} measurements was not possible in these subjects as the asthma patient did not take the test, and the COPD patient who did take the test was also a smoker and displayed no detectable levels of FE_{NO} . While no conclusive results can be made regarding either of these identified signals, given the small number of subjects in which these compounds were detected, improved instrument sensitivity may allow their significance to be more thoroughly examined.



Figure 4.8 An example of a m/z 73 exhalation profile from a COPD patient, with the acetone profiles also displayed for comparison.

The report by Senthilmohan et al. [10] on the detection of haloamines in breath was particularly interesting given that these compounds were derived from both eosinophils and neutrophils, and that these compounds were potentially detectable using CIR-MS; all reported haloamines have been found to undergo fast proton transfer with H_3O^+ reagent ions or charge transfer with O_2^+ reagent ions. However, no protonated product ions for these species were observed in the breath of the subjects in the current study, which may have reflected either that these compounds were not present in the breath samples or that they were present in levels below the limit of detection of the CIR-MS instrument. Senthilmohan reported the detection of bromamine (BrNH₂) in a healthy volunteer (~ 100 ppbv) and chloramine (ClNH₂) in the breath of a COPD patient (~ 50

ppbv), although these were both determined using O_2^+ reagent ions with no specific mention of whether these products were detected in breath when using H_3O^+ reagent ions.

4.3.3 Breath VOC distributions

4.3.3.1 General comments

It is important to establish the normal concentration ranges of breath VOCs, so that deviations in diseased states can be identified. The present study provided the first opportunity to investigate VOC concentrations in a reasonably large number of individuals. The aim of the following section is to present the distributions in the measured concentrations for a number of breath VOCs, and to investigate the various factors that may influence the exhaled concentrations. Quantitative breath VOC measurements were obtained through the application of the experimentally determined sensitivity values (Section 4.3.2) to the end-exhaled average signals. Calibrations using the double ²⁴¹Am ion source arrangement were only performed at 100% relative humidity, so in order to provide estimates of ambient air concentrations for comparison, the single source sensitivity values listed in Table 2.6 (0% RH) were applied.

All 203 breaths collected during the study were used to construct VOC concentration distributions for the 5 VOCs listed in Table 4.4. The concentration data for all 5 breath VOCs were not normally distributed, as confirmed by an Anderson-Darling test for normality (p < 0.05, Minitab). All histograms were positively skewed and appeared to be close to log-normal distributions (as marked by the curve on each histogram in the following sections), which was similarly reported by Smith et al. for a number of VOC concentration distributions in healthy subjects, including methanol, acetone and isoprene [26]. The study by Smith involved a smaller number of volunteers (n = 30) in comparison to the present work, but accumulated a larger number of breath concentrations (n > 400) through repeated measurements over a six month period. A log-transformation of the current data found only dimethyl sulphide and isoprene concentrations to form true log-normal distributions (p = 0.59 and 0.45 respectively, Anderson-Darling). As the concentration distributions were not normal, the data were described by the median and quartiles, and analysed using non-parametric statistical methods such as the Spearman's rank test for correlation, the Mann-Whitney test for the equality of two medians or the Kruskal-Wallis test for the equality more than two medians. The Spearman's rank test was carried out using SPSS, and all other statistics were carried out in Minitab.

	Concentration (ppbv)					
VOC	Median	Lower quartile	Upper quartile	Minimum	Maximum	
Methanol	350	250	498	126	1719	
Acetonitrile	3	1	6	0	322	
Acetone	484	326	683	163	4264	
Dimethyl sulphide	11	8	14	4	32	
Isoprene	193	150	264	65	575	

Table 4.4 Descriptive statistics for the measured breath VOC concentrations (n = 203).

For the comparison of VOC concentrations within the different subgroups of the study (study group, age, gender, BMI), all 'outliers' from these distributions were left out of the analysis so that only trends within the main body of the data were identified. Outliers were determined by Minitab as being a value outside of the interquartile range (i.e. less than the lower quartile or greater than the upper quartile) by more than 1.5 times the interquartile range. For comparison of the groups where these extreme values may have more significance, such as the investigation of acetonitrile in smokers or acetone in diabetics, all data were included, with the exception of a small number of uncertain samples. These samples, which consisted of two asthmatics and two healthy controls, were all collected on one particular day. These subjects provided some of the highest breath VOC concentrations, for acetone and acetonitrile in particular, even though none claimed to be current smokers. Slightly higher ambient levels were also recorded in many mass channels on this day, although all subjects still provided positive exhalation profiles well in excess of the ambient levels. Whether the high levels in these

subjects were the result of exogenous interference was uncertain, so while the data for these individuals were not excluded from the construction of the concentration distributions, they were left out of any subsequent analysis.

4.3.3.2 Methanol

The breath methanol concentration distribution showed a median concentration of 350 ppbv and a range of 126 - 1719 ppbv (Figure 4.9/Table 4.4). Overall this was in reasonably good agreement with the distribution reported by Smith et al. with a median concentration of 461 ppbv and a range of 32 - 1684 ppbv [26]. The methanol concentrations in the ambient air over the duration of the study were estimated to be between 10 - 40 ppbv.



Figure 4.9 The distribution of breath methanol concentrations (n = 203).

Figure 4.10 compares the exhaled methanol concentrations measured within the different subgroups of the study to investigate potential factors that may influence breath VOC concentrations. The results of the tests to determine any statistically significance differences between the exhaled methanol concentrations are presented in Table 4.5.



Figure 4.10 Comparison of breath methanol concentrations in the different subgroups of the study (outliers excluded).

Table 4.5 Significance test results for the breath methanol data presented in Figure 4.10.

Median Methanol Concentration (ppbv) p				
Asthma $(n = 99)$	COPD(n=15)	Control $(n = 78)$		
292	312	415	< 0.05	
Male (<i>n</i> = 93)	Female $(n = 99)$	-		
383	280	-	< 0.05	
Age < 30 (<i>n</i> = 37)	Age $30 - 50$ ($n = 80$)	Age > 50 (<i>n</i> = 75)		
456	330	323	< 0.05	
BMI < 25 (<i>n</i> = 92)	BMI 25 – 30 (<i>n</i> = 53)	BMI > 30 (<i>n</i> = 33)		
359	350	280	0.10	

According to the statistical tests, significant differences were found between the exhaled methanol concentrations of males and females, the different study groups and the different age groups. A Spearman's rank test also suggested a significant negative correlation between age and breath methanol concentration ($r_s = -0.230$, p < 0.05). No significant trends were observed between BMI and the methanol concentration in breath, through either median testing or correlation ($r_s = -0.115$, p = 0.13). Given the mainly dietary origin of methanol, the reason for the difference in the exhaled methanol concentrations between the study groups was uncertain. Smith et al. similarly reported higher methanol levels in males in comparison to females, however in contrast to the current study a significant negative correlation with BMI was observed [26]. It was suggested that the link with BMI may relate to differences in dietary intake. Despite the current study finding no significance between the methanol concentrations that were grouped according to BMI, the data presented in Figure 4.10 could be viewed as showing slightly lower methanol concentrations in subjects with a BMI over 30. Smith et al. reported that methanol showed no correlation with age, while this data suggested that methanol may tend to decrease with age.

4.3.3.3 Acetone

The breath acetone concentration distribution displayed a median concentration of 484 ppbv and a range of 163 - 4264 ppbv (Figure 4.11/Table 4.4). During the study period, ambient acetone concentrations were variable, but were generally estimated to be between 20 - 80 ppbv. While the median acetone concentration was in reasonably good agreement with previously reported values, the maximum concentrations were slightly higher than most. It is worth noting that the concentration distribution was constructed using all of the breath samples, and therefore included those samples from the diabetic patients. However, the highest acetone concentrations were actually measured in the breath of healthy control subjects, and not only those that were within the group of uncertain samples described in Section 4.3.3.1. The breath acetone concentration distribution reported by Smith et al. had a median concentration of 462 ppbv and a range of 148 - 2744 ppbv [26]. Schwarz et al. found the acetone concentrations in

mixed expiratory breath to have a median concentration of 559 ppbv and a total range of 280 - 1269 ppbv, and also determined the concentration distribution in overnight fasted subjects, which was found to have a median concentration of 609 ppbv and a total range of 177 - 3490 ppbv, therefore displaying a much higher upper concentration limit than the non-fasted distribution [27]. While the maximum concentrations observed in the current study were greater than those in the previously described distributions, acetone levels of almost 8 ppmv have been observed in the alveolar air of healthy subjects [28].



Figure 4.11 The distribution of breath acetone concentrations (n = 203) and the distribution observed in the lower concentration range (outliers excluded, n = 182).



Figure 4.12 Comparison of breath acetone concentrations in the different subgroups of the study (outliers and diabetic data excluded).

Table 4.6 Significance test results for the breath acetone data presented in Figure 4.12.

Median Acetone Concentration (nnhy)				
	P			
Asthma $(n = 92)$	COPD ($n = 12$)	Control ($n = 72$)		
378	539	461	< 0.05	
Male (<i>n</i> = 83)	Female $(n = 93)$	-		
510	405	-	< 0.05	
Age < 30 (<i>n</i> = 39)	Age $30 - 50$ ($n = 77$)	Age > 50 ($n = 60$)		
611	446	373	< 0.05	
BMI < 25 (<i>n</i> = 87)	BMI 25 – 30 (<i>n</i> = 47)	BMI > 30 (<i>n</i> = 24)		
484	471	267	< 0.05	

Figure 4.12 displays the exhaled acetone concentrations for each of the different subgroups, with the significance test results presented in Table 4.6. In addition to the exclusion of any outliers, any remaining data from diabetic subjects were also removed from the analysis because of the influence of diabetes on exhaled acetone concentrations. The results in Table 4.6 suggested that significant differences existed in all of the subgroups. Smith et al. reported significantly higher acetone concentrations in the breath of males in comparison to females (mean: males 558 ppbv/females 406 ppbv) [26]. These values were similar to the levels found in the present work (median: males 510 ppbv/females 405 ppbv). Smith et al. suggested that the difference between males and females may relate to the greater average energy consumption of males, and that a diet controlled group would be required to assess if this difference would be applicable to the wider population. Schwarz et al. analysed acetone in the breath of both uncontrolled diet and fasted groups, and found no significant difference between males and females in either groups [27].

Both Smith et al. and Schwarz et al. reported no significant correlation between age and exhaled acetone concentration [26, 27]. Schwarz et al. did find a slight age dependency for females in the fasted group, although after taking into account the rate of increase and the variability in the data, concluded that this trend was practically irrelevant unless comparing data from distant age groups [27]. Španěl et al. compared exhaled acetone concentrations over a wide age range (4 – 83 years) and found that the acetone concentrations did not vary greatly [29]. In the current study, the breath acetone concentrations in the age-grouped data were found to be significantly different, and a significant negative correlation was also observed ($r_s = -0.228$, p < 0.05), suggesting that acetone concentrations may decrease with age. However, given that this finding was not consistent with the previous reports, it may have only represented a trend within this data set.

Schwartz et al. found no correlation between breath acetone concentration and BMI in males or females [27], and Smith et al. found no significant correlation with BMI in females, but found a negative correlation in males [26]. The results in Table 4.6

suggested that there was a significant difference between the breath acetone concentrations of the different BMI groups (combined male and female data), although no significant correlation was found between breath acetone concentration and BMI ($r_s = -0.151, p = 0.06$). However, if the male and female data were analysed separately, the correlation with BMI was found to be significant for males, but not for females (males: $r_s = -0.256, p < 0.05$, females: $r_s = -0.167, p = 0.12$), which was consistent with the observations reported by Smith et al.

Four patients within the asthma and COPD groups had diabetes, a condition that can result in elevated acetone concentrations in breath. In type I diabetes the body is unable to produce insulin, while the more common type II diabetes is associated with either insufficient insulin production or a decrease in the sensitivity of cells to insulin (insulin resistance) [30]. Insulin is a hormone that signals to cells to take up glucose from the blood, so low or absent insulin causes blood glucose levels to rise and the body has to use fat as a source of energy instead, resulting in the increased production of ketone bodies [31]. However, breath acetone levels in controlled diabetes can be comparable to that found for healthy subjects. Ueta et al. found breath acetone concentrations in 21 controlled type II diabetics to range between 190 - 660 ppbv, which is within the normal range reported for healthy subjects (for example [26]), however the concentrations measured in one uncontrolled patient were slightly higher, ranging between 920 - 1200 ppbv [32]. Deng et al. reported higher breath acetone concentrations of 1760 - 3730 ppbv in 15 type II diabetics, in comparison to 220 - 800ppbv in 15 healthy controls, although the level of control in the diabetic subjects was not specified [33]. For insulin-dependent (type I) diabetics, even while adequately controlled, breath acetone levels between 2000 - 5000 ppbv have been observed [21]. Of the four diabetic subjects in the current study, one was a type II diabetic and the others did not specify. One of the diabetic subjects belonged to the group of individuals who provided the uncertain samples described in Section 4.3.3.1 and showed breath acetone concentrations greater than 2000 ppbv, although this was no higher than the non-diabetic subjects who provided samples on the same day. Out of the remaining diabetics, the 3-breath median acetone concentrations were 487 ± 16 ppbv, 529 ± 30

ppbv and 1752 ± 101 ppbv, of which the middle value represents that obtained from the type II diabetic subject. Overall these values gave a higher median breath acetone concentration for diabetics in comparison to non-diabetics (median: diabetics/non-diabetics 529/458 ppbv, p < 0.05, Figure 4.13), although the diabetic group was very small. While two of the diabetic subjects showed reasonably normal breath acetone concentrations, the other subject displayed relatively high concentrations, although without full information on the type of diabetes or level of control at the time of sampling for these subjects, it is not possible to make any further comment.



Figure 4.13 Comparison of breath acetone concentrations in non-diabetic (n = 182) and diabetic individuals (n = 9). All data were included except for the uncertain samples discussed in Section 4.3.3.1.

4.3.3.4 Dimethyl sulphide

The breath dimethyl sulphide concentration distribution was found to have a median concentration of 11 ppbv and a range of 4 - 32 ppbv, although most breath samples displayed concentrations between 8 - 14 ppbv, corresponding to a narrow interquartile range of 6 ppbv (Figure 4.14/Table 4.4). Even with such low concentrations in breath, the exhaled dimethyl sulphide levels were still in excess of the levels found in the ambient air, which were typically around 1 ppbv. A study by van den Velde et al. found that while dimethyl sulphide concentrations greater than 100 ppbv could be observed in the breath of healthy subjects, the majority of the breath concentrations ranged between

8 - 24 ppbv [28], which was in good agreement with the results observed in this study. Taucher et al. reported slightly higher dimethyl sulphide concentrations in the breath of healthy subjects, with a mean concentration of 28 ppbv and a range of 20 - 40 ppbv, however concentrations up to 90 ppbv were also observed in one individual several hours after the consumption of garlic [25], so diet may therefore be responsible for the higher dimethyl sulphide concentrations in the breath of some individuals.



Figure 4.14 The distribution of breath dimethyl sulphide concentrations (n = 203).

The statistical analysis (Figure 4.15/Table 4.7) showed that no significant differences were observed between the exhaled dimethyl sulphide concentrations of males and females, or of the different age groups. However, significant differences were found between the breath dimethyl sulphide concentrations of the different study groups and of the different BMI groups, and exhaled dimethyl sulphide concentrations were found to show a significant negative correlation with BMI ($r_s = -0.167, p < 0.05$). Even though these results suggest that the observed trends were statistically significant, the true significance may have been somewhat less given that the median values of these groups differ by a few ppbv at most, yet the variability observed in the measurement of dimethyl sulphide in breath can often be much greater (see Section 4.3.4).



Figure 4.15 Comparison of breath dimethyl sulphide concentrations in the different subgroups of the study (outliers excluded).

Table 4.7 Significance test results for the breath isoprene data presented in Figure 4.15.

Median Dimethyl Sulp	р		
Asthma (<i>n</i> = 103)	COPD (<i>n</i> = 15)	Control $(n = 79)$	
10	13	12	< 0.05
Male (<i>n</i> = 99)	Female $(n = 98)$	-	
11	10	-	0.21
Age < 30 (<i>n</i> = 42)	Age $30 - 50$ ($n = 86$)	Age > 50 (<i>n</i> = 69)	
11	11	10	0.18
BMI < 25 (<i>n</i> = 89)	BMI 25 – 30 (<i>n</i> = 58)	BMI > 30 (<i>n</i> = 33)	
11	10	10	< 0.05

4.3.3.5 Isoprene

The breath isoprene concentration distribution displayed a median concentration of 193 ppbv and a range of 65 - 575 ppbv (Figure 4.16/Table 4.4). The estimated ambient air concentrations during the study period were typically found to be below 10 ppbv. The breath isoprene concentration distribution reported by Smith et al. showed a median concentration of 106 ppbv and a range of 0 - 474 ppbv [26], which were slightly lower than the concentrations in the current study. Taucher et al. reported a mean breath isoprene concentration of 240 ppbv in a group of healthy adults, but a much larger range of 0 - 1400 ppbv [34], while Kushch et al. found breath isoprene concentrations in mixed expiratory breath to have a geometric mean of 99 ppbv and a smaller range of 5 - 275 ppbv [35].



Figure 4.16 The distribution of breath isoprene concentrations (n = 203).

Out of the different subgroups within the study, the only statistical difference was observed between the exhaled dimethyl sulphide concentrations of males and females (Figure 4.17/Table 4.8). Lechner et al. also observed significant differences between the mean breath isoprene concentrations of males (81 ± 34 ppbv) and females (57 ± 28 ppbv) [36]. The isoprene concentrations reported by Lechner et al. were lower than

those observed in the current study and in comparison to other reports, and may have resulted from the use of concentrations that were calculated from PTR-MS measurements, which if only based on the protonated ion signal measured at m/z 69 may not have accounted for any isoprene fragmentation. In some PTR-MS instruments the signal measured at m/z 69 has been shown to account for less than 50% of the total isoprene product ions [35]. In contrast, Smith et al. found no significant differences between breath isoprene concentrations in males and females [26], as was similarly reported by Kushch et al. [35]. These same studies also reported finding no significant correlation between breath isoprene concentrations and BMI [26, 35], which was consistent with the current results.

Some studies have reported an age dependency for breath isoprene concentrations. Lechner et al. reported significant differences between the concentrations found in younger adults (< 30 years old, 40 ± 24 ppbv) and those found in older adults (> 30 years old, between 67 ± 29 ppbv – 75 ± 29 ppbv depending on the age group) [36], however other studies have reported finding that concentrations show no correlation with age in adult subjects [37, 38]. Taucher et al. observed no age dependency in adults, but found that concentrations in a small number of children were lower (< 200 ppbv) [34]. The breath isoprene concentrations in the current study, which consisted largely of adults, did not show any significant age dependency based on the analysis of the different group medians (Table 4.7) or correlation ($r_s = -0.030$, p = -0.67). It was worth noting that the youngest subjects in the study (16 years old) did not display the lowest breath isoprene concentrations.

Isoprene is present in cigarette smoke in much greater concentrations than in alveolar air [39], so smokers may be expected to have elevated breath isoprene levels. Whilst Euler et al. reported elevated breath isoprene concentrations shortly after smoking, baseline levels were found to return within 10 minutes [39]. In the present study, no difference was found between breath isoprene concentrations in non-smokers, exsmokers and current smokers (respective medians: 189 ppbv, 173 ppbv, 165 ppbv, p = 0.37), which was similarly reported by Kushch et al. [35].



Figure 4.17 Comparison of breath isoprene concentrations in the different subgroups of the study (outliers excluded).

Table 4.8 Significance test results for the breath dimethyl sulphide data presented in Figure 4.17.

Median Isoprene Conc	р		
Asthma ($n = 101$)	COPD ($n = 15$)	Control $(n = 83)$	
195	172	185	0.77
Male (<i>n</i> = 97)	Female (<i>n</i> = 102)	-	
212	172	-	< 0.05
Age < 30 (<i>n</i> = 42)	Age $30 - 50$ ($n = 86$)	Age > 50 (<i>n</i> = 69)	
181	208	181	0.06
BMI < 25 (<i>n</i> = 92)	BMI 25 – 30 (<i>n</i> = 59)	BMI > 30 ($n = 30$)	
182	201	196	0.31



Figure 4.18 Comparison of breath isoprene concentrations in non-smokers (n = 146), ex-smokers (n = 30) and smokers (n = 15). All data were included except for the uncertain samples discussed in Section 4.3.3.1.

4.3.3.6 Acetonitrile

Acetonitrile is an exogenous compound that is commonly observed in the breath of smokers. In the current study, exhaled acetonitrile levels in most non-smoking individuals were found to be equivalent to that measured in the ambient air, i.e. no exhalation profile was observed. Ambient acetonitrile concentrations throughout the study period were estimated to be less than 3 ppbv. The breath acetonitrile concentration distribution displayed a median concentration of 3 ppbv, but a wide range of 0 - 322 ppbv (Figure 4.19/Table 4.4) because of the influence of smoking. Previous studies have found that acetonitrile concentrations in the breath of non-smokers are generally below 20 ppbv [20, 40, 41], while smokers could have concentrations as high as 200 ppbv [20]. Acetonitrile has a long residence time in the body in comparison to other smoking-related compounds such as benzene, and acetonitrile concentrations in the breath of smokers who have been prevented from further smoking can take almost one week to return to non-smoker levels [20]. Figure 4.20 shows a comparison of the acetonitrile concentrations observed in the breath for non-smokers, ex-smokers and current smokers measured during the present study and, as would be expected, a

significant difference was found between the groups (respective medians: 2 ppbv, 2 ppbv, 18 ppbv, p < 0.05).



Figure 4.19 The distribution of breath acetonitrile concentrations (n = 203).

As shown in Figure 4.20, some non-smoking and ex-smoking individuals showed relatively high acetonitrile concentrations in their breath, which were more indicative of concentrations that would have been observed for current smokers. There are currently no other known species or fragments reported to occur at m/z 42 [42], so the assignment of the signal in this mass channel to acetonitrile can be made with some confidence. Classification of smokers, ex-smokers and non-smokers was based on patient declaration, so it was possible that some people were not honest with their smoking status, especially for those within the asthmatic group and maybe some of the younger subjects. Another option that was considered was the effect of passive smoking. Based on the measurement of acetonitrile in breath, Prazeller et al. found that a person who remained in a smoke contaminated environment for an 8 hour duration suffered from the equivalent of smoking 1 - 2 cigarettes [43]. If any of the non-smoking subjects, for example, lived in a smoking household, then the exposure may have been sufficient to elevate the acetonitrile concentrations in their breath. The patient questionnaire only asked about the smoking status of the patient and not whether they were regularly

exposed to second-hand cigarette smoke. While this information was not available during the current study, it would be worth including in the patient questionnaires of future studies. Acetonitrile does have other sources in the urban environment, such as automobile exhaust [44, 45], although this seemed to be an unlikely cause of the high concentrations observed in the breath of some of these subjects.



Figure 4.20 Comparison of breath acetonitrile concentrations in non-smokers (n = 146), ex-smokers (n = 30) and smokers (n = 15). All data were included except for the uncertain samples discussed in Section 4.3.3.1.

4.3.4 Variability in breath measurement

With the exception of one subject who had one of their breath samples excluded, all volunteers who participated in the study provided triplicate breaths. For an uncontrolled vital capacity breath collection, the variability in the triplicate end-exhaled breath concentrations were generally found to be between 5 - 11% for methanol, 3 - 6% for acetone, 13 - 28% for dimethyl sulphide, and 12 - 26% for isoprene (based on the lower and upper quartile values). This is summarised graphically in Figure 4.21. The variability was defined as the interquartile range divided by the median of the three individual end-exhaled concentrations (expressed as a percent).



Figure 4.21 The variability (%) observed in the triplicate breath measurements (n = 68) for the following parameters: V – exhalation volume, F – exhalation flow rate, m/z 33 – exhaled methanol concentration, m/z 59 – exhaled acetone concentration, m/z 63 – exhaled dimethyl sulphide concentration and m/z 69 – exhaled isoprene concentration.

Some of the variability may have resulted from the averaging method used to obtain the end-exhaled values, as it was possible that not all exhalation profiles were the same shape or perfectly aligned to the acetone profile, but this represents a limitation of the data processing rather than of the breath measurements, and would be correctable with the right data program. Without the use of a dedicated computer program, the peak height method (Section 3.3.4.3) would not have been practical for use with a data set of this size. As an example, to have determined end-exhaled concentrations for five VOCs in the 203 breath samples collected, over 1000 exhalation profiles would have needed to be analysed.

The lack of a controlled sampling procedure can have a significant effect on the variability of breath measurements. Breath isoprene concentrations are sensitive to changes in heart rate and breathing rate [46], which could have lead to variable isoprene measurements if not the subject is not at rest during breath collection. Isoprene concentrations have also shown a dependency on the duration of exhalation [47]. Some researchers believe that highly water soluble trace gases interact with the airway mucosa

such that breath concentrations will depend upon factors such as exhaled volume and flow rate [47, 48]. Suggestions for a more controlled on-line sampling method based on modifications of the current sampling apparatus were made in Section 3.3.4.3, and while this was not possible to implement at the time of the current study, it remains a focus of future work.

4.3.5 Longitudinal measurements

One healthy subject (female, non-smoker) provided repeat breath measurements at regular intervals over the 4 week study period to assess the intra-individual variation in breath VOC concentrations. All measurements took place in the morning between 9 am and 12 pm, and on some occasions two sets of measurements were given on the same day to provide both morning and afternoon measurements. Figure 4.22 shows the variation in the breath VOC concentrations over the duration of the study. Median breath concentrations for dimethyl sulphide and isoprene remained reasonably constant over the study period, while methanol and acetone concentrations were more variable. The concentration of acetone in breath can change over the course of a day depending on the amount of carbohydrates received in the diet [49]. However, on the few occasions where two sets of breaths were given on the same day, no substantial difference was observed between the morning and afternoon breath concentrations. Also shown in Figure 4.22 are the measurements that were made for another healthy subject (male, non-smoker) who provided breath samples on three occasions during the first half of the study at various times of day. The variation in the breath VOC concentrations were not as great in this subject in comparison to the previous individual, but this may have been a reflection of the much smaller number of samples.



Figure 4.22 The variation in breath VOC concentrations for one female subject over a 4 week period (red circles) and one male subject who provided samples on three occasions (blue squares). Each point represents the triplicate breath median and the error bars display the interquartile range. The horizontal lines mark the overall median concentration in each individual.

In addition to presenting the breath VOC concentrations as a function of time, the same data can be used to construct concentration distributions to provide a better representation of the intra-individual variation (Figure 4.23).



Figure 4.23 Breath VOC concentration distributions for one individual monitored over a 4 week period (n = 57).

Table 4.9 Descriptive statistics for the measured breath VOC concentrations in one individual monitored over a four week period (n = 57).

	Concentration (ppbv)				
VOC	Median	Lower quartile	Upper quartile	Minimum	Maximum
Methanol	454	353	569	219	1127
Acetone	473	408	963	261	1708
Dimethyl sulphide	11	10	15	7	22
Isoprene	166	145	195	95	278

The histograms in Figure 4.23 displayed similar skewed distributions to those found for the study group as a whole (Section 4.3.3.2 – Section 4.3.3.5). The descriptive statistics for the distributions are displayed in Table 4.9. The exhaled methanol and acetone concentrations were found to range between 219 - 1127 ppbv and 261 - 1708 ppbv respectively. Comparable variability for the concentrations of these VOCs in breath was also observed by Turner et al. when monitoring a group of subjects over a 6 month period [50, 51]. The exhaled dimethyl sulphide and isoprene concentrations were found to range between 7 - 22 ppbv and 95 - 278 ppbv respectively. For the breath isoprene concentrations, the variability was again comparable to that observed during the monitoring of subjects over a 6 month period [52]. Exhaled dimethyl sulphide concentrations do not appear to have been previously monitored during a longitudinal study. Monitoring of VOCs in the breath of the same individual removes any effects of age, gender or BMI on the exhaled concentrations, so the observed intra-individual range was likely to have resulted from either the variability in the breath measurement or reflected true variations within the body. Given that the triplicate breath variability for methanol and acetone was small in comparison to the observed intra-individual variability, this suggested that these VOCs can show considerable range within the body.

4.4 Summary

This chapter saw the CIR-MS instrument successfully relocated to perform its first study in a clinical setting, and demonstrated the effectiveness of the breath sampling protocol in terms of dealing with a reasonably large number of people. The objective of this study was to examine VOCs in the exhaled breath of asthma and COPD patients, and to search for potential markers of airway inflammation. While none were immediately apparent, a small number of signals were observed in certain individuals, although it was not clear whether these signals resulted from compounds of relevance to asthma or COPD. Fortunately a reasonably large number of subjects participated in the trial, which provided an opportunity to investigate the variation in the concentrations of a number of breath VOCs. The VOC concentrations were found to have distributions that were close to log-normal, and were generally in good agreement with those of other published studies. The large amount of data generated by this study highlighted the need for dedicated software to automatically process and display the data; real-time analysis ideally requires real-time results.

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Chapter 5.

Off-line breath collection and its application to the analysis of the exhaled breath in a group of female cancer patients

5.1 Introduction

Until now the focus has been on the on-line, real-time analysis of breath. Direct, on-line analysis is preferred since there are no intermediate steps that increase the chance of contamination or loss of the sample. However, if it is not possible to perform breath sampling and analysis at the same location, then a number of off-line methods are available. For clinical studies it can often be simpler to collect breath samples in some form of container in that the analytical instrument does not need to be relocated to the site of the study (as described in Chapter 4) or alternatively require the patients to be brought to the laboratory, providing that the samples can be adequately stored and transported from the site of breath collection to the site of analysis. In addition to the standard breath collection requirements (Section 3.1), off-line sampling has the added concern of how to preserve sample composition during collection and storage.

Containers for off-line breath collection include stainless steel canisters, adsorbent traps and polymer bags [1-3]. Canisters offer durability, so even though the initial cost is higher their working lifetime will be longer, although canisters require a slightly more technical approach to sampling. The 'single breath canister method' involves a 1 L canister to first be evacuated, and then an end-exhaled breath sample is provided via a small Teflon tube until atmospheric pressure is achieved [4]. It has been claimed that canister samples are stable for storage periods of 30 days [4], although others have shown that this is not the case for some compounds such as thiols and alcohols [5]. A wide variety of sorbents are available for the trapping of VOCs [2], although there is no one sorbent that is applicable to all, so multi-bed sorption traps are required for the analysis of VOCs in complex samples such as breath [6, 7]. Collection into polymer bags has become a popular method for off-line breath collection. Polymer bags are easy to use, moderately priced and reusable to a degree, although there are some issues with permeation, adsorption and contaminant emissions. Commonly used bag materials include Tedlar (polyvinylfluoride, PVF), Teflon (polytetrafluoroethylene, PTFE), Nalophan (polyethyleneterephthalate, PET), and Mylar (aluminium-coated PET), of which several studies have investigated the suitability of these materials for the storage of VOCs taking into account losses, contaminants and ease of cleaning [8-11]. Tedlar bags are the most commonly used with regard to breath analysis (see for example [12-18]). However, Tedlar bags are not designed for long-term storage, so the amount of time available between breath collection and analysis has to be determined. Reports on the stability of both synthetic mixtures and real breath samples contained in Tedlar bags are variable, ranging from a few hours to one week, and usually depend on the analyte of focus [8-10, 12, 13, 18-20].

During storage in Tedlar bags, VOCs can be lost either through adsorption to the inner walls or valve fittings, permeation through the bag walls or partitioning into condensed water vapour in the case of humid samples [8, 21]. Steeghs et al. simultaneously measured the loss of VOCs from a black-layered Tedlar bag and the corresponding increases in a cuvette surrounding the bag [22]. For most of the VOCs, the concentration increase in the cuvette corresponded well with the observed decrease from the bag, supporting the idea of loss via diffusion. However, for methanol only 20% of the losses from the bag were accounted for in the cuvette suggesting that some adsorption had also occurred. Samples contained in black-layered Tedlar bags have been found to display higher losses in comparison to those contained in standard Tedlar bags, which was presumed to result from the adsorption of compounds to the carbon layer [9, 23]. Also, older Tedlar bags have been found to show higher losses in comparison to new bags owing to micro-damage of the polymer film [9]. Diffusion

losses are thought to be dependent on the size of the bag, with greater losses being observed for bags with larger surface area-to-volume ratios [24]. While Groves et al. observed lower VOC recoveries from 'wet' bags in comparison to 'dry' bags, the quantity of water required to produce this difference was greater than that expected to condense from a typical breath sample, so it was concluded that the loss to condensed water would not be significant [21]. Another problem with the use of Tedlar bags is the contamination of the contents as a result of compounds that originate from the bag material. The main contaminants have been identified as *N*,*N*-dimethylacetamide and phenol [22, 25], which are assumed to result from the production process [22]. Other lower intensity contaminants have also been observed [9, 25].

An opportunity arose to perform a pilot study examining the exhaled breath from a group of female cancer patients. During this study, the instrument was not relocated to a hospital, and the breath samples were instead collected off-line in Tedlar bags and transported to the laboratory for analysis. This chapter initially focuses on the testing performed in preparation for the study to evaluate the storage properties of Tedlar bags, especially with regard to the determination of acceptable storage times for the period between sample collection and analysis. This is then followed by the details and results of the study.

5.2 Tedlar bags

5.2.1 Sample stability and contaminant measurements

A number of experiments were performed to determine sample recoveries with respect to time and the degree of contamination of the bag contents. For the collection of breath samples, 10 L bags were thought to be the most suitable in terms of providing a sufficient sample volume for CIR-MS analysis, whilst still being a size that a patient could comfortably fill with breath. In addition to 10 L bags, 5 L and 40 L bags were also tested to investigate size-related effects. All 5 L, 10 L and 40 L Tedlar bags (SKC Ltd., UK) came with a single polypropylene combined hose/valve and septum fitting,

and had a film thickness of 2 mil (approximately 0.05 mm). The dimensions along the inner seal of a deflated bag and approximate surface area-to-volume ratios, where the volume was taken as the maximum capacity of the bag, were as follows: $5 \text{ L} (31.5 \times 30 \text{ cm}, 378:1 \text{ cm}^2 \text{ L}^{-1})$, $10 \text{ L} (47.5 \times 30.5 \text{ cm}, 290:1 \text{ cm}^2 \text{ L}^{-1})$ and $40 \text{ L} (61.5 \times 60 \text{ cm}, 185:1 \text{ cm}^2 \text{ L}^{-1})$. All new bags were flushed at least 3 times with high-purity nitrogen before use, as recommended by the manufacturer. A single flush consisted of filling the bag with nitrogen, leaving for approximately 5 minutes, and then evacuating the contents using a small diaphragm pump. For analysis, the Tedlar bags were connected to a $\frac{1}{8}$ inch PFA sampling line using a section of Marprene flexible tubing, as shown in Figure 5.1. The bag contents were analysed at a sampling rate of 220 sccm, and CIR-MS analysis was performed at 1 minute integration times (12 - 200 u) and an *E/N* of 80/170 Td.



Figure 5.1 The experimental arrangement for the analysis of Tedlar bag samples.

The flushing process for a new Tedlar bag was monitored and repeated over several days (Figure 5.2). The Tedlar contaminants N,N-dimethylacetamide and phenol were observed at m/z 88 and m/z 95 respectively. Flushing of the bag with nitrogen only

temporarily reduced the levels of these compounds and as the contaminants are released from the bag material, the levels were found to return over time. Traces of methanol at m/z 33 were also detected in the new bag, but the flushing process was found to reduce the levels at least on longer timescales, if not permanently. Ideally each bag would have been flushed immediately before the addition of any sample, although depending on the sampling location and therefore the availability of a pure gas supply and pump, this was not always an option. In addition to these compounds, a low level contaminant was also observed at m/z 46, which again was only temporarily reduced by the flushing process, indicating that it originated from the bag material. This may have corresponded to dimethylamine, which has been suggested as a hydrolysis product of N,Ndimethylacetamide [25].



Figure 5.2 Background levels of *N*,*N*-dimethylacetamide (m/z 88), phenol (m/z 95), methanol (m/z 33) and m/z 46 measured in the consecutive flushes of a new Tedlar bag with nitrogen on 3 separate days. On each day 5 consecutive flushes were performed, which are labelled F1 – F5. Data points represent the average signal (5 scans) and error bars display the standard deviation.

To test the stability of VOCs during storage, 6 Tedlar bags $(2 \times 5 L, 2 \times 10 L, 2 \times 40 L)$ were filled with a standard mixture containing a selection of VOCs of interest to breath research and monitored over a 2 day period. The mixture was generated using the gas standards generator (Section 2.3.7) and consisted of acetonitrile (53 ppbv), acetaldehyde (16 ppbv), acetone (120 ppbv), dimethyl sulphide (4 ppbv), methanol (22 ppbv) and isoprene (7 ppby). The isoprene concentration was estimated using Equation 2.29, as the permeation tube had to be added to an oven that was set 5°C above the certified temperature in order to incorporate the compound into the mixture. Although some VOC concentrations in the mixture were lower than that found in exhaled breath, the dilution settings of the gas standards generator were chosen as a balance between achieving high VOC concentrations, which requires a low dilution flow rate, and the time required to fill the bags. Under the chosen settings the bags took between 5 - 10minutes to fill. Direct measurements from the gas standards generator were made both before and after all of the bags were filled. The mixture contained within the bags was analysed shortly after filling (0 hours), and again after 6, 24 and 48 hours. During this time, all bags were stored at room temperature. In order to make this number of measurements, the sample and therefore water vapour flow rates into the CIR-MS instrument during this experiment had to be reduced to 100 sccm and 15 sccm respectively. All other settings were maintained as described previously.

One study, which focussed on organochloride compounds and toluene, suggested that adsorption to the bag fittings could affect recovery [26], although another study found that, in terms of VSCs, no differences were observed for bags fitted with either Teflon or stainless steel fittings [9]. Visual inspection of the measurements made directly from the gas standards generator in comparison to those made of the same mixture from the Tedlar bags indicated that there was no immediate loss of the test VOCs to the fittings or the Tedlar material, under both dry and humid conditions. While the test compounds were not representative of all VOCs, it provided reassurance for some of the important breath VOCs that were included in the standard mixture.

The VOC levels measured during storage in the Tedlar bags are displayed in Table 5.1, and illustrated in Figure 5.3 for the 10 L Tedlar bags. Acetonitrile showed on average between 82 - 88% recovery after the first 6 hours of storage, and between 63 - 73% recovery after 48 hours. Beauchamp et al. found acetonitrile to show a fast rate of loss with 67% recovery after 10 hours and only 9% after 70 hours from a 3 L Tedlar bag that initially contained 72 ppbv [8]. Diffusion was suggested to be the main cause of loss, given the small size and linear shape of acetonitrile molecules. The bags in the study by Beauchamp et al. were also stored at 45°C, which probably enhanced diffusion and may explain the faster rates of loss observed in comparison to the current results. Ambient levels were not measured at the time of analysis, but inspection of previously collected data showed that acetonitrile levels in the laboratory were usually much lower than that initially contained in the bag mixture, so it was likely that there was a sufficient concentration gradient for diffusion to occur, supporting this as the route of loss.



Figure 5.3 The VOC levels (relative to 0 hours) measured during storage in a dry gas mixture contained in 10 L Tedlar bags. Data points represent a two bag average and error bars display the relative standard deviation (%).

Table 5.1 The VOC levels (relative to 0 hours) measured during storage in a dry gas mixture contained in 5 L, 10 L and 40 L Tedlar bags. Values represent a two bag average with the relative standard deviation (%) given in brackets. * The $H_3O^+/H_3O^+(H_2O)$ ratio is presented in the standard form.

			Relative Signal (%)			
			0 h	6 h	24 h	48 h
5 L	m/z (19/37) [*]	${\rm H_{3}O^{+}\!/H_{3}O^{+}\!(H_{2}O)}$	20	10	9	9
	<i>m/z</i> 33	Methanol	100 (8)	97 (7)	119 (8)	145 (8)
	<i>m/z</i> 42	Acetonitrile	100 (5)	82 (6)	74 (4)	63 (5)
	<i>m/z</i> 45	Acetaldehyde	100 (6)	92 (6)	95 (6)	94 (2)
	<i>m/z</i> 59	Acetone	100 (3)	91 (3)	95 (3)	92 (3)
	<i>m/z</i> 63	Dimethyl sulphide	100 (8)	96 (14)	97 (9)	97 (13)
	<i>m/z</i> 69	Isoprene	100 (10)	78 (17)	87 (12)	81 (13)
10 L	m/z (19/37)*	$H_3O^+/H_3O^+(H_2O)$	21	10	9	9
	<i>m/z</i> 33	Methanol	100 (7)	95 (5)	105 (5)	121 (7)
	<i>m/z</i> 42	Acetonitrile	100 (3)	85 (3)	79 (3)	71 (3)
	<i>m/z</i> 45	Acetaldehyde	100 (4)	94 (4)	99 (5)	97 (4)
	<i>m/z</i> 59	Acetone	100 (2)	93 (2)	98 (2)	97 (2)
	<i>m/z</i> 63	Dimethyl sulphide	100 (14)	104 (8)	104 (8)	106 (7)
	<i>m/z</i> 69	Isoprene	100 (10)	79 (16)	86 (13)	83 (19)
40 L	$m/z (19/37)^*$	$H_3O^+/H_3O^+(H_2O)$	20	11	9	9
	<i>m/z</i> 33	Methanol	100 (7)	96 (6)	110 (9)	124 (4)
	<i>m/z</i> 42	Acetonitrile	100 (3)	88 (4)	81 (3)	73 (4)
	<i>m/z</i> 45	Acetaldehyde	100 (5)	95 (5)	99 (5)	97 (6)
	<i>m/z</i> 59	Acetone	100 (2)	94 (2)	97 (1)	94 (2)
	<i>m/z</i> 63	Dimethyl sulphide	100 (18)	91 (9)	94 (13)	86 (11)
	<i>m/z</i> 69	Isoprene	100 (13)	71 (16)	72 (17)	70 (12)

Table 5.2 The VOC levels (relative to 0 hours) measured during storage in a humid gas mixture contained in 10 L Tedlar bags. Values represent a two bag average with the relative standard deviation (%) given in brackets. ^{*} The $H_3O^+/H_3O^+(H_2O)$ ratio is presented in the standard form.

			Relative Signal (%)		Relative ppbv (%)	
			0 h	48 h	0 h	48 h
10 L	<i>m/z</i> (19/37) [*]	${\rm H_{3}O^{+}\!/H_{3}O^{+}\!(H_{2}O)}$	3	9	-	-
	<i>m/z</i> 33	Methanol	100 (4)	146 (2)	100	121
	<i>m/z</i> 42	Acetonitrile	100 (3)	102 (2)	100	84
	<i>m/z</i> 45	Acetaldehyde	100 (5)	141 (3)	100	90
	<i>m/z</i> 59	Acetone	100 (2)	116 (2)	100	108
	<i>m/z</i> 63	Dimethyl sulphide	100 (15)	114 (12)	100	111
	<i>m/z</i> 69	Isoprene	100 (15)	170 (17)	100	72

Isoprene appeared to show some loss over the first 6 hours and no further loss thereafter, although it was uncertain whether this reflected a true decrease since there was between 10 - 19% variability in the measurements. Isoprene levels in the standard gas mixture were only marginally higher than those typically found in the ambient air, whereas isoprene concentrations in breath are typically tens to hundreds of ppbv, so a greater diffusional loss of isoprene may occur during the storage of breath samples in Tedlar bags. Using an initial concentration of 76 ppbv, Beauchamp et al. reported isoprene recoveries of 81% and 61% after 10 and 70 hours respectively, although the samples were contained in smaller 3 L bags that were again stored at 45°C [8]. These losses were attributed mainly to diffusion through the Tedlar film with minimal amounts of adsorption. Breath isoprene concentrations have been found to remain stable for up to 6 hours in 8 L Tedlar bags, but not for longer periods [13].

Acetaldehyde and dimethyl sulphide showed no major change over the 48 hour monitoring period. Since the acetaldehyde and dimethyl sulphide levels within the bag were greater than that normally found in the ambient air there would have been a sufficient concentration gradient for diffusion to occur, therefore these compounds were assumed to remain relatively stable during short-term storage. Similar observations have also been reported for these compounds. Beauchamp et al. found 76 ppbv of acetaldehyde to remain stable over a 10 hour period, and to still show 85% recovery after 70 hours [8]. The measured losses were thought to have been caused by the adsorption to the inner walls as acetaldehyde levels were found to increase upon heating the bag. In 1 L Tedlar bags containing an initial concentration of 53 ppbv, Mochalski et al. found that dimethyl sulphide showed good short-term recovery and greater than 80% recovery even after 3 days of storage [9]. In 12 L Tedlar bags with an initial concentration of 420 ppbv, Sulyok et al. found that dimethyl sulphide to show greater than 80% recovery even after 1 week [23].

Acetone also appeared to show no major change over the duration of the monitoring period. However, acetone concentrations in the laboratory environment are highly variable and levels comparable to that contained within the bag have been observed, so

it was difficult to assess the true stability of acetone from these experiments without knowledge of the ambient levels at the time of analysis. Beauchamp et al. found 76 ppbv of acetone to remain stable over 70 hours of storage, although similar problems were reported in that the ambient concentrations were found to be equal to that contained within the bag [8]. Deng et al. reported acetone concentrations to remain stable in 3 L Tedlar bags for storage times of up to 4 hours at 40°C, but for no longer than 6 hours [12]. In this example the bags contained 600 ppbv of acetone, which was much higher than that used by Beauchamp et al. and the current experiment, although this is more representative of the concentrations that are found in breath. Acetone losses may therefore be expected over time, if there is a sufficient concentration gradient between the sample and the surrounding environment.

Methanol levels were actually found to increase over time, showing an increase of between 5 – 19% after 24 hours and 21 - 45% after 48 hours, although the levels remained reasonably constant for the first 6 hours. Laboratory methanol levels are typically double that initially contained within the bag mixture, so the diffusion of methanol into the bag was possible. Lipari et al. found that over 70% of the initial methanol concentrations in a 10 L Tedlar bag were lost after 6 hours of storage [27], while Andino et al. reported only a 3% loss from a 60 L bag containing ppmv levels of methanol over the same period, and less than 10% loss after 25 hours [24]. Andino et al. concluded that the different recoveries reported in the two studies resulted from the use of different-sized bags, which therefore had different surface area-to-volume ratios. Beauchamp et al. found 85 ppbv of methanol to remain stable over 10 hours of storage in 3 L Tedlar bags at 45°C, although only 67% recovery was achieved after 70 hours, with the losses thought to result from a combination of diffusion and adsorption [8]. The increase observed in the current experiment compared to the losses observed by other researchers could be explained by their use of higher initial concentrations, which were presumably greater than that in the environment surrounding the bags, therefore leading to outward diffusion. Had the methanol increases in this experiment been a result of emission from the bag material, it would have been expected to show an increase within the first 6 hours, as was observed for the N,N-dimethylacetamide and phenol

contaminants (Figure 5.3), and also that it would have been identified in previous studies.

In terms of surface area-to-volume ratio effects, the loss of acetonitrile and the increase in methanol after 48 hours was slightly greater for the 5 L bag in comparison to the larger bags, but no substantial difference was observed between the 10 L and 40 L bags. This may have reflected that the 40 L bag was not filled as close to its maximum capacity as were the 5 L and 10 L bags. In terms of breath sampling, 10 L probably would have been the largest acceptable volume of breath that a patient could have been asked to provide, which ruled out the use of larger bags even though they may have offered longer storage times. Both 5 L and 10 L bags seemed acceptable for short storage times, although a 10 L sample was more preferable as this provided a sufficient volume for analysis without having to reduce the sample inlet flow rates.



Figure 5.4 The change in the $H_3O^+/H_3O^+(H_2O)$ ratio, m/z (19/37), with time for one 10 L Tedlar bag initially filled with a dry gas mixture and another 10 L bag initially filled with a saturated gas mixture.

To test for the effect of humidity on VOC stability during storage, similar experiments were performed using two 10 L bags containing a humid standard mixture with the same initial VOC concentrations as that of the dry mixture used in the previous experiment. On the basis of the m/z (19/37) ratio, water vapour displayed the greatest

changes in both the dry-filled and humid-filled bags, which occurred mostly within the first 6 hours (Table 5.1 and Table 5.2). Water vapour appeared to permeate through the walls of the bag until equilibrium with the ambient levels was reached (Figure 5.4), an observation which has also been made by other researchers [8, 20]. In terms of the actual humidity readings, which were estimated from the m/z (19/37) ratio, there was a much larger drop in the relative humidity of the humid bag in the first 6 hours (57% decrease) than the increase in the dry bag (24% increase). This was presumably the result of the larger humidity gradient between the humid bag and the ambient air (m/z (19/37) = 9, ~ 35% RH) in comparison to the dry bag and the ambient air.

For the humid-filled bags, increases were observed in the levels of all of the VOCs over time except for acetonitrile for which no change was observed (Table 5.2), contrasting with the results observed in the previous dry experiments. This difference could probably be explained by the humidity-dependent sensitivities of some VOCs, and the change in the sample humidity with time. This effect would have been greater for the humid-filled bags in comparison to the dry-filled bags of the previous experiment owing to the greater change in humidity. This can be illustrated using acetonitrile as an example, a compound that is known to have a humidity-dependent sensitivity (Section 2.4.6). The m/z (19/37) ratio can be used to estimate sample humidity, and then the signals can be converted to concentrations with the use of the correct sensitivity value. After 48 hours the mixture was estimated to have a relative humidity of around 35%, although the lack of calibration data at this humidity meant that the concentration had to be estimated using a sensitivity value that was obtained under dry conditions. After estimation of the concentration at 0 and 48 hours, acetonitrile was found to show an overall loss, as observed in the dry experiments. Even though the overall recovery values for each VOC under dry and humid conditions were not in exact agreement, the same overall trends were observed. Therefore, these results suggested that VOC loss was largely unaffected by the sample humidity. Steeghs et al. also reported that relative humidity did not have any significant effect on VOC recovery [28].

As would be expected, the Tedlar contaminants N,N-dimethylacetamide (m/z 88) and phenol (m/z 95) were found to increase over time (Figure 5.3), owing to their release from the Tedlar film. The high levels of these contaminants (up to 10^5 ncps for m/z 88 and 10^4 ncps for m/z 95) not only obscured any signals in these mass channels, but also in the isotopic mass channels at m/z 89 (5% of m/z 88) and m/z 96 (7% of m/z 95), and to a lesser extent m/z 90 and m/z 97. This could be a potential problem for breath analysis as a compound at m/z 89 was observed in the previous breath studies, but owing to the overlap with the contaminant signal it will be difficult to measure this compound in Tedlar bag samples. These compounds were also found to 'stick' in the gas inlet system. For example, Figure 5.5 shows how N,N-dimethylacetamide and phenol contamination affected an on-line breath experiment that was carried out following the analysis of a Tedlar bag sample. The humid breath sample seems to have acquired the contaminants on passage through the inlet system, which then appeared as an exhalation profile in the resulting breath data even though these compounds were not present in the breath itself. The levels were found to decrease with each subsequent breath as the contaminant levels in the inlet system were depleted. Care was therefore needed to ensure that the inlet system was clean to minimise the contamination of any experiments performed after the analysis of a Tedlar bag sample.



Figure 5.5 An example of *N*,*N*-dimethylacetamide (m/z 88) and phenol (m/z 95) contamination during a breath experiment that was carried out following a Tedlar bag analysis. Also shown are the acetone (m/z 59) exhalation profiles for comparison.

In order to best preserve the original composition of a sample during storage, it was clear that Tedlar bags were only suitable for relatively short-term containment as timedependent changes were observed for some of the test compounds, especially those of low mass. These compounds were tested at relatively low concentrations, so greater diffusional losses may be expected for those VOCs that are found in higher concentrations in breath. Based on this work, and the reported findings of other research groups provided in this section, a maximum storage time of 6 hours was allocated between the collection and analysis of breath samples in Tedlar bags.

5.2.2 Tedlar bag heating device

Cleaning procedures are important for both new and used Tedlar bags. The reuse of bags requires a suitable cleaning procedure, which must be efficient at removing previously stored compounds while not causing damage to the bag. The procedure also needs to be a reasonably quick process, so that the rate of sample collection is not limited by the availability of clean bags. Various cleaning methods have been suggested, which generally consist of flushing with either synthetic air or nitrogen, and sometimes include a period of heating. Flushing of the sample bags is a simple yet effective cleaning method, although adsorbed compounds usually need some form of heating to assist their removal. Steeghs et al. reported the use of Tedlar bags with two valve fittings to allow for the continual flushing of clean gas [28]. Beauchamp et al. reported suggested cleaning guidelines for Tedlar bags, which recommended heating at 75°C for at least one hour [8], however Mochalski et al. found that some polymer bags including Tedlar emitted VSCs at temperatures greater than 50°C [9].

A heating device was required for the impending study in order to minimise the risk of sample contamination during the reuse of the Tedlar bags. All preparation for the study had to be performed on a short timescale in order to maximise the available study time. Heating protocols commonly involve placing the bags inside an oven for a period of time, but for the current work an oven was not available that could accommodate 10 L bags or be located in the laboratory to allow the heating process to be monitored by the

CIR-MS instrument. A custom-built heated blanket similar to that used with the standard on-line breath apparatus would have provided an alternative, although this was not an option with the time available prior to the start of the study. The solution was to construct a simple lamp heater, as shown in Figure 5.6. The lamp heater was made out of two 500 W tungsten halogen lamps. The lamps were low cost (\sim £5) and widely available from hardware stores. The lamps were attached to a stand of variable height and situated above a curved aluminium base. The lamp heater also had the advantage of being transportable, which was useful when requiring a location near the pump for evacuating the contents whilst being heated or near to the CIR-MS instrument for analysis of the bag contents during heating.



Figure 5.6 A simple lamp heater designed for the baking of Tedlar bags.

Surface temperatures were monitored using a thermocouple (type K), which was attached to the upper side of a test bag. During heating the bag was filled to approximately one third of its capacity. The lamps were found to heat the bag up to 90°C, providing a suitable temperature range for use with Tedlar bags since the manufacturer recommends that bags with polypropylene fittings do not exceed

temperatures of 93°C [29]. A target temperature of 63°C was chosen, which taking into account the variability (~ \pm 2°C) maintained the temperature in the range of 61 – 65°C. This value was chosen based on the literature reports, balancing the need for a sufficiently high temperature to release adsorbed VOCs, but without causing thermal degradation of any of the bag components. A cardboard enclosure was placed around all four sides of the heater to shield from the effects of the air conditioning in the laboratory and additionally to screen the bright lamps from view. The lamps generated more heat with time, so the intensity of the lamps had to be manually reduced using a variable transformer to maintain the target temperature. The transformer typically ranged from 100% initially down to 70%, depending on the length of time that the lamps were on. After running the lamps for 30 minutes, the temperatures were found to remain reasonably stable within the target range with the transformer set to 70% intensity.

The heater originally used only a single lamp, but it was found that while the centre of the bag directly under the lamp was suitably heated, the outer areas of the bag were not. A significant improvement was found in moving to the two lamp arrangement. While some temperature gradient was still observed, the majority of the bag was maintained above 60°C. The uniformity of the temperature could probably be further improved through the addition and arrangement of more lamps, but the current design was considered adequate for the current work.

Some halogen lamps emit ultraviolet radiation that can lead to the degradation of certain polymers, and so an assessment of whether the lamp heater was exposing the bags to ultraviolet radiation had to be made. While Tedlar shows good stability in the ultraviolet range [29], the bag fitting was made of polypropylene, which can undergo photooxidation to form volatile products such as acetone, methanol and acetic acid [30]. Therefore, a quick experiment was performed to check the degree of ultraviolet emission from the lamps. The emission spectrum of the halogen lamp was measured using a UV-VIS spectrometer (Type 2 Matchbox Spectrometer, Spectroscopic and Analytical Developments, UK). The experiment was carried out inside a dark box with the lamps positioned on an optical bench, 30 cm from the optical fibre of the

spectrometer. Figure 5.7 shows that there was very little light emitted in the ultraviolet range, as most was probably blocked by the glass panel in front of the bulb.



Figure 5.7 The emission spectrum of a tungsten halogen lamp at varying intensity.

5.2.3 Cleaning procedure

In order to determine a suitable cleaning procedure for the Tedlar bags, the effectiveness of a series of flushing and heating stages after the containment of a synthetic 'breath' mixture was monitored. A new 10 L bag, which had undergone the standard flushing process, was filled with high-purity nitrogen and the contents were analysed to provide the background of the bag. The bag was emptied and then filled with a humidified mixture (100% RH) containing a selection of breath-relevant compounds from the gas standards generator. The mixture consisted of acetonitrile (91 ppbv), acetaldehyde (44 ppbv), acetone (209 ppbv), dimethyl sulphide (6 ppbv), methanol (43 ppbv) and isoprene (15 ppbv). The mixture was used to simulate the conditions from which the bags would need to be cleaned after the containment of a breath sample. The contents were analysed as before, and the remaining mixture pumped out. The cleaning process

began with a series of three nitrogen flushing cycles, with the contents of the bag analysed during each fill. During the heating stage that then followed, a nitrogen-filled bag was positioned under the lamp for 20 minutes, after which a sample was analysed and then the bag emptied whilst being maintained under heat. This was repeated 3 times and then followed by a final set of flushing cycles, again with the bag contents analysed during each fill. CIR-MS analysis was carried out as described in Section 5.2.1.

The measurements made during each of the stages outlined above are presented for a selection of compounds in Figure 5.8. For methanol (Figure 5.8 (a)), the first set of flushing cycles were shown to reduce the residual levels that were detected following the removal of the VOC mixture. Levels after this stage were found to be lower than the background levels that were measured before the humid sample was added. During the heating stage a rise in methanol levels was observed, suggesting that some adsorption had occurred which was being released upon heating. This was seen to decrease with each subsequent heating. Comparison of the methanol levels measured just before the heating stage to those measured just after showed only a slight overall reduction, whilst comparison of the initial background at the start of the experiment to those at the end of the entire procedure showed a 70% reduction.

For acetone (Figure 5.8 (b)), a similar pattern was initially observed, with the residual levels following the removal of the humid sample showing a reduction after the first set of flushing cycles. Unlike methanol, acetone showed no sign of desorption during the heating stage, but a gradual decline was observed each time the bag was flushed with nitrogen. Overall there was a 50% reduction in comparison to the initial background. This same trend was also observed for acetonitrile, acetaldehyde, dimethyl sulphide and isoprene, each showing little response to the heating stage and displaying an overall reduction of between 20 - 50% by the end of the cleaning process.



Figure 5.8 Monitoring VOC levels throughout the Tedlar bag cleaning process as illustrated by (a) methanol (m/z 33), (b) acetone (m/z 59), (c) H₃O⁺/H₃O⁺(H₂O) ratio as a measure of relative humidity, and (d) the contaminants *N*,*N*-dimethylacetamide (m/z 88) and phenol (m/z 95). The *x*-axis labels refer to the following measurement conditions: 'nitrogen' is the initial measurement of the background of the bag, 'humid VOC' is the measurement of the humidified VOC mixture from the bag, 'flush' refers to the bag background after the removal of the previous sample and the refilling with nitrogen (repeated 3 times), 'heat' refers to the measurements as before. Each condition was monitored for 10 minutes with measurements made with 1minute integration times.

Referring back to Section 5.2.1, Beauchamp et al. reported that both methanol and acetaldehyde showed some degree of adsorption during storage in Tedlar bags [8]. The release of methanol during the heating stage of the cleaning process was consistent with this observation, however acetaldehyde showed no sign of desorption upon heating.

Comparison of the methods used by Beauchamp et al. to those used in the current experiment, aside from the initial concentrations used, the main differences were that the VOC-filled bags were stored for 70 hours before cleaning and the heating stage was carried out at 95°C over a 10 hour period. The VOC mixture during this experiment was stored for approximately 30 minutes prior to cleaning, so short-term storage may therefore help to minimise adsorption losses. Even though the heating stage during the current experiment was performed at a lower temperature for a shorter duration, it was still sufficient to release adsorbed methanol.

The humidity of the sample contained within the bag was found to increase during the heating stage (i.e. the m/z (19/37) value decreased; Figure 5.8 (c)), which was thought to result from the increased rate of diffusion of water vapour from the ambient air into the drier, nitrogen-filled bag. It was possible that increased diffusion into the bag may also have been responsible for the observed rise in methanol levels during the heating stage, although a constant rate of diffusion and therefore comparable levels during each period of heating would have been expected (as was observed for water vapour), rather than the observed decline that was suggestive of progressive cleaning. This indicated that at least some of the observed methanol increase occurred as a result of desorption. As would be expected, the levels of the *N*,*N*-dimethylacetamide and phenol contaminants were found to increase during the heating stage as they were released from the bag material (Figure 5.8 (d)). Whilst an overall decrease in the levels of these contaminants was found following the cleaning procedure, no substantial reduction was observed.

In terms of defining a cleaning procedure for use during the breath study, a heating stage did seem beneficial, although a reduction in the length of the heating period meant that the entire procedure would be quicker without particularly compromising the cleaning efficiency. Therefore it was decided that the cleaning procedure would involve 3 flushing cycles with high-purity nitrogen immediately after use, a single 20 minute heating period, and then another 3 flushing cycles (total cleaning time per bag < 1 h). The shorter time requirement of the cleaning stage would be useful during clinical

studies where large numbers of samples are collected and a quick turnover of bags is needed.

5.3 Preliminary study of the exhaled breath of female cancer patients

5.3.1 Introduction

Reports of cancer diagnosis after canine scent detection [31] suggest that cancers may produce distinctive odours. Dogs, which are thought to have odour thresholds in the pptv range [32], have been applied to the detection of lung and breast cancers through the scent of breath samples [32], and the detection of bladder cancer from that of urine samples [33]. Rather than a canine nose, some studies have applied electronic noses or colorimetric sensor-array technology to show that the lung cancer breath has distinct chemical characteristics [34-37].

Mass spectrometry techniques have tried to identify some of the VOCs present in the breath of cancer patients, of which many are aliphatic or aromatic hydrocarbons, or oxygenated compounds. Phillips et al. distinguished between patients with lung cancer and those without using a group of 22 breath VOCs that included alkanes, methylated alkanes, benzene derivatives, alkenes, and the aldehydes hexanal and heptanal [38], and in a later study based on a smaller group of 9 alkanes and methylated alkanes [39]. Gaspar et al. similarly found that a group of $C_{14} - C_{24}$ linear and branched hydrocarbons had the power to distinguish between healthy subjects and lung cancer patients [18]. Phillips et al. also used the measurement of alkanes and methylated alkanes in breath to identify breast cancer patients [40]. Elevated concentrations of hexanal and heptanal have been found in lung cancer blood [41], and hexanal, along with 1-octen-3-ol and octane, were suggested to have diagnostic value in the blood of liver cancer patients [42]. Poli et al. identified a group of aliphatic and aromatic hydrocarbons in breath that could classify 80% of their lung cancer patients before surgery from various control groups [43]. In an extension of this work, Poli et al. analysed the breath of patients one month and three years after the surgical removal of cancerous lung tissue, and found that surgery influenced the exhaled concentrations of various VOCs [44]. The aromatic compound o-toluidine has been identified as being elevated in the breath of lung cancer patients [45] and those with other forms of cancer [46], although its reliability as a marker has been questioned [47]. O'Neill et al. found that out of 28 compounds that were observed in the breath of more than 90% of their lung cancer patients, only 9 oxygen-containing compounds were potential markers [48]. The prediction of breast cancer has also been based on a group of 5 oxygen-containing breath VOCs which included heptanal and 2-propanol [49]. Wehinger et al. identified 2 breath VOCs that best discriminated between lung cancer patients and healthy controls, which were tentatively assigned to formaldehyde and 2-propanol [17]. Formaldehyde has also been detected in the breath of breast cancer patients [50] and has been identified in the urine headspace of bladder and prostate cancer patients [51]. 1-Butanol and 3-hydroxy-2butanone were found to be elevated in the breath of patients with lung cancer [52]. Cancer cells have been found to release VOCs in vitro; Smith et al. detected acetaldehyde in the headspace of lung cancer cells, which was found to be proportional to the number of cancer cells in the medium [53].

A common trend in the previously mentioned studies is the use of combinations of VOCs coupled with statistical analysis to identify the cancer group, emphasising that the 'diagnosis' may rely on VOC fingerprinting rather than the presence of an individual marker. The ability of TOF-MS to simultaneously monitor a given mass range on a short timescale and to record a comprehensive VOC profile makes it ideally suited to the identification of complex chemical fingerprints [54]. While most researchers have focused on the analysis of breath from lung cancer patients, there should be no reason why exhaled breath cannot yield information on disease in other parts of the body, since any volatile markers released into the systemic circulation could eventually pass into breath. The aim of this pilot study was to investigate whether exhaled markers could be identified in the breath of females with endometrial or ovarian cancer, and to assess the effect of different physiological conditions (e.g. menstrual cycle phase, pregnancy) on the exhaled VOC profiles.

5.3.2 Breath apparatus for use with Tedlar bags

The sampling apparatus that was coupled to the Tedlar bags for the collection of exhaled breath is shown in Figure 5.9. The apparatus consisted of a disposable mouthpiece and bacterial filter attached to a non-return valve and a Teflon adapter holding a piece of Marprene flexible tubing. The Marprene tubing, which was connected to the adapter using PFA fittings, attached over the polypropylene fitting of the Tedlar bag to form a good seal. The non-return valve allowed exhaled breath into the bag but prevented any loss of the bag contents during inhalation. The DVT was of limited use with one directional flow as it could only provide a total cumulative volume, so was not included in the apparatus.



Figure 5.9 The breath sampling apparatus that was coupled to the Tedlar bags for the collection of exhaled breath.

5.3.3 Breath collection and analysis

Breath samples were collected by medical staff at Leicester Royal Infirmary between August – November 2008. All samples were collected in 10 L Tedlar bags with standard polypropylene fittings. A total of 18 bags were available for the study, all of which were

new and had undergone the standard flushing process before use. All bags were labelled for identification to track its use and cleaning.

The patients were rested for at least 10 minutes before collection. A clean Tedlar bag was attached to the collection apparatus and the polypropylene tap opened. Patients were asked to provide an end-exhaled breath by breathing into the mouthpiece with full and repeated exhalations, after excluding the first portion of breath (the dead space air) each time, until the Tedlar bag was filled. The bags were sealed before the removal of the apparatus. Some resistance was experienced because of the small inner diameter of the standard bag fitting, although all subjects were able to provide the sample without difficulty. Sample bags were not heated during collection as it was not be possible to store and transport the bags whilst maintaining the same temperature, so for consistency the bags were kept at ambient temperature. Each patient provided a single breath sample.

For every group of breath samples an ambient air sample was also collected using a small diaphragm pump to fill the bag. The collection time of both the breath and the air samples were noted. The samples were collected from Leicester Royal Infirmary and transported to the laboratory at the University of Leicester for CIR-MS analysis. Breath collection typically took place between 9:00 am and 2:30 pm, leaving sufficient time for analysis within the pre-determined 6 hour time-window. All samples were attached to the CIR-MS instrument and analysed as described in Section 5.2.1 for a total of 20 minutes.

Immediately after analysis, any remaining sample was evacuated and the bags flushed 3 times with high-purity nitrogen. Before reuse, all of the bags were filled with nitrogen to approximately one third of their capacity and baked for 20 minutes at $63 \pm 2^{\circ}$ C using the lamp heater described in Section 5.2.2. The bags were emptied whilst being heated, and then another set of 3 flushes was performed. After cleaning, the bags were stored evacuated.

5.3.4 Patient details

In this preliminary study, 4 female cancer patients and 10 healthy female subjects were recruited (Table 5.3). The cancer group consisted of 2 patients with endometrial cancer and 2 patients with ovarian cancer. The control group consisted of 2 individuals who were pregnant and 8 individuals who were categorised according to being in either the proliferative or secretory phase of their cycles at the time of breath collection, of which 4 subjects provided breath samples during both phases. Healthy subjects ranged between 20 - 42 years of age with BMIs of 17.7 - 28.0, while the cancer patients ranged between 56 - 63 years of age with BMIs of 22.3 - 32.7. None of the patients were diabetic, smokers, or had any respiratory, thyroid, liver or renal disease. All subjects were on a normal diet. The study was approved by the Derbyshire Research Ethics Committee and all subjects provided written informed consent.

Patient ID	Patient Description	Age	BMI
A1-a	Normal proliferative phase	34	19.9
A1-b	Normal secretory phase	-	-
A2-a	Normal proliferative phase	42	28.0
A2-b	Normal secretory phase	-	-
A3	Normal secretory phase	23	20.3
A4	Pregnant 2 nd trimester	28	24.2
A5-a	Normal secretory phase	39	-
A5-b	Normal proliferative phase	-	-
A6	Normal secretory phase	37	27.6
A7	Normal secretory phase	20	19.4
A8-a	Normal proliferative phase	31	21.2
A8-b	Normal secretory phase	-	-
A9	Pregnant 1 st trimester	26	21.0
A10	Normal proliferative phase	29	26.9
B1	Ovarian cancer (stage IIIc)	56	26.9
B2	Endometrial (stage Ic)	61	26.8
B3	Ovarian cancer (stage IIIc)	63	22.3
B4	Endometrial cancer (stage Ib)	61	32.7

Table 5.3 Demographics of the healthy control group (A1 - A10) and the cancer group (B1 - B4). Patient IDs labelled 'a' and 'b' denote different samples that were provided by the same individual.

5.3.5 Results and discussion

During this study, most of the samples were analysed within 3 hours of collection, and all were analysed within 5 hours of collection, so time-dependent VOC losses and contamination should have been minimal. Water vapour diffusion through the walls of Tedlar bags occurred rapidly until ambient levels were reached. By the time of analysis, the ambient air samples had m/z (19/37) ratios between 7 – 14 and the breath samples between 3-6, which provided estimated relative humidity values of 18-42% and 45-94% respectively. The variability in the relative humidity of the samples made data interpretation and absolute quantification more difficult. Calibration data over the range of humidities were not available for all of the tentatively assigned compounds, and would have required a reasonable amount of time to obtain. As a result, all data reported in this section are presented as the recorded CIR-MS signal in ncps. This can be similarly problematic when comparing samples of varying humidity as the signal intensity for any given concentration can vary for VOCs with a humidity-dependent sensitivity. Even though the relative humidities of the breath samples were reported to range from 45 - 94%, the majority of the samples were estimated to be between 60 - 94%80% by the time of analysis, so this effect during should not have been too pronounced during this particular study.

For analysis, all of the breath and the ambient air measurements were averaged. Following inspection of the data, 17 m/z values of interest were selected on the basis of either showing elevated levels in the breath of three or more individuals or in at least one cancer patient, in comparison to the levels in the corresponding ambient air samples taken from the site of breath collection. Figure 5.10 summarises the data for each of the selected m/z values, displaying the levels measured in the breath of each individual with the corresponding ambient levels. Figure 5.10 also includes a plot of the m/z (19/37) ratios for all of the breath and air samples.



Figure 5.10 Plots displaying the measured signals (*y*-axis, ncps) in all of the breath samples for the 17 selected m/z values. Also shown in the first plot is the m/z (19/37) ratio (*y*-axis, unitless) that provides an indication of the sample humidity. The first fourteen bars of each plot display the measurements for the healthy control subjects and the four bars on the far right display those of the 4 cancer patients. The black markers over each bar represent the corresponding ambient air levels.

The measured intensities for the breath signals that were common to all test subjects are summarised in Table 5.4.

Table 5.4 Comparison of the median signals (ncps) and the upper and lower quartile values for the common VOCs within ambient air, the breath of control subjects, and the breath of those with cancer. See text for m/z assignments.

_	Air $(n = 9)$		Control $(n = 14)$		Cancer $(n = 4)$	
m/z	Median	Lower/Upper Quartiles	Median	Lower/Upper Quartiles	Median	Lower/Upper Quartiles
(19/37)*	10	8-11	4	4 – 4	4	4-5
33	1082	830 - 1213	10597	9183 - 13929	4535	3776 - 6475
45	619	472 - 747	972	931 - 1286	1250	1151 - 1482
51	24	23 - 32	332	277 - 534	137	108 - 164
59	4744	4121 - 4986	32356	30506 - 45588	79961	57802 - 95364
63	30	27 – 33	544	470 - 638	433	353 - 541
69	109	92 - 136	2100	1814 - 2782	2575	2348 - 2781

* unitless ratio

Methanol (m/z 33) levels were significantly lower in the breath of the cancer group in comparison to that of the control group (one-sided Mann-Whitney test, p < 0.05, Minitab), and as would be expected, this trend was also mirrored by the levels of the methanol-water cluster ions (m/z 51). The clinical significance of the lower methanol levels in this situation was uncertain, and the result may just have been an effect of the small size of the cancer group. Alternatively, if there is a true trend between exhaled methanol concentrations and age (Section 4.3.3.2), then the lower levels observed may have been a reflection of the older average age of the cancer group. As similarly found during the cystic fibrosis trial (Section 3.3.2), the methanol levels in some of the ambient air samples were much higher than normal and often exceeded the levels measured in the corresponding breath samples. It was uncertain whether the high ambient levels affected the breath measurements given that the exhaled levels appeared to fall within the range typically observed for methanol in this study.

Even though the presence of acetaldehyde in the breath of healthy individuals has been reported, it has not been routinely observed during on-line CIR-MS breath analysis, probably because the low concentrations in breath are close to the limit of detection for real-time measurement. In analysing breath samples from Tedlar bags, there is the benefit being able to use longer integration times, which improves the limits of detection in comparison to real-time analysis and should allow for the detection of a larger number of VOCs. Acetaldehyde (m/z 45) levels in the breath of the cancer group were slightly higher in comparison to the control group, but this was found not to be statistically significant (one-sided Mann-Whitney test, p = 0.08).

A number of subjects within the cancer group displayed relatively high levels of acetone (m/z 59) in their breath, and while the overall levels in the cancer group were higher than the control group, this was found not to be statistically significant (one-sided Mann-Whitney test, p = 0.09). It is thought lipid peroxidation can contribute to endogenous acetone levels [55], so the higher levels in some of the cancer patients may have reflected increased oxidative stress [56]. However, given the influence of diet on exhaled acetone concentrations and the high variability even in healthy subjects, this was difficult to assess without the measurement of other lipid peroxidation markers [57]. No significant differences were found between the levels of dimethyl sulphide (m/z 63) or isoprene (m/z 69) in the breath of the cancer and the control groups (one-sided Mann-Whitney test, p = 0.11 and p = 0.21 respectively).

For the 17 selected m/z values, no obvious differences were observed between the exhaled levels of the different subgroups, although the number of subjects in each subgroup was insufficient to allow this to be comprehensively studied. The only minor observation was that the highest levels of dimethyl sulphide were found in breath samples belonging to subjects within the secretory phase subgroup (Figure 5.11), although in comparison to the proliferative phase subjects there was no statistically significant difference between the groups (one-sided Mann-Whitney test, Sec. = 547 ncps vs. Pro. = 541 ncps, p = 0.31). Higher concentrations of sulphur-compounds in the oral cavity have been previously observed in females during the pre-menstrual

(secretory) phase and menstrual phase, in comparison to females in the proliferative phase and male subjects [58]. While the current observation may just reflect the degree of variability that can be observed in exhaled dimethyl sulphide concentrations (Section 4.3.4), this observation may become more significant should the study be extended to a larger group.



Figure 5.11 Comparison of acetone levels (m/z 59) and dimethyl sulphide levels (m/z 63) in the breath of different patient subgroups. Pro. = proliferative phase controls (n = 5), Sec. = secretory phase controls (n = 7), Pre. = pregnant controls (n = 2), Can. = cancer patients (n = 4).

Smith et al. observed higher acetone concentrations in the headspace of urine around the time of ovulation, which was not found in the urine samples from postmenopausal women [59, 60]. However, the same trend was not reflected in parallel breath acetone measurements, and it was suggested that the accumulation of acetone in the urine had removed acetone from the blood, and therefore exhaled acetone levels appeared normal. For completeness, the acetone levels in the breath of the proliferative and secretory groups were compared. Acetone levels were found to be slightly higher in two subjects within the secretory phase group, who also happened to be two of the subjects that displayed the higher dimethyl sulphide levels, although this may again just be a reflection of the normal breath acetone variability. A proper investigation of the observation made by Smith et al. would have been difficult since the window of occurrence was very small in comparison to the sampling frequency used in this study. Comparison of the secretory phase and the proliferative phase breath acetone levels

(Figure 5.11) found no statistically significant difference (one-sided Mann-Whitney test, Sec. = 38055 ncps vs. Pro. = 31622 ncps, p = 0.21).

Pregnancy did not seem to influence the measured breath signals, although the group only consisted of two individuals who were both at different stages of pregnancy. One individual was in the early stages of the first trimester, so the breath VOC composition at this stage may not necessarily be expected to differ substantially from the healthy controls. A larger group size is essential to allow this to be properly investigated, but should this be achieved, this work could then be extended to investigate conditions associated with complicated pregnancy such as preeclampsia; Moretti et al. demonstrated through breath alkane measurements that there is greater oxidative stress in women with preeclampsia than in uncomplicated pregnancy and non-pregnant control subjects [61].

Returning to the comparison of the cancer and the control groups, for the remaining 11 of the 17 selected m/z values that were not common in the breath of all of the subjects, there was no one signal that was found to be elevated in the breath of all of the cancer patients, however there were a number of signals that were elevated in one or two of the cancer samples. One such example is ammonia (m/z 18), which was found to be clearly elevated in one of the endometrial cancer samples. In most of the other breath samples, the ammonia levels were equivalent to that measured in the ambient air. The signal at m/z 47, which was elevated in one ovarian cancer patient and one healthy control, is usually assigned to ethanol, although there are other possibilities for the signal in this mass channel [16]. Methanethiol is usually assigned to m/z 49, which was found to be present in greater levels in the breath of all but one of the cancer patients and also in one control. The signal at m/z 61 was only observed in the breath samples of the endometrial cancer patients, although the level in one sample was only marginally above the level in the background air. This may be an isomer of propanol or acetic acid, although propanol usually fragments to m/z 43 [17], which in this case was below the ambient air levels.

Most previous studies have focused on the analysis of breath from lung caner patients, so there are few results that are of direct relevance to the current clinical application. However, one study by Rieder et al. analysed the breath of various cancer patients, including those with ovarian and endometrial cancer [46]. The aromatic compound *o*-toluidine was found to be higher in the breath of patients collectively forming a gynaecological cancer group in comparison to the healthy controls and intensive care patients, although greater levels were found in separate group of patients with other forms of cancer. In the current study, none of the breath samples from either the cancer or healthy control subjects displayed levels in the appropriate mass channel for *o*-toluidine (m/z 108) that were greater than that measured in the ambient air samples, although the reliability of this marker has been questioned owing to the wide variability observed for m/z 108 in the breath of hospital staff [62].

As illustrated by many of the studies mentioned in Section 5.3.1, it is possible that the action of disease may affect multiple VOCs in breath, so rather than focussing on individual compounds, the overall pattern of exhaled compounds may be more informative for the assessment of differences between breath samples. A principal component analysis (PLS Toolbox) was performed on the data presented in Figure 5.10 and the resulting biplot is shown in Figure 5.12, which presents the second and third principal components as this provided the best presentation of the groups within the study. The biplot demonstrated that on the basis of the overall breath profile formed by the group of selected m/z values, the breath samples of the endometrial cancer patients were sufficiently different to be separated from the samples belonging to the healthy controls. The breath samples of the ovarian cancer patients were not so well differentiated from the controls, however the samples still displayed enough similarity to that of the endometrial cancer patients to be grouped in close proximity. There appeared to be some degree of subgrouping within the control samples, although this did not relate to the menstrual cycle phase or pregnancy. While the significance of these results was uncertain in such a small group of patients, the results from this pilot study suggested that there may be subtle differences between the breath profiles of cancer patients and healthy controls. Despite the small samples size, encouraging results were obtained that justify further investigation.



Figure 5.12 A principal component analysis biplot showing the grouping of exhaled breath samples from endometrial and ovarian cancer patients and healthy controls. The plot displays the second principal component against the third principal component. The principal component analysis was performed using 17 m/z values and the data were pre-processed by autoscaling.

While the sample protocol implemented was adequate for this pilot study, there were a number of improvements that could be made if off-line measurements become routinely used. To maximise the available study time, standard sampling bags and fittings were used because of the quicker delivery time for 'off-the-shelf' products. Ideally custom bags would be required, featuring a wide-bore fitting for the breath inlet to reduce the resistance to exhalation. Also including an additional standard port would be beneficial in terms of having the ability to continually flush the bags during cleaning, as described by Steeghs et al. [22]. Assuming that custom bags would come at a higher cost, the need for a suitable cleaning procedure becomes more important to get the maximum use out of the bags. The experiments presented here suggested that bags could be efficiently

cleaned in a relatively quick time-frame to allow reuse without compromising future samples. During the study, the collection of alveolar breath was based on the ability of the patient to judge the volume of dead-space air to exclude. When real-time VOC measurements are not made, the use of real-time carbon dioxide measurement is recommended to reliably collect alveolar air [63, 64], however this would have required additional instrumentation. O'Hara et al. reported the use of an isothermal rebreathing method as a step towards a standardised off-line sampling protocol [14]. The required apparatus is relatively simple, comprising a suitable bag with a comparatively widebore inlet and a heating device to maintain the bag at a constant temperature. The rebreathing technique requires the subject to initially provide a mixed-expiratory sample into the bag, from which they then inhale and exhale over a series of cycles. This allows the lung air and pulmonary blood to equilibrate, and is thought to provide a better representation of alveolar composition as it minimises the interaction of highly soluble gases with the airways [65]. Whilst this method could be adopted to satisfy the sampling issues, the rapid loss of humidity with storage time and the effect that the variable sample humidity has on quantification would still be a problem unless the time between collection and analysis is further reduced.

5.4 Summary

The use of Tedlar bags for off-line breath analysis was investigated, and while having the benefit of being able to acquire samples and analyse them off-site, the method was only suitable if the sample could be adequately stored between collection and analysis. It has been shown that even for relatively short storage times, samples within Tedlar bags suffer from unavoidable internal contaminants and time-dependent changes in composition. The internal contaminants were a particular problem as they appeared in significantly high levels, meaning that the data in the associated mass channels and their isotopic channels were effectively lost. Alternative materials could be explored; Flexfilm bags have been advertised as a better alternative to Tedlar with lower total VOC background and longer storage stability [29], although similar testing would be required for verification. A pilot study investigating the off-line analysis of breath from
a small number of endometrial and ovarian cancer patients showed that, while no single species displayed a clear correlation with cancer, the overall breath profile appeared to be sufficiently different to distinguish between the endometrial cancer patients and the healthy controls, demonstrating that the chemical 'fingerprinting' of exhaled breath has diagnostic potential. While this study represented a very small sample set, the results were encouraging and could be used to initiate future studies in this area.

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Chapter 6. Headspace analysis of MVOCs emitted from bacteria and fungi

6.1 Microbial volatile organic compounds

Microbial VOCs (MVOCs) are produced by the metabolic processes of bacteria and fungi. MVOCs are responsible for the odours associated with microbial colonies, such as the 'musty' and 'earthy' odours often reported in fungal-contaminated environments [1], or the grape-like smell produced by *Pseudomonas aeruginosa* cultures [2]. MVOCs can be produced during both primary and secondary metabolism. Primary metabolism usually refers to the important processes involved in growth, such as energy generation and the synthesis of nucleic acids, proteins, lipids and carbohydrates, whereas secondary metabolism usually occurs towards the end of the growth phase and can result in the production of a wide variety of compounds [3]. Many MVOCS have been detected in laboratory-cultured samples of bacteria [1, 2, 4-14] and fungi [1, 15-28], of which the most commonly occurring compounds include alcohols, ketones, aldehydes, esters, terpenes and various nitrogen-containing and sulphur-containing compounds.

MVOC production is greatly dependent on factors that affect microbial metabolism such as species, growth substrate, growth phase, temperature, pH and humidity [26, 27, 29, 30]. Numerous studies have reported the effect of growth substrate on both the abundance and the type of MVOCs produced [21, 24, 31]. For example, one study of a single *Penicillium* species on various growth media found terpene production to be higher on agar-based substrates, while alcohol production was greater on cereal-based substrates [21]. The on-line monitoring of microbial headspace has revealed the extent

of temporal changes in MVOC emission during growth [11, 13, 14]. Using PTR-MS, Bunge et al. monitored the emission of MVOCs from various bacteria over a 24 hour incubation period and found that while a few signals correlated with bacterial cell numbers, most displayed complex time-dependent behaviour, with some showing only transient production [11]. Also using PTR-MS, O'Hara et al. monitored MVOCs in the headspace of *Staphylococcus aureus* cultures grown in different broths and found that whilst the MVOCs produced in each case were consistent, the time-dependent emission patterns and the measured intensities varied between the different growth media [14].

It is thought that particular microbial species may produce characteristic MVOCs, and the use of unique species-specific markers or chemical fingerprints has been investigated as a form of bacterial and fungal identification [19, 25, 32, 33]. This may have application in the diagnosis of infection in clinical samples. Using conventional culture methods, the diagnosis of a bacterial infection can take at least 24 - 48 hours, and even longer for the diagnosis of a fungal infection [34]. Based on the analysis of MVOCs, the rapid detection of bacterial growth 6 - 8 hours after the inoculation of blood culture bottles has been demonstrated using SIFT-MS [4-6]. The detection of MVOCs emitted in vivo through the analysis of exhaled breath is being explored as a non-invasive tool for the rapid diagnosis of infection. Some studies have compared the MVOCs produced by microbial cultures in vitro to the VOCs observed in the breath of infected individuals. The most abundant MVOCs measured in the headspace of Mycobacterium tuberculosis cultures were found to be structurally similar to a group of compounds identified in breath as being the best discriminators between patients with pulmonary tuberculosis and those without [10]. A number of fungal cultures were found to produce 2-pentylfuran, which was also observed in the breath of Aspergillus-infected patients but not in healthy subjects [25]. Higher hydrogen cyanide concentrations have been observed in the headspace of *Pseudomonas aeruginosa* cultures in comparison to other bacterial cultures, and in the breath of cystic fibrosis patients infected with Pseudomonas aeruginosa in comparison to asthmatics [12, 35]. Breath tests have been developed for the diagnosis of Helicobacter pylori infection, a bacterium that can infect the gastrointestinal system. H. pylori produce urease enzymes which break down urea

into ammonia and carbon dioxide, so positive diagnosis is provided by an increase in ${}^{13}\text{CO}_2$ (~ 26 ppmv) after the ingestion of a ${}^{13}\text{C}$ -urea solution [36].

The analysis of MVOCs from microbial cultures in vitro may provide an indication of those produced by microorganisms in vivo, and therefore those that may be present in the exhaled breath of infected individuals. If a characteristic MVOC or group of MVOCs for a specific pathogen can be identified, then the measurement of these compounds in exhaled breath may have the potential for the rapid and non-invasive diagnosis of infection. This chapter reports the preliminary investigation of MVOCs can be detected in the headspace of these cultures using CIR-MS, and examining species-specific differences in the measured MVOC profiles.

6.2 Bacteria

6.2.1 Initial Pseudomonas aeruginosa analysis

The first bacterial headspace experiments were carried out in preparation for the cystic fibrosis study (Section 3.2.2) and focussed on *Pseudomonas aeruginosa* since it was originally thought that this would be the main cause of infection in the test group. To determine the best form of sample for analysis, *P. aeruginosa* cultures on three different types of growth media were tested. The cultures were contained either in liquid blood culture bottles (BacT/ALERT, bioMérieux), in nutrient agar slope bottles (Oxoid) or on blood agar plates. All cultures were supplied by Leicester Royal Infirmary.

Blood culture bottles contained the bacterial cultures in a sealed environment and benefited from ease of handling. For analysis, the septum of the bottle was pierced using a needle which was directly connected to the instrument inlet by a standard PFA sample line. As the headspace was sampled, the pressure inside the bottle was maintained using a second needle open to the laboratory air. Most of the volatiles that were detected in the headspace were found to originate from the liquid growth medium.

Given the small volume of the headspace compared to the sampling rate of the CIR-MS instrument, it was likely that the headspace MVOCs were rapidly removed. Even though the sample flow rate could have been reduced, it was not possible to do so on the scale that would have been required with the equipment that was available.

Two types of solid growth media were tested. The agar slope bottles were not sealed like the blood culture bottles, but were covered by laboratory film and a removable screw top. The samples were analysed using a method similar to that described for the blood culture bottles, where the headspace was sampled using a needle pierced through the laboratory film. Again very little was detected, perhaps owing to the small surface area of the culture growth (estimated to be ~ 7 cm²) or the small headspace volume. The headspace of the agar plate (plate diameter ~ 9 cm and surface area ~ 65 cm²) was sampled whilst the plate was contained inside a press-seal bag, which was sealed except for a small opening to allow a PFA sampling line through to the culture. The agar plates were detected. However, a better sampling method was needed to remove the background interference of laboratory air. Knowing that culture plates provided the best form of sample for analysis, a more suitable method could be designed around this.

6.2.2 Further bacterial headspace analysis

The bacterial headspace analysis was later repeated with 4 different species of bacteria. The sample set consisted of *Haemophilus influenzae, Streptococcus pneumoniae, Staphylococcus aureus* and *P. aeruginosa*, which were originally chosen because of their significance in cystic fibrosis. Five plates of each species were cultured on either blood agar (*H. influenza, S. pneumonia*) or Luria-Bertani agar (*S. aureus, P. aeruginosa*) in standard Petri dishes at 37°C. All samples were supplied by the Department of Infection, Immunity and Inflammation at the University of Leicester.

A 2.5 L anaerobic jar (also supplied by the Department of Infection, Immunity and Inflammation) was modified for use as the sampler container (Figure 6.1). Two PFA

panel mount unions were fixed though the lid, onto which PFA taps were attached for the isolation of the container. Owing to the size of the container, all 5 plates of each species were analysed together to ensure that sufficient MVOC concentrations were obtained. The 5 plates with their lids removed were stacked inside the container, which was sealed and left for approximately 20 minutes during which time the samples were contained in laboratory air. The taps were then opened and the container was flushed with high-purity nitrogen. The headspace was sampled at a flow rate of 250 sccm and analysed for 30 minutes (1 minute integration times, 12 - 200 u) at an *E/N* of 90/190 Td. Prior to the addition of the bacterial cultures, the empty container was flushed with nitrogen and similarly analysed to obtain background measurements.



Figure 6.1 The apparatus used for the headspace analysis of bacterial cultures.

Nitrogen is the standard carrier gas used in the CIR-MS system and was automatically used as a means to remove the volatiles from the bacterial headspace without consideration of the effect that the anaerobic environment may have had on the cultures during the sampling period. *H. influenzae, S. aureus* and *S. pneumoniae* are classified as facultative anaerobic organisms [37], which means that these bacteria switch between aerobic or anaerobic processes depending on the availability of oxygen. Even though *P. aeruginosa* is classified as an aerobic organism [37], which therefore requires oxygen, it is thought to adapt to anaerobic conditions [38]. Whilst the cultures should have been able to exist within the anaerobic conditions created inside the sample container, the microbial metabolism, and therefore the MVOC production, may have been changed as a result.

All of the test species were found to produce high intensity signals at m/z 18, which was assigned to ammonia. Although ammonia is actually an inorganic compound, it is grouped under the MVOC title throughout this chapter. In the headspace of the *S. aureus* and *P. aeruginosa* cultures, the ammonia concentrations were high enough to significantly deplete the number of H₃O⁺ reagent ions, to the extent that the measured signal at m/z 18 was greater than that at m/z 19. In comparison to *H. influenzae*, which was found to produce the lowest amount of ammonia out of the 4 species, the raw H₃O⁺ count rates recorded during the *P. aeruginosa* analysis were an order of magnitude lower. Applying the standard normalisation process to these samples resulted in a large normalisation factor that caused the data across all mass channels to scale to artificially high levels in comparison to the other samples. In order to correct for this effect, the data for all samples were normalised to 10^6 counts with respect to the sum of H₃O⁺, H₃O⁺(H₂O) and ammonia, i.e. $\Sigma(m/z 19 + 37 + 18)$. This did not assume that NH₄⁺ was a reagent ion, but that the number of reagent ions, in terms of H₃O⁺ and H₃O⁺(H₂O), would have been approximately equal to this value prior to proton transfer to ammonia.

After the initial experiments demonstrated that MVOCs could be detected in the headspace of bacterial cultures, the aim of the current experiments was to gain an understanding of the types of MVOCs produced and their abundance, and to assess the degree of MVOC variation between the different species of bacteria. At this stage there was no plan to monitor the evolution of MVOCs over time, so relatively short

experiments were performed. However, even over the 30 minute analysis period, the MVOC measurements were found to produce some form of time-dependent profile as a result of the sampling process. This is illustrated in Figure 6.2, using the MVOCs measured in the headspace of the *H. influenzae* cultures as an example. Since it took 10 minutes for the sampling container to be entirely flushed through with nitrogen and therefore to remove any traces of the laboratory air, some reduction in VOC levels over this time may have been expected. After this time it was assumed that any VOCs detected were originating from the bacterial cultures. Some MVOCs were found to display a decrease even after this point, meaning that either the rate of production was slower than the rate of headspace removal, or the metabolic processes responsible for the production of these compounds may have been changing in response to the sampling conditions. Some MVOCs were also found to show an increase with time, which may have reflected that these compounds were originating from an anaerobic process.



Figure 6.2 An example of the MVOC changes over time during the headspace analysis of *H. influenzae* cultures. The horizontal lines display the average background levels in the corresponding mass channel and the vertical line marks the point after which the container had been completely flushed with nitrogen.

The recorded signals were averaged over the final 10 minutes of measurements to allow for the flushing of the container with the carrier gas and the adjustment to anaerobic conditions. Table 6.1 summarises the signals that were observed in the culture headspace of the different bacterial species. At this stage no attempt was made to obtain absolute concentrations, so the data are presented in terms of the ratio of the average signal measured in the headspace of each sample to the average background signal. A ratio of 1 meant that levels in the sample headspace were approximately equal to that observed during the blank experiment, where a ratio greater than 1 showed that the signal was originating from the sample. For simplification, only those mass channels with a sample/blank ratio of 3 or greater in at least one of the species are shown in Table 6.1. An indication of the intensity of the original signal is also provided.

Table 6.1 A summary of the MVOC signals measured in the headspace of different species of bacteria. The sample/blank ratio refers to the ratio of the average signal measured in the bacterial headspace (ncps) to the average signal measured in the blank experiment (ncps). The results are also shown in terms of the original signal intensity measured in the bacterial headspace where trace $(tr) = 10^1$ ncps, $+ = 10^2$ ncps, $+ = 10^3$ ncps and $+++ = 10^4$ ncps or greater.

		Sample	e/Blank			Signal Intensity							
m/z	H. inf.	S. pne.	S. aur.	P. aer	-	H. inf.	S. pne.	S. aur.	P. aer				
18	6	54	133	164	-	+++	+++	+++	+++				
33	4	2	1	2		++	+	+	+				
36	4	11	22	24		tr	tr	+	+				
39	3	2	1	0		+	+	+	tr				
42	2	3	4	3		+	+	+	+				
45	21	21	0	0		++	++	+	tr				
46	2	3	4	4		+	++	++	++				
49	2	28	5	65	65		+	tr	+				
57	3	4	1	0		+	+	+	tr				
59	2	4	1	0		++	+++	++	++				
63	3	3	1	299		tr	tr	tr	++				
70	246	6	4	3		+	tr	tr	tr				
71	2	2	3	0		+	+	+	tr				
73	3	5	1	2		+	+	+	+				
79	6	3	1	18		tr	tr	tr	+				
89	5	3	3	2		+	+	+	+				
95	1	1	1	5		tr	tr	tr	+				
107	25	9	0	0		++	+	tr	tr				
109	3	3	8	4		tr	tr	+	+				
118	51	10	4	4		+	tr	tr	tr				
125	3	1	1	1		+	tr	tr	tr				
136	1	0	0	4		tr	tr	tr	+				

Even though the CIR-MS technique does not provide the absolute identification of compounds, the large amount of literature in this field can assist the tentative assignment of many signals. While not a focus of the current study, knowledge of possible MVOC identities may be of interest since it could provide information on the microbial metabolic processes occurring. As previously mentioned, the most abundant compound produced by all of the species was ammonia, with the highest levels being observed in the headspace of the *P. aeruginosa* and *S. aureus* cultures. Ammonia has been previously observed in *P. aeruginosa* cultures on blood agar and *Pseudomonas*-selective media [12], and in *P. aeruginosa* and *S. aureus* in liquid blood culture media [5]. Given that the ammonia concentrations were so high, the signal at m/z 36 was thought to reflect the formation of ammonia-water cluster ions of the form NH₄⁺(H₂O), which made up 0.02% of the m/z 18 signal.

Methanol is usually responsible for the signal at m/z 33, which was observed in the headspace of all of the species except S. aureus. Little information relating to methanol production by the test bacteria could be found, although the same mass channel and assignment has been reported in the headspace of other bacterial cultures [11] and microbial spoiled meat [39], both measured using PTR-MS. Ethanol is often observed in the headspace of bacterial cultures [5, 6, 12, 13], although no ethanol production was detected in the cultures in this study. Even though the CIR-MS instrument has poor sensitivity towards ethanol (Section 2.4.6), headspace concentrations in the ppmv range are not uncommon [5, 6, 12] and should therefore be detectable by CIR-MS at the current integration times. There are numerous factors that may account for the lack of ethanol in these cultures, such as differences in the growth stage or the growth substrate. The signal at m/z 57, which was only present in the headspace of the *H*. influenzae and S. pneumoniae cultures, may have represented a $C_4H_9^+$ fragment ion from the dehydration of a C₄ alcohol. No signal was observed at m/z 75, the mass channel in which the protonated ion of a C₄ alcohol would have occurred, however the dehydration of protonated alcohol ions becomes more common as the size of the alcohol increases [40] and therefore the fragment ion may have represented the only product. The signal at m/z 71, which was present in the headspace of all of the species except *P. aeruginosa*,

may have arisen under similar circumstances and represented the $C_5H_{11}^+$ fragment ion of a C_5 alcohol. Both C_4 and C_5 alcohols have been identified in the headspace of bacterial cultures [7-9, 41]. All of the cultures showed a signal at m/z 89, the mass channel in which the protonated ion of a C_5 alcohol would have been observed, although other MVOCs may have been responsible for the signal in this mass channel, such as acetoin (3-hydroxy-2-butanone) [7-9, 13, 42] or a C_4 carboxylic acid [42].

Some of the observed signals were thought to indicate the presence of carbonyl compounds. The signal at m/z 45 was assigned to acetaldehyde and was only found to be produced by *H. influenzae* and *S. pneumoniae*. The strong signal at m/z 59, which was again only found in the headspace of the *H. influenzae* and *S. pneumoniae* cultures, was thought to result from acetone. Previously published work has reported the presence of acetone rather than its aldehyde counterpart propanal [11, 14], although the assignments made in these studies were again only tentative. The signal at m/z 73 was observed in the headspace of all of the bacterial cultures except *S. aureus*, and probably represented a C₄ ketone or aldehyde; 2-butanone has been found to be produced by *Pseudomonas* species including *P. aeruginosa* [7-9], and signals in this mass channel have been reported for the PTR-MS analysis of other bacterial cultures [11].

A number of possible nitrogen-containing MVOC peaks were observed, characterised by the even-mass protonated ion signals. The signal at m/z 42 was assigned to acetonitrile, and was found in the headspace of all of the test species. The production of acetonitrile by *P. aeruginosa* cultures on blood agar has been previously reported [12]. The signal at m/z 46 was again present in all of the samples, although the identity of this signal was uncertain as there are a number of possible amine or amide compounds that could occur in this mass channel [43]. In this instance, the m/z 46 peak was not an isotopic peak of m/z 45 as the signal intensities in the two mass channels did not correlate. Various nitrile-compounds may have caused the signal at m/z 70 [43], which was highest in the headspace of the *H. influenzae* cultures. The emission of hydrogen cyanide from *P. aeruginosa* cultures on blood agar and *Pseudomonas*-selective media has been reported [12], but no signal at m/z 28 was observed in the headspace of the *P*. *aeruginosa* cultures in the current experiment. However, the detection of hydrogen cyanide using PTR-MS can be difficult owing to the low proton affinity of this compound [44].

A number of signals were assigned to sulphur-containing compounds. The signal at m/z 49 was assigned to methanethiol and was present in the headspace of all four of the test species, with the highest levels being observed in the *S. pneumoniae* and *P. aeruginosa* cultures. The presence of methanethiol in the headspace of *S. aureus* and *S. pneumoniae* in blood culture bottles has been previously reported [4, 5], and for *P. aeruginosa* after longer incubation times [5]. Methanethiol has also been observed in the headspace of *S. pneumoniae* cultured under anaerobic conditions [6]. Dimethyl sulphide was assigned to the signal at m/z 63 and was produced in much greater levels by the *P. aeruginosa* cultures. The signal at m/z 95 was assigned to dimethyl disulphide, which was only observed in the headspace of the *P. aeruginosa* cultures. The production of dimethyl disulphide by *P. aeruginosa* on various culture media has been previously reported [4, 5, 7, 9, 12, 41, 42].

Low level signals at m/z 39 were found in the headspace of the *H. influenzae* and *S. pneumoniae* cultures. This may have represented a C₃H₃⁺ fragment ion, which can be produced by the fragmentation of protonated isoprene [45]. Table 6.1 does not show a signal at m/z 69 for protonated isoprene or its allyl fragment ion at m/z 41, although examination of the data did reveal signals in these mass channels that were not included owing to the filter process (i.e. the signal/blank ratios were less than 3). Signals at m/z 39 have been observed in the PTR-MS analysis of the headspace of *S. aureus* cultures [46], and the production of isoprene by various bacteria has been reported [41].

The signal at m/z 79 in CIR-MS/PTR-MS is usually assigned to benzene, and while some bacteria are known to produce benzene-derived compounds [47], benzene itself is not often reported. The higher mass signals at m/z values greater than 100 are most likely to represent aromatic compounds. A number of aromatic compounds have been identified for various bacterial cultures on blood agar-based media, including all of the species that were analysed in the current experiment [42]. The aromatic compounds that corresponded to the signals listed in Table 6.1 included benzaldehyde (m/z 107), benzylalcohol (m/z 109) and indole (m/z 118). The identity of the signal at m/z 125 was uncertain owing to the large number of possible compounds. The signal at m/z 136, which was only observed in the headspace of the *P. aeruginosa* cultures, was assigned to 2-aminoacetophenone, a compound that has been identified as being responsible for the characteristic odour associated with *P. aeruginosa* cultures [2]. In one study of 6 different *Pseudomonas* species, only *P. aeruginosa* was found to produce 2-aminoacetophenone [9].

Preti et al. used GC/MS to analyse MVOCs in the headspace of various bacterial cultures, including those species analysed in the current CIR-MS study, all of which were cultured on blood agar or chocolate blood agar [42]. Aminoacetophenone, dimethyl disulphide and dimethyl sulphide were amongst the MVOCs identified as being characteristic for *P. aeruginosa*, which was in good agreement with the tentative assignments made in the current study. In the CIR-MS analysis, aminoacetophenone and dimethyl disulphide were only detectable in the headspace of the P. aeruginosa cultures, and while dimethyl sulphide was found to be produced by the other species, the levels produced by *P. aeruginosa* were much greater. Similarly, the MVOCs that Preti et al. found to be characteristic of H. influenzae included indole, benzaldehyde and benzylalcohol, while those for S. pneumoniae included benzaldehyde, benzylalcohol and methanethiol, which were again in reasonably good agreement with the current data. However, for S. aureus the characteristic compounds were not so well matched, and those identified by Preti et al. consisted largely of organic acids and hydroxy ketones. While some of these identified compounds may have been responsible for the signal observed at m/z 89, this signal was also found to be present in similar levels in all of the other test species and therefore could not really be classed as being characteristic of S. aureus in these experiments. Since MVOC production can be affected by the choice of growth substrate, it is perhaps the case that the compounds identified by Preti et al. were more specific to S. aureus cultures on blood agar, which differed from the growth medium used for the S. aureus cultures in the current study.

A previous CIR-MS investigation examined whether the analysis of VOCs in exhaled breath could be used to identify lung infection in individuals with cystic fibrosis (Section 3.3.2). In this small study, two signals at m/z 71 and m/z 89 appeared to correlate with the presence of infection, displaying elevated levels in the breath of three individuals with S. aureus infection and two individuals with fungal lung infection, which may have related to compounds directly released from the infecting microorganisms. However, the link between these signals and the presence of infection was complicated by the observation of elevated levels in the breath of one cystic fibrosis subject who was thought to be free of infection, although it was possible that these signals were instead associated with other factors of cystic fibrosis disease. Comparison of the signals observed in the exhaled breath of the infected subjects to those measured in the headspace of the corresponding bacterial culture may support the assignment of signals as being of bacterial origin. In the current investigation, both signals were measured in the headspace of the S. aureus cultures and that of some of the other species, and while this does not necessarily prove that the signals observed in breath resulted from infection, it demonstrated that S. aureus bacteria are capable of producing MVOCs in these mass channels, which may potentially be produced by S. aureus colonies in vivo. Exhaled ammonia levels may have been expected to be elevated in individuals with bacterial lung infection since it was found to be produced in such large concentrations by cultures in vitro. However, no differences were observed in the exhaled ammonia levels of the infected and the non-infected individuals, which may have been a reflection of differences between microbial metabolism in vitro and in vivo, or the bacterial ammonia production in vivo was too small to detect.

In terms of a future clinical application, while a breath test may offer a quicker diagnosis in comparison to current culture methods, it would need to accurately identify the specific cause of infection so that the appropriate antibiotic treatment can be initiated. Many of the MVOCs listed in Table 6.1 were found to be produced by more than one of the bacterial species examined, albeit in varying abundances. Assuming that the bacteria display similar MVOC production in vivo, if it is not possible to identify the presence of a particular species on the basis of a specific MVOC in breath, the

diagnosis may instead be made through the measurement of characteristic MVOC patterns. To assess the differences between the MVOC profiles of the different bacterial species in the current investigation, the signals measured over for the last 10 minutes of the headspace analysis for the mass channels shown in Table 6.1 were entered into a principal component analysis (PLS Toolbox). The resulting biplot is shown in Figure 6.3, which showed good separation between the measurements of the different species, demonstrating that bacterial cultures can produce distinctive MVOC patterns.



Figure 6.3 A principal component analysis biplot showing the separation of different bacterial species based on the MVOCs measured in the culture headspace. The biplot displays the first principal component against the second principal component. The principal component analysis was performed using 22 m/z values and the data were pre-processed by autoscaling.

The main outcome of this investigation was the demonstration that different bacterial species can produce characteristic MVOC profiles, and if such chemical fingerprints are produced in vivo then the rapid, non-invasive diagnosis of infection based on the measurement of these compounds in breath may become possible. However, the current investigation does not take into account how 'characteristic' these profiles may be amongst a wider population of microorganisms, or how the difference between in vitro and in vivo conditions may affect MVOC production. Preti et al. reported that the headspace of bacterial cultures and sinus mucus infected with the same bacteria contained some similar characteristic volatiles, however the relative amounts of these compounds and the other types of compounds present were found to differ, which was thought to reflect the different growth substrate and the presence of competing organisms in the natural environment [42]. While this may limit the conclusions that can be drawn from analysing in vitro cultures, the analysis of these species on various growth media may identify MVOCs that are consistently produced. For any further analysis of microbial cultures, the experimental conditions should be modified by switching the carrier gas from nitrogen to synthetic air, such that oxygen concentrations are maintained during the change from the ambient conditions in which the cultures were grown and initially contained within the sample container to the conditions encountered during sampling, therefore removing the effect that the changing oxygen concentrations may have on MVOC emission. In the current study, background measurements were taken as the empty sample container, however the analysis of the uninoculated growth media should also be performed to ensure that the observed VOCs were originating from the cultures and not the growth medium.

6.3 Fungi

6.3.1 Initial fungal headspace analysis

The microbiological culture results for the patients in the cystic fibrosis trial illustrated that it is not just bacteria that infect the lungs of these individuals, as some of the subjects were found to be infected with the fungus *Aspergillus fumigatus*. Also, two of

the subjects who participated in the asthma study (Chapter 4) were known to suffer from allergic bronchopulmonary aspergillosis (ABPA), a condition where *Aspergillus* fungi residing in the airways cause a constant source of allergy [48]. The measurement of MVOCs in breath may also have potential for the non-invasive identification of fungal airway infection. Therefore, the aim of the following experiments was to investigate whether MVOCs could be similarly detected in the headspace of fungal cultures, in particular that of *Aspergillus* fungi.

Preliminary analysis was performed to assess the degree of MVOC production from fungal cultures. The initial measurements were made by analysing the headspace of a selection of *Penicillium* and *Aspergillus* cultures provided by the Department of Infection, Immunity and Inflammation at the University of Leicester. A single culture of *P. spinulosum* (EPA 447), *P. brevicompactum* (EPA 435), *P. chrysogenum* (NCPF 2715), *P. citrinum* (EPA 448), *P. fellutanum* (EPA 431), *A. niger* (EPA 88) and *A. fumigatus* (NCPF 7097 and EPA 526) were provided for the investigation. All samples were cultured at room temperature on standard Petri dishes containing potato dextrose agarose growth medium (Oxoid) supplemented with antibiotics (34 mg/mL Chloramphenicol (Sigma) and 50 mg/mL Gentamycin (Sigma)).

After the large amount of MVOC production observed in the headspace of the bacterial cultures, it was decided to scale down the analysis by headspacing single culture plates. As the volume of the container used for the bacteria experiments was too large for a single plate analysis, a basic sample container was constructed out of a polypropylene box (volume 450 mL) with a clip tight lid. The lid was modified by adding two PFA panel mount connectors to act as the inlet and outlet for the gas flow. To reduce the rate at which the headspace was removed, the sample flow rate into the drift tube was lowered to 150 sccm and the water vapour flow rate to 20 sccm in order to maintain the usual sample/vapour flow ratio. Consequently, the reduction in gas flow into the drift tube resulted in a lower drift tube pressure, so the amount of gas being pumped out of the drift tube had to be restricted to compensate for this (as described in Section 2.3.3). This meant that drift tube was operated at the normal pressure of 6 mbar and at an E/N

of 80/170 Td. The culture plate with the lid removed was sealed inside the container and flushed for 5 minutes with the synthetic air (BTCA 178, BOC) to remove any traces of laboratory air. The contents were then analysed for 60 minutes at 1 minute integration times (12 - 200 u). Background measurements were similarly made by analysing the empty sample container prior to the addition of a sample. The H₃O⁺ ion count was found to be unaffected by the levels of MVOCs produced during the analysis of the single culture plates, so the data were normalised using the standard method (Section 2.3.6).



Figure 6.4 Stacked mass spectra displaying the combined fungal MVOC measurements (after background subtraction).

Figure 6.4 shows the combined MVOC profile after the data for each individual sample were averaged over the final 30 minutes of measurements and the background subtracted. The initial analysis revealed a number of MVOCs in the headspace of the fungal cultures, with some MVOCs appearing to be common to most of the fungi although in varying abundance (m/z 33, m/z 59) and others that seemed to be produced by only a few species (m/z 45). It was interesting to note that the two different strains of *A. fumigatus* showed differences in their MVOC profiles (Figure 6.5). One *A. fumigatus*

strain (EPA 526) showed a greater production of m/z 33 in comparison to other strain (NCPF 7097), and was also found to produce low levels of m/z 45 and m/z 73, for which no detectable levels were observed in the headspace of the other culture. Although this was a small sample set and each species was only represented by a single culture plate, it confirmed that MVOCs could be detected from fungal cultures and showed that the overall profiles of each species were different.



Figure 6.5 A comparison of the mass spectra obtained from the headspace analysis of two different strains of *A. fumigatus* (after background subtraction).

6.3.2 Headspace analysis of 2-pentylfuran

A recent publication by Syhre et al. identified 2-pentylfuran as being produced by a number of *Aspergillus* species, including *A. fumigatus*, which was also found to be detectable in the breath of individuals with *A. fumigatus* colonisation [25]. The authors also reported the analysis of a number of bacterial species, including all of those analysed in the previous bacterial headspace experiments, but found only *S. pneumoniae* to produce 2-pentylfuran. Given the potential of this compound as a marker of fungal

airway infection, and that this compound had not been previously analysed using the CIR-MS technique, the pure chemical (Sigma-Aldrich, CAS: 3777-69-3, 97% purity) was headspaced to determine the product ion distribution that would assist the identification of this compound in future measurements. The headspace analysis of 2-pentylfuran (C₉H₁₄O, 138 u) was performed by flushing high-purity nitrogen through a glass Dreschel bottle containing a single drop of the pure chemical, dispensed using a glass Pasteur pipette. The headspace was analysed for 20 minutes (1 minute integration times, 12 - 200 u) at an *E/N* of 80/170 Td. Prior to the addition of the sample, background measurements were made by passing nitrogen through the empty flask.



Figure 6.6 The mass spectrum of 2-pentylfuran at an E/N of 80/170 Td (after background subtraction and the base peak set to 100% relative intensity).

The initial headspace concentrations were sufficiently high to deplete the entire H_3O^+ reagent ion supply, but over the course of the 20 minute sampling period the sample evaporated and standard PTR-MS conditions were resumed. The resulting mass spectrum after background subtraction showed that 2-pentylfuran produced a single MH⁺ peak at *m*/*z* 139 (Figure 6.6), and did not display any fragmentation at 80/170 Td.

6.3.3 Further fungal headspace analysis

Following the positive results obtained from the initial fungal headspace experiments, a second study was conducted using a larger sample set. The following fungal cultures were provided by the Department of Infection, Immunity and Inflammation at the University of Leicester: *Aspergillus flavus* (EPA 532), *Aspergillus fumigatus* (EPA 7097), *Aspergillus niger* (EPA 88), *Aspergillus restrictus* (EPA 458), *Aspergillus ustus* (EPA 427), *Aspergillus versicolor* (EPA 524), *Penicillium brevicompactum* (EPA 435), *Penicillium chrysogenum* (NCPF 2715), *Penicillium spinulosum* (EPA 447) and *Alternaria alternata* (NCPF 7147). All samples were cultured at room temperature on standard Petri dishes containing potato dextrose agarose growth medium, as described in Section 6.3.1. For each species, 4 culture plates were provided, resulting in a total of 40 plates for analysis. To test for the emission of VOCs from the culture medium, 2 uninoculated plates were similarly analysed.

In order to gain an understanding of the consistency of MVOC production, each of the culture plates were analysed individually. A custom glass container with an approximate volume of 570 mL was built to accommodate a single culture plate (Figure 6.7). Prior to the addition of any sample, background measurements of the empty container were performed. For the analysis of the fungal cultures, a culture plate with the lid removed was placed into the sample container, and the upper and lower sections of the container were secured using rubber bands. The container was flushed for 5 minutes with synthetic air and then the headspace was analysed as described in Section 6.3.1, except that each culture was monitored over a shorter period of 30 minutes owing to the larger number of samples.



Figure 6.7 The apparatus used for the headspace of fungal cultures.

During the analysis of the uninoculated culture media, a signal at m/z 47 and lower intensity signals at m/z 45 and m/z 33 were observed, which were assigned to ethanol, acetaldehyde and methanol respectively. With the exception of one sample, in which the levels were much greater than that measured in the uninoculated growth medium, ethanol and acetaldehyde were not observed in the headspace of the fungal cultures and the levels were comparable to that found during the blank measurements. This suggested that these volatiles were no longer emitted from the growth medium once the culture was established. Therefore, in the following section 'blank' measurements refer to those of the empty container and not of the uninoculated culture media.

Since the H_3O^+ reagent ion signal suffered no depletion during these experiments, the data were normalised using the standard method described in Section 2.3.6. The resulting data were averaged over the last 20 minutes of measurements and converted to a ratio of the average signal for the sample to the average blank signal. Table 6.2 presents the data for each individual sample.

								<i>m/z</i>							
Species	Sample	15	18	33	41	43	45	47	51	57	69	71	73	111	
A. flavus	1	0	_	•		-			0			-	•		
	2		•												
	3	-	0	•	0	0		•	0	0		0	0		
	4		•						-						
A. fumigatus	1	0	0	•					•						
	2	0	•	•					0				-		
	3	0	0	•					0						
	4	0	•	•					0				-		
A. niger	1			0	-	-			-		-				
	2			-	-	-									
	3		0	•	-	-			0		0			0	
	4		-	0	-	-			-		0			-	
A. restrictus	1		0	0					-						
	2		-	0					-				•		
	3		•	0					-						
	4		0	0				-	0				•		
A. ustus	1		0												
	2		-												
	3		0						-						
	4		0												
A. versicolor	1		•	0					-						
	2		•	0					-						
	3	•	0						•						
	4	•	0			-			•						
P. brevicompactum	1	0	-	•					0				-		
	2	0		•					•		0				
	3	0		•					0		0				
	4	0		•					0		0				
P. chrysogenum	1		-	0	-				-						
	2	-	-	0	-				0						
	3		-	0	-	-			0						
	4		-	0	-	-			0						
P. spinulosum	1		-										-		
	2														
	3		-										-		
	4		0										0		
A. alternata	1		0						_						
	2		٠												
	3		٠						-						
	4		0												

Table 6.2 A summary of the MVOC signals measured in the headspace of different species of fungi. Table symbols: - = sample/blank ratio of 2, \circ = sample/blank ratio of 3 - 9, \blacklozenge = sample/blank ratio of 10 - 100, \blacksquare = sample/blank ratio greater then 100.

Many of the signals that were detected in the fungal culture headspace were previously observed during the analysis of the bacterial cultures. Ammonia $(m/z \ 18)$ was again detected, and was produced in varying abundance by most of the fungal cultures. While not often reported, ammonia production from fungal cultures has been previously observed [31]. The signals at m/z 33, m/z 15 and m/z 51 were all assigned to methanol; in addition to the protonated ion, fragment and cluster ions were also observed for many of the species owing to the high methanol concentrations produced by these cultures. Especially high methanol levels were found in the headspace of the A. fumigatus and A. *versicolor* cultures, although the production of methanol by these fungi does not appear to have been previously reported. It should be noted that, in most cases, the methanol levels detected in the fungal culture headspace were much higher than that found in the headspace of the uninoculated growth medium, so the methanol emissions were therefore assumed to result from the cultures. The signal at m/z 71 was only observed in the headspace of two of the A. flavus cultures, which may have resulted from crotonaldehyde (but-2-enal), a compound which has been reported to be emitted by A. flavus cultures [24], or as discussed previously, the signal could have related to the fragment ion of a C₅ alcohol. Of the C₅ alcohols, the methylbutanol isomers are commonly reported as being produced by many fungi including the Aspergillus species [16, 19, 20, 26, 49, 50]. As before, m/z 73 could be attributed to a C₄ ketone or aldehyde, of which 2-butanone is the most commonly reported for Aspergillus and *Penicillium* fungi [16, 22, 31, 50]. The signals at m/z 41 and m/z 43, which were mainly observed in the headspace of the A. niger and P. chrysogenum cultures, are usually assigned to fragments of higher mass compounds. Isoprene (m/z 69) may have been responsible for the fragment ion at m/z 41 (Section 2.4.4), which was detected in the headspace of the A. niger cultures, but not in the P. chrysogenum cultures, suggesting that other compounds were also contributing to this mass channel. The production of isoprene by various Aspergillus and Penicillium fungi has been reported [18, 26, 31]. Low intensity signals at m/z 111 were only observed in the headspace of two of the A. niger cultures. This was the only signal that was detected in the fungal headspace that showed no production in any of the bacteria, and may have represented a fragment of the commonly reported fungal MVOC 1-octen-3-ol [20, 26, 27, 31], which in being a

large alcohol is likely to undergo dehydration. 1-Octen-3-ol, octan-3-ol and octan-3-one are produced by many fungi and are referred to as the 'C₈ complex'[17]. Another C₈ compound that may appear in this mass channel is 1,3-octadiene, which has been observed in the headspace of *Aspergillus* fungi [26].

For some of the fungal species, considerable variability was observed in the MVOC production of the individual cultures. For the A. flavus cultures, a number of strong signals were emitted from one particular sample, and while this result was not consistent with the other A. flavus samples, it showed that this species was capable of producing these compounds. The most intense signals at m/z 45 and m/z 47 were assigned to acetaldehyde and ethanol, both of which have been previously observed in the headspace of this species [24]. For the A. versicolor cultures, the differences in the volatiles measured in the headspace appeared to reflect the differences observed in the physical appearance of the samples (Figure 6.8). The two 'pale' samples were found to produce more methanol, while the two 'red' samples were found to produce more ammonia. While A. versicolor is known to display variable colony colours [51], the reason as to why this would affect MVOC production is currently uncertain, but since the cultures were of the same strain, experienced the same growth conditions and were of the same age, the observed differences could not be attributed to these factors. These findings may just reflect the natural variability that can occur in microbial cultures, but highlighted that the determination of characteristic MVOC patterns will require the examination of multiple cultures, so that the compounds that are consistently produced by each species can be identified. In comparison to the bacterial cultures, the fungal cultures showed less variation in terms of the total number of MVOCs observed. The variety may have been more limited given that all of the fungal species were cultured on the same growth media, and that the majority of the species belonged to one of two genera, whereas the bacterial species were all of different genera.



Figure 6.8 The visual differences observed for two A. versicolor cultures.

As described in Section 6.3.2, headspace analysis was performed on 2-pentylfuran to evaluate its product ion distribution with the aim of identifying this compound in the headspace of the *Aspergillus* fungi, and in particular that of *A. fumigatus*. Very low intensity signals (sample/blank ratio < 2) at m/z 139 were observed in two of the *A. versicolor* cultures, but it was not detected in any of the *A. fumigatus* cultures or that of any of the other species. Whilst Syhre et al. did not use the same growth medium as the current experiment, *A. fumigatus* was consistently found to produce 2-pentylfuran on various types of growth media [25]. Perhaps the presence or absence of 2-pentylfuran is dependent upon the age of the cultures, as both the measurements made by Syhre et al. and that in the current experiment were made at single points in time, and as yet there is no information on the temporal emission of this compound from *A. fumigatus*. 2-Pentylfuran was not found in the breath of any of the asthma patients with ABPA or the cystic fibrosis patients with *A. fumigatus* infection, although it was possible that the concentrations were below the limit of detection for the one second timescale used during breath measurement.

The fungi samples used in the current experiments were only intended for a single analysis in case the sampling procedure induced any changes in the cultures. However, after the main headspace analysis had been completed, an exploratory experiment was performed to investigate potential sampling effects. Given that fungi prefer humid conditions, there was concern over the drying effect of the carrier gas over the course of a headspace experiment. After the initial analysis, the cultures were stored in the laboratory at room temperature (~ 25°C) and re-analysed 2 weeks later using one of two different sampling conditions. For each species, two of the four plates were analysed as before using a dry flow of synthetic air to remove the culture headspace, while the other two plates were analysed using a humid carrier gas. To generate the flow of humid synthetic air, a glass Dreschel bottle containing deionised water was added in-line before the sample container, through which the synthetic air flow was bubbled. Using this method the relative humidity was estimated to be between 65 – 70% on the basis of the *m/z* (19/37) ratio.

The results of the two sets of experiments are displayed in Table 6.3. The results focus on the presence rather than the abundance of MVOCs, where the presence of a signal was defined by a sample/blank ratio of greater than 3. Table 6.3 displays the results for the dry fungal headspace experiments, which was compared using the same format against the corresponding results of the original dry fungal headspace experiments. Some of the MVOCs showed an increased production in comparison to the original measurements, such as methanol (m/z 33) in the P. spinulosum cultures, whilst a number of cultures actually showed a reduction in MVOC production. For example, the high acetaldehyde and ethanol levels produced by one of the A. flavus cultures were not present during the second analysis. These observations probably reflected the differing ages of the cultures between the first and the second analysis. Table 6.3 similarly displays the results of the humid fungal headspace experiments. In comparison to the original dry measurements for these samples, the humid conditions seemed to increase the amount of MVOCs detected. Had this been an effect of the age of the cultures, a similar result may have been expected in the dry repeat experiments, so the difference was assumed to arise from the use of a humid carrier gas. This suggested that more MVOCs may have been detectable during the original measurements had the fungi been contained in more preferable conditions (i.e. humidity). The increased levels observed with the use of a humid carrier gas suggested that the experimental conditions may have had a significant impact upon the activity of the fungi such that future experiments should be performed using a humidified carrier gas, although a dedicated set of experiments with fresh cultures should ideally be performed to confirm this finding.

Table 6.3 Comparison of the original fungal headspace analysis performed using a dry carrier gas to the repeat analysis performed using a dry (samples 1 and 3) or humid carrier gas (samples 2 and 4). Table symbols: • = both cultures show a sample/blank ratio > 3, \circ = only one culture shows a sample/blank ratio > 3.

	z/m	<i>A. fla.</i> - original	A. fla repeat	A. fum original	A. fum repeat	A. nig original	A. nig repeat	A. res original	A. res repeat	A. ust original	A. ust repeat	A. ver original	A. ver repeat	P. bre original	P. bre repeat	P. chr original	P. chr repeat	P. spi original	P. spi repeat	A. alt original	A. alt repeat
	15	0		•	•							0		•	•						
	18	0	٠	•	•	0	•	•	٠	•	٠	•	٠						•	•	•
	33	٠		•	•	•	•	•	0			•	•	٠	٠	٠	•		•		
	41	0					•														
	42																				
	43	0																			
eat	45	0																			
kep	46																				
уF	47	0																			
/D1	51	•		•	•	0						0		•	•	0					
ina	56										0										
rig	57	0																			
y O	59																				
Dr	62																				
	69					0								0	0						
	71	0																			
	73	٠													0				0		
	81																				
	87																				
	111					0															
	15			•	•		0				0	0	•	•	0						
	18	٠	٠	•	•		0	0	٠	0	•	•	٠					0	•	•	•
	33			•	•	0	•	•	0		0	•	0	•	•	•	•				
	41						•		0						0		•				
	42		0		•		•		•				0		0		•		•		0
at	43						•										0				
epe	45																				
l Re	46		٠		•				•		٠		•								
mic	47																				
Ηu	51			•	•		0	0	0			0	0	•	•	•	•				
al/.	56				0						•								•		
igir	57																				
Dry Ori	59				•		0								0		0		٠		
	62		0						٠												
	69				•	0	0							٠	0						
	71																				
	73				•			•	•						•		0	0	•		
	81		0										•								
	87				•												0		0		
	111						0														
6.4 Summary

The current experiments represent the preliminary investigation of MVOC production from microbial cultures in vitro using CIR-MS. The headspace analysis of bacterial and fungal cultures showed that a number of MVOCs could be detected. The analysis of the bacterial cultures demonstrated that different species can produce characteristic MVOC profiles, and should similar patterns be produced in vivo then the analysis of exhaled breath may provide a means for the rapid and non-invasive identification of infection. The headspace analysis of individual fungal cultures highlighted the degree of natural variability in MVOC production that can occur in some species, and an exploratory experiment suggested that the experimental conditions may have an effect on the detection of MVOCs, so further development of the sampling procedure may be required. This preliminary investigation focussed on how MVOCs vary between different bacterial and fungal species, however once the sampling procedure is finalised the effect of factors such as growth substrate and growth phase on MVOC output can be studied. Another interesting possibility for future work is the growth and analysis of mixed culture plates to investigate whether MVOC production is changed in response to the presence of other microorganisms.

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Chapter 7. Summary and future direction

7.1 Introduction

This thesis described the application of CIR-TOF-MS to the analysis of exhaled breath. The development of the CIR-MS system for breath analysis has come a long way over the time-course covered in this thesis, from the design of a suitable sampling apparatus and the first CIR-MS measurements of breath, to the participation in clinical trials and the search for potential markers of disease. Most of the work performed until now has been on a 'proof-of-principle' basis, which has provided some interesting results that could be used as a starting point to initiate further investigations.

7.2 CIR-MS performance

For the application of CIR-MS to the analysis of exhaled breath, high sensitivity on a short timescale was required. The CIR-MS instrument was optimised through a series of calibration experiments, where varying the drift cell E/N and sample humidity allowed the determination of the settings that provided the greatest sensitivity for the compounds of interest. At these settings, the CIR-MS sensitivity for a range of chemical species was determined. With the exception of ethanol, for which CIR-MS/PTR-MS has poor sensitivity owing to fragmentation processes, the sensitivities at 100% relative humidity were determined to be between 3.4 - 169.5 ncps ppbv⁻¹, which corresponded to limits of detection between 1 - 159 ppbv at 1 second integration times, as would be used during real-time breath measurement.

The effect of the sample carbon dioxide concentration on VOC measurements [1] highlights the need to perform further calibration experiments using gas standards containing 5% carbon dioxide, since this could impact on the accuracy of the sensitivity values applied to breath measurements. While the current performance allows most of the common breath VOCs to be analysed simultaneously in real-time, increased sensitivity may allow more species to be measured. One of the ways in which this could be achieved is through improvements to the ion source. Modification of the current ion source through the addition of a second americium strip was found to increase the H_3O^+ count rate, and also the sensitivity for a number of VOCs. As part of the ongoing instrument development within the research group, a new discharge ion source is under construction, which has the potential to generate higher H_3O^+ count rates than the radioactive ion source, which may therefore improve upon the current sensitivity.

The exploration of the multi-reagent capability of the CIR-MS instrument for the analysis of exhaled breath was briefly described. While the instrument has previously demonstrated the ability to generate alternative chemical ionisation reagent ions, the benefit of these alternative reagents for the analysis of breath has not yet been established since the high humidity caused significant interference from H_3O^+ and its ionisation products. The use of alternative reagent ions such as NO^+ and O_2^+ may provide a means to analyse breath VOCs that cannot be ionised by H_3O^+ or those where the measurement of the protonated ion is complicated by other isobaric species, and is therefore worthy of further investigation. However, a method for suppressing the H_3O^+ interference would first be required.

7.3 Breath sampling

The first requirement for the analysis of exhaled breath was the construction of a sampling device. A sampling apparatus was developed, which coupled directly to the CIR-MS instrument to allow the on-line collection and real-time analysis of breath. The final apparatus was built out of standard, wide-bore components, which produced a comfortable, low resistance design. The incorporation of a Digital Volume Transducer

(DVT) allowed valuable information on breathing patterns to be monitored and logged. The apparatus itself was found to cause no detectable volatile emission or analyte loss which, with the use of a heated blanket to prevent soluble losses to breath condensation, ensured that sample integrity was maintained during collection.

The first CIR-MS breath measurements were made using a tidal breath collection over a defined length of time. The apparatus and collection method was tested with groups of healthy subjects and individuals with reduced lung function, who found no difficulty in providing repeated tidal exhalations over a 5 minute period. However, several problems with this method were found, including the variability in breathing pattern, the risk of hyperventilation, and the difficulty in monitoring the performance of the procedure during breath collection. This prompted the change to a single breath procedure which, in combination with instrument improvements and apparatus changes, resulted in the ability to view real-time exhalation profiles for VOCs whose levels in exhaled breath exceeded that of the ambient air.

While the breath collection method described was sufficient for the preliminary investigations performed throughout this thesis, there are a number of ways that on-line breath collection could be improved. Suggestions for a more controlled exhalation procedure were made, which utilized the DVT and a modification of the software to display target flows and volumes. A recent publication reported a buffered end-tidal (BET) method for on-line breath sampling [2], whereby an exhaled breath is buffered in a collection tube such that it allows the use of longer integration times, which will benefit from lower limits of detection. A new apparatus could be designed that incorporates elements of the BET-apparatus, whilst retaining the DVT featured in the current apparatus. However, for application to the CIR-MS system, a reduction in the sample inlet flow into drift tube would be required. Also, a specially designed computer program to automatically process and display the data for instant results would be beneficial.

For the situations where the CIR-MS instrument was not able to be located at the site of breath collection, a method for off-line breath collection was required. The use of Tedlar bags for the collection of breath was described. Testing of the Tedlar bags showed that contaminants from the bag material and the diffusion of VOCs through the bag walls made it difficult to maintain sample integrity during storage, especially over longer periods of time. In order to minimise these effects, it was decided that all samples should be analysed within 6 hours of collection. Given the time constraints for the development of an off-line collection method, Tedlar bags with standard fittings were used and a basic apparatus was designed, which allowed repeated end-exhaled breaths to be collected. However, the collection of end-exhaled breath was based on an individual discarding the initial portion of each exhalation, with no control of the volume excluded or the number of breaths used to fill the bag, so a more reproducible method would be required for future work. A rebreathing technique for the off-line collection of breath has been reported [3], and would improve on the current method without the need for expensive equipment, although the use of wide-bore bag fittings would be required, and the investigation of alternative bag materials would also be recommended.

7.4 Normal breath composition

As the ultimate goal of breath research is to identify exhaled markers of disease, the natural variation in exhaled compounds in healthy subjects needs to be qualitatively and quantitatively determined, so that deviation as a result of disease can be identified. There were several occasions throughout this thesis where the breath of healthy individuals was examined, which allowed the identification of a group of VOCs that appeared to be common to all subjects. Focussing only on the compounds that were present in levels in excess of that found in the ambient air, the commonly observed VOCs were tentatively assigned to methanol, acetone, dimethyl sulphide and isoprene. Acetaldehyde was also found to be present in the breath of healthy subjects, although this was only measured during off-line analysis, since this benefitted from the use of longer integration times whereas breath acetaldehyde concentrations are likely to be

close to the limit of detection during real-time analysis. Acetonitrile was observed in the breath of some individuals, a compound that is usually associated with smoking.

Breath VOC concentrations in a group of 68 subjects (203 individual breaths) were examined, which allowed the overall concentration distributions for the common VOCs to be determined. The median concentrations and concentration ranges were determined to be 350 ppbv (126 - 1719 ppbv) for methanol, 484 ppbv (163 - 4264 ppbv) for acetone, 11 ppbv (4 – 32 ppbv) for dimethyl sulphide and 193 ppbv (65 – 575 ppbv) for isoprene. The variability in the VOC concentrations for an uncontrolled triplicate breath collection for the 68 subjects was typically found to be between 5 - 11% for methanol, 2-6% for acetone, 13-28% for dimethyl sulphide and 12-26% for isoprene. Various factors that may influence the exhaled concentrations were also investigated, which included age, gender and BMI, and a number of results were found to be statistically significant in the group of individuals studied, although it is likely that some of these may not be applicable in a wider population. The true significance of these findings may become apparent as further studies are performed. Monitoring of VOCs in the breath of the same individual allowed the variation to be assessed without the influence of these factors. Longitudinal measurements were performed for one healthy subject over a 4 week period to assess the intra-individual variation in breath VOC concentrations. The median concentrations and concentration ranges were determined to be 454 ppbv (219 -1127 ppbv) for methanol, 473 ppbv (261 - 1708 ppbv) for acetone, 11 ppbv (7 - 22ppbv) for dimethyl sulphide and 166 ppbv (95 - 278 ppbv) for isoprene, which indicated that some VOCs can display considerable range even within a single individual.

7.5 Clinical studies

The first clinical trial analysed the breath of a small number of individuals with cystic fibrosis (9 cystic fibrosis children, 4 healthy children) and aimed to investigate whether the presence of microbial lung infection would cause detectable changes in the exhaled VOC profiles. Two signals at m/z 71 and m/z 89 were found to be elevated in the breath

of cystic fibrosis patients who were thought to have lung infection at the time of breath sampling. However, the link between these signals and the presence of infection was complicated by the observation of elevated levels in the breath of one cystic fibrosis subject who was thought to be free of infection, although it was possible that these signals were instead associated with other factors of cystic fibrosis disease, such as inflammation or oxidative stress. Given the small numbers of participants in this study, the significance of these elevated signals is yet to be determined. Should the opportunity arise to continue this study, it would be recommended that a much larger number of subjects are recruited, and for the cystic fibrosis patients it would be useful to obtain samples for microbiological culture on the same day as the breath collection since this would allow the confident assignment of infection status and consequently a better correlation with the VOCs observed in breath. A continuation of this study would also benefit from the improvements made to the sampling protocol and the CIR-MS instrument since the original study.

A larger study that focussed on the examination of VOCs in the breath of asthma patients (35 asthmatic subjects, 5 COPD, 28 healthy controls) saw the CIR-MS instrument positioned in a clinical setting for the first time. The objective of the study was to search for potential markers of airway inflammation and while none were immediately apparent, a small number of signals were observed in certain individuals, although it was uncertain whether they resulted from compounds of relevance to asthma. One of these signals was observed at m/z 89, for which there are a number of compounds that may be responsible. It would be interesting to identify this signal to determine whether it is the same compound that is present in the breath of the asthmatic subjects and the cystic fibrosis patients. This could be achieved through combined CIR-MS and GC/MS analysis. GC/MS would require breath to be collected off-line, although collection into Tedlar bags would not be suitable for CIR-MS analysis since the measurement of the signal at m/z 89 would be affected by the isotopic peak of the Tedlar contaminant N,N-dimethylacetamide, so an alternative bag material would be needed. The positive identification of the compounds present in breath and knowledge

of their potential sources may allow their relevance in these studies to be better assessed.

The final study represented a preliminary investigation of the VOCs in the exhaled breath of a small number of female cancer patients (2 ovarian cancer patients, 2 endometrial cancer patients, 10 healthy female controls). While no single species displayed a clear correlation with the presence of cancer, the overall breath profile formed by a group of 17 *m*/*z* values appeared to provide some degree of discrimination between the endometrial cancer patients and the healthy controls, although the ovarian cancer patients were not so well differentiated. This study demonstrated that the chemical 'fingerprint' of exhaled breath may have diagnostic potential, but the size of this preliminary investigation was too small for any firm conclusions to be drawn. Nevertheless, the preliminary results have provided a good basis on which to expand the study to involve a greater number of subjects, which may then allow the variation of breath VOC patterns with cancer stage to be investigated, so that the point at which any differences between healthy individuals and those with cancer can be determined, and the potential of breath VOC measurement for the early diagnosis of cancer can be assessed.

7.6 Microbial headspace analysis

While the studies of bacteria and fungi in vitro moved away from the direct analysis of breath, the MVOCs produced by these microbial cultures may provide an indication of those produced by microorganisms in vivo, and therefore those present in the breath of infected individuals. The headspace analysis of *Haemophilus influenzae* and *Streptococcus pneumoniae* cultures on blood agar and *Staphylococcus aureus* and *Pseudomonas aeruginosa* cultures on Luria-Bertani agar revealed that these bacteria can produce a range of MVOCs which, based on the tentative assignments, included alcohols, aldehydes, ketones, nitriles, ammonia, sulphur-containing compounds and aromatic compounds. Principal component analysis of the bacterial headspace measurements demonstrated that, on the basis of 22 m/z values, the MVOC profiles

were sufficiently different to distinguish between the 4 species, suggesting that the measurement of these characteristic profiles may provide a means of identification should similar patterns be produced in vivo.

Following the initial analysis of a small number of *Aspergillus* and *Penicillium* fungi, a larger set of headspace experiments were performed on individual cultures of 6 *Aspergillus* species, 3 *Penicillium* species and a single *Alternaria* species all on potato dextrose agarose growth medium. A smaller range of MVOCs were observed in the headspace of the fungal cultures in comparison to the bacterial cultures, which may have reflected that the fungi were all cultured on the same growth medium, and that the majority of the species belonged to one of two genera. The analysis of individual cultures showed that MVOC production in some species can vary considerably, indicating that future investigations will require the analysis of multiple cultures to identify those MVOCs that are consistently produced by each species. An exploratory experiment found that the use of a humidified carrier gas for sampling the culture headspace appeared to increase the amount of MVOCs detected in comparison to the dry carrier gas used throughout this study, however this should be properly investigated with a dedicated set of experiments.

Once the sampling method has been optimised, further CIR-MS experiments should be performed to investigate how MVOC production is affected by various growth factors, such as growth phase and substrate, and to identify those MVOCs that are consistently produced and characteristic of a particular microbial species. The investigation could be extended to the analysis of mixed culture plates to investigate whether MVOC production is changed in response to the presence of other microorganisms. The ultimate goal would be the measurement of these characteristic MVOC patterns in the breath of infected individuals or in other biological samples such as sputum, thus providing a means to diagnose infection more rapidly than traditional culture methods.

7.7 Final comments

The work covered here represents exploratory studies that tested the capabilities of CIR-TOF-MS for breath analysis research. While a number of interesting preliminary results have been reported, follow-up investigations are required to substantiate these early findings. Long-term studies would allow much larger groups of participants to be recruited and may enable repeat visits so that variations in an individual's breath can be monitored over time. The development of standardised breath sampling protocols is currently one of the key goals for the field of breath research. This is not just a necessity for breath tests to progress into clinical practice, but will also allow the comparison of data between different research groups and may promote multicentre research trials. The pursuit of bringing breath analysis into a clinical setting is demanding, but ultimately worthwhile in the hope that it will become routinely used as a legitimate medical diagnostic. It may eventually form the basis of screening programs for conditions such as cancer, potentially saving lives in the process through early detection and treatment. As such, breath analysis is both a highly interesting and highly rewarding field of research.

References

1. L. Keck, C. Hoeschen, U. Oeh (2008) Effects of carbon dioxide in breath gas on proton transfer reaction-mass spectrometry (PTR-MS) measurements. *International Journal of Mass Spectrometry*, 270 (3) 156-165.

2. J. Herbig, T. Titzmann, J. Beauchamp, I. Kohl, A. Hansel (2008) Buffered endtidal (BET) sampling - a novel method for real-time breath gas analysis. *Journal of Breath Research*, 2 (3) 1-9.

3. M. E. O'Hara, S. O'Hehir, S. Green, C. A. Mayhew (2008) Development of a protocol to measure volatile organic compounds in human breath: a comparison of rebreathing and on-line single exhalations using proton transfer reaction mass spectrometry. *Physiological Measurement*, 29 (3) 309-330.

Appendix

Seminars Attended	Date
Aspects of chiral surface chemistry: An electrochemical perspective Gary Attard	17/10/05
Airway disease research: The first fifteen years Ian Pavord	01/11/05
New chemistry and physics of drug transport Richard Templer	07/11/05
Density functional based molecular dynamics simulation of redox reactions in solution Michiel Sprik	30/01/06
What we can learn from current and future satellite observations of tropospheric composition Paul Palmer	01/02/06
Explosives and explosive detection Dan Wood	03/02/06
Ion mobility spectrometry: shaping up for structural and trace analysis Colin Creaser	20/02/06
<i>The sweet smell of clean chemistry</i> Andrew Abbott	21/02/06
Soft ionisation mass spectrometry for quantitative studies of non- covalent interactions Renato Zenobi	08/03/06
Nanoparticle toxicology Paul Howes	13/03/06
Chemistry and giant thunderstorms Geraint Vaughan	07/03/07

Seminars Attended (continued)	Date
Meteors and their impact on the atmosphere John Plane	16/05/07
Directed self-assembly within systems of subcomponents Jonathan Nitschke	06/11/07
Baltimore and Beijing: a tale of two cities Russell Dickerson	08/11/07
Exploring the dynamics of single aerosol particles Jonathan Reid	12/11/07
Laser studies of photofragmentation dynamics Grant Richie	26/11/07
I spy with my little eye – what remote sensing has done for us Heiko Baltzer	04/12/07
Synchrotron radiation studies of processes near electrode surfaces Trevor Rayment	10/12/07
<i>Fingerprinting air: decoding the atmosphere</i> Paul Monks	12/02/08
On-line sensor systems to capture the chemical signature of brain injury Martyn Boutelle	18/02/08
Correlated quantum dots David Logan	12/05/08
<i>The Diamond Project</i> Colin Norris	20/10/08

Conferences/Meetings Attended	Date
Tri-Universities Gas Phase Chemical Physics Meeting University of Leicester, Leicester	26/10/05
RSC The Use of Elemental and Molecular Mass Spectrometry in the Life Sciences University of Leicester, Leicester	16/03/06
International Mass Spectrometry Conference (IMSC) Prague Congress Centre, Prague	27/08/06 - 01/09/06
Scientific Meeting of the International Association for Breath Research (IABR) - Breath Analysis in Physiology and Medicine J. Heyrovsky Institute of Physical Chemistry, Prague	04/09/06 - 05/09/06
RSC Environment, Sustainability and Energy Forum: Trace measurements and sensors Zoological Society, London	15/11/07

Presentations	Date
Poster Presentation: Breath Analysis using Chemical Ionisation Reaction Time-of-Flight Mass Spectrometry IMSC 2006, Prague	31/08/06
Poster Presentation: Breath Analysis using Chemical Ionisation Reaction Time-of-Flight Mass Spectrometry IABR 2006, Prague	04/09/06
Poster Presentation: <i>Breath Analysis for Medical Diagnosis</i> Open Day, University of Leicester	26/04/08
Oral Presentation: <i>Catch your breath: the development of real-</i> <i>time, multichannel analysis of exhaled breath using chemical</i> <i>ionisation reaction time-of-flight mass spectrometry</i> Departmental Research Day, University of Leicester	23/05/08

Publications

K. P. Wyche, R. S. Blake, K. A. Willis, P. S. Monks, A. M. Ellis (2005) Differentiation of isobaric compounds using chemical ionization reaction mass spectrometry, *Rapid Communications in Mass Spectrometry*, 19 (22) 3356 – 3362

R. L. Cordell, K. A. Willis, K. P. Wyche, R. S. Blake, A. M. Ellis, P. S. Monks (2007) Detection of chemical weapon agents and simulants using chemical ionization reaction time-of-flight mass spectrometry, *Analytical Chemistry*, 79 (21) 8359 – 8366

K. P. Wyche, C. Whyte, R. S. Blake, R. L. Cordell, K. A. Willis, A. M. Ellis, P. S. Monks (2007) Atmospheric monitoring with chemical ionisation reaction time-of-flight mass spectrometry (CIR-TOF-MS) and future developments: Hadamard transform mass spectrometry, In *Advanced Environmental Monitoring*, 64 – 76

P. S. Monks, K. A. Willis, A. M. Ellis (2010) Taking your breath away: real-time mass spectrometric analysis of breath, *Mass Matters*, Edition 61, 12 - 13

P. S. Monks, K. A. Willis, Chemical analysis of breath – a key to your health, *Education in Chemistry, In press*