# Metabolite Profiling of Biological Specimens Using Small Molecular Weight Volatile Organic Compounds by Proton Transfer Reaction Time-of-Flight Mass Spectrometry

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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 2010 and September 2013. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

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# Metabolite Profiling of Biological Specimens Using Small Molecular Weight Volatile Organic Compounds By Proton Transfer Reaction Time-of-Flight Mass Spectrometry

**Sharmilah Kuppusami**

Metabolite profiling is an analytical study of metabolites that are of low molecular weight which results from normal and pathological cellular processes, using high throughput analytical technologies. This thesis documents the development of the analytical technique of proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) for the analysis of small molecular weight volatile organic compounds (VOCs) in different biological specimens. The work explored the challenges associated with sampling, analysis, and metabolite profiling and identification in microbiology and clinical studies. Initial work focused on the VOCs produced in the headspace of ten *Clostridium difficile* ribotypes in an attempt to metabolically profile *C. difficile* at the ribotype level. The *C. difficile* ribotypes were successfully distinguished from one another. The metabolite profiles suggested that VOC profiling may provide a useful indicator for the identification of the ribotypes. The PTR-ToF-MS system was applied to two clinical trials. The first was a genitourinary clinical trial of patients with sexually transmitted infections (STI), which explored the VOCs emitted from vaginal, cervical and throat swabs to support the hypothesis that metabolite profiling has the potential to identify the presence of infection. The second involved the analysis of exhaled breath to examine the VOCs in the breath of individuals with ovarian cancer (7 female cancer patients, 12 healthy female controls, 5 female with benign cysts) using offline breath collection technique. The premise is that the VOCs in the breath are representative of the VOCs in blood; therefore specific VOCs may be produced in the body caused by tumour cells and these can be detected in breath. Within these applications, the PTR-ToF-MS was able to demonstrate that metabolite profiles are promising biomarkers for disease/infection identification, as well as providing information of cell mechanism and alterations in cells.

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## List of Abbreviations

| | |
|---|---|
| **bVOC** | biogenic volatile organic compound |
| **CI-MS** | Chemical ionisation - mass spectrometry |
| **EI** | Electron ionisation |
| **FID** | Flame ionisation detector |
| **GC** | Gas chromatography |
| **GC-MS** | Gas chromatography - mass spectrometry |
| **KE** | Kinetic energy |
| **LoD** | Limit of detection |
| **MFC** | Mass flow controller |
| **MCP** | Multichannel plate |
| **ppbV** | parts per billion by volume |
| **ppmV** | parts per million by volume |
| **pptV** | parts per trillion by volume |
| **PA** | Proton affinity |
| **PTR-MS** | Proton transfer reaction - mass spectrometry |
| **PTR-ToF-MS** | Proton transfer reaction - time of flight - mass spectrometry |
| **RF** | Radio frequency |
| **RSD** | Relative standard deviation |
| **SIFT-MS** | Selected ion flow tube - mass spectrometry |
| **ToF-MS** | Time of flight - mass spectrometry |
| **ToF** | Time of flight |
| **TDC** | Time to digital converter |
| **VOC** | Volatile organic compound |

# Chapter 1

# LITERATURE REVIEW: INTRODUCTION TO METABOLOMICS

## 1.1 Introduction

Metabolomics is the most recently developed 'omics' that analyses final cellular metabolites by using high throughput analytical technologies such as gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR) and high performance liquid chromatography-mass spectrometry (HPLC) [1,2,3]. Researches are allowed to perform qualitative and quantitative analysis of low and high molecular weight compound analysis in biological experiments [4].

Volatile organic compounds (VOCs) are carbon based molecules which are naturally volatile in ambient temperature and are produced as parts of plants, humans, animals, and microorganism's metabolic pathways [1]. VOCs can be categorised in several groups including fatty acids, aromatic compounds, nitrogen containing compounds and sulphur containing compounds.

There are four different approaches in metabolomics [5,6]: targetted analysis, metabolite profiling, metabolomics, and metabolic fingerprinting. Targetted analysis is the process of determination and quantification of a small set of known metabolites (targets) using one particular analytical technique of best performance for the compounds of interest. Metabolite profiling aims at the analysis of a larger set of compounds, both identified and unknown with respect to their chemical nature. This approach has been applied for many different biological systems using GC-MS, including plants (7), microbes (8), urine (9), and plasma samples (10).

Metabolomics employs complementary analytical methodologies, for example, LC-MS/MS, GC-MS, and/or NMR, in order to determine and quantify as many metabolites as possible, either identified or unknown compounds. Metabolic fingerprinting is a "signature" or mass profile of the sample of interest generated and then compared in a large sample population to screen for differences between the samples. When signals that can significantly discriminate between samples are detected, the metabolites are identified and the biological relevance of that compound can be elucidated, greatly reducing the analysis time. Metabolomics can therefore be seen as providing a more comprehensive view of how cells function, as well as identifying novel or striking changes in specific metabolites.

Metabolites, especially secondary metabolites, are extremely important for most organisms to defend themselves from stressful environments or predators. Although primary metabolites involved in central metabolism can be used to determine nutritional and growth status, secondary metabolite profiles may better reflect the differentiation of species and their complex response to environmental factors and other organisms. The suite of secondary metabolites in an organism can be astonishingly complex, and while certain compounds may be found in different organisms, a vast number of compounds are very species-specific.

### 1.2 Outline of Thesis

The proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) instrument described in this thesis was originally built for atmospheric applications to measure trace volatile organic compounds (VOCs) and was shown to be effective in monitoring VOCs in complex mixtures with low ppbv detection. The PTR-MS technique was later expanded into other research fields which shows the versatility of this technique and its capabilities. This thesis represents a further expansion of the PTR-MS technique into the field of metabolite profiling, the study of small molecule VOC metabolites. In my thesis, my goals are to develop the analytical technique of PTR-ToF-MS for the analysis of metabolite of small molecule VOCs in different biological specimens - which includes bacterial cultures, genitourinary swab specimen and

exhaled breath analysis; in systems biology and clinical studies. To the best of my knowledge, the studies (Chapter 3 to 5) reported in this thesis were attempted for the first time using PTR-ToF-MS technique.

Chapter 2 describes the PTR-TOF-MS instruments used in this thesis. It focuses on the theoretical principles, instrumental operation and arrangement, together with an evaluation of the instrument performances through calibration performed using the identified instrumental arrangements.

Chapter 3 details the application of PTR-ToF-MS technique in identifying VOCs in the headspace of clinically important *Clostridium difficile* ribotype cultures and the ability to distinguish between different ribotypes of *C. difficile*. This was an attempt to metabolically profile *C. difficile* at ribotype or genomic DNA level by analysing VOCs released by the bacteria using PTR-ToF-MS.

Chapter 4 moves the focus from systems biology to clinical study. This chapter discusses a genitourinary clinical study involving a group of patients with sexually transmitted infections (STI); consisting of gonorrhoea, bacterial vaginosis, trichomonas, chlamydia and candida. The headspace VOCs of vaginal, cervical and throat swabs were measured to investigate whether patients with STI can be distinguished from healthy controls, and to determine whether identification of species-specific infections was possible.

Chapter 5 details the use of offline breath analysis and its application in a cohort study of exhaled breath from a group of ovarian cancer patients. The premise is that specific VOCs may be produced in the body because of tumour growth and these can be detected in breath. This pilot study investigated whether patients with ovarian cancer can be discriminated from healthy subjects using exhaled breath VOCs. This study also assessed the difference between cancerous ovarian cells and benign cysts found in the patients by biopsy using the exhaled VOC profiles.

Chapter 6 summarises the findings of the work presented in this thesis and discusses the suggestions for improvement and the future direction of the studies conducted in this thesis.

### 1.3 Key Instrumentations

The rapid, non-invasive and online measurement of VOCs plays an increasingly important role in various research fields. Many biological processes constantly release VOCs. These VOCs are important because many can be linked to important characteristics and properties of processes and products, they provide relevant and important information on biological processes, *e.g.* metabolism [1] and can impact greatly on the human health in the early detection of diseases [4], even though their presence are often at trace levels.

An ideal analytical method for detecting VOCs would be rapid, non-invasive, non-destructive and selective with capabilities of real time detection in complex mixtures at trace level concentrations. A rapid analysis would allow samples introduced directly to the mass spectrometer without any pre-treatment *e.g.* sample pre-concentration or clean up. VOC monitoring also relies greatly on high time resolution and high sensitivity as the compounds of interest may well be present in the sub-parts per trillion (pptv) range. The technique must be capable of monitoring simultaneously a large number of compounds over a large range of concentrations. Such a technique would allow new advancements in fast process monitoring and rapid characterisation of metabolites and products. It would also allow timely assessment of medical and environmental conditions that would permit immediate response, decision and corrective actions in addressing medical situations for example.

In a routine and conventional technique of VOC detection, a mass spectrometer is usually chosen, preceded either by a gas chromatography (GC) or a liquid chromatography (LC) separation step. GC-based methods [101] are the standard analytical technique used for VOC identification and quantification. Although GC-MS is highly valuable, it is not designed to monitor the rapid changes of VOC

concentrations occurring in biological processes. Often, a pre-treatment phase or pre-concentration step [101] introduces time averages of the concentration of the measured sample mixture. Because of these drawbacks, other methods with simple sampling, high sensitivity, high time resolution, fast and direct on-line monitoring of VOCs have been developed over the years to complement GC/LC-MS-based methods.

The analysis of VOCs is challenging for various reasons; (1) they are often complex, volatile mixtures; (2) their concentrations may change rapidly with time; and (3) they may be present in trace amounts. Routine MS-based analytical techniques such as GC-MS and LC-MS are not entire suitable to address these challenges.

A number of techniques have been developed over the years, from sensor arrays to spectroscopic techniques, with high sensitivity and fast monitoring while avoiding the drawbacks of chromatographic separation and sample pre-treatment methods. Some of the most important approaches are discussed in this section including atmospheric pressure chemical ionisation-MS (APCI-MS) [102, 103], proton transfer reaction-MS (PTR-MS), selected ion flow tube-MS (SIFT-MS) and e-noses. These techniques vary significantly in terms of sampling, inlet and ionisation/detection principles.

### 1.3.1 Atmospheric Pressure Chemical Ionisation (APCI)

Atmospheric Pressure Chemical Ionisation (APCI) is an ionisation technique that uses gas-phase ion-molecule reaction at atmospheric pressure. In this technique, primary ions are produced by a corona discharge across a solvent spray. APCI is mainly used for polar compounds with molecular weights up to about 1500 amu [104]. The principle for an APCI source is shown in the Figure 1.1.

The analyte in solution comes from a direct inlet probe or a liquid chromatography elutes. It is directly introduced into a pneumatic nebulizer where it is converted into a thin fog by a nebulizer gas. Normally nitrogen is used as a nebulizer gas. Droplets are

then displaced by the gas flow through a heated quartz tube called a desolvation chamber. The heat transferred to the spray droplets allows the vaporisation of the mobile phase and of the sample in the gas flow. The temperature of this chamber is controlled. After the desolvation, the mixture is carried past a corona discharge electrode, where ionization occurs.

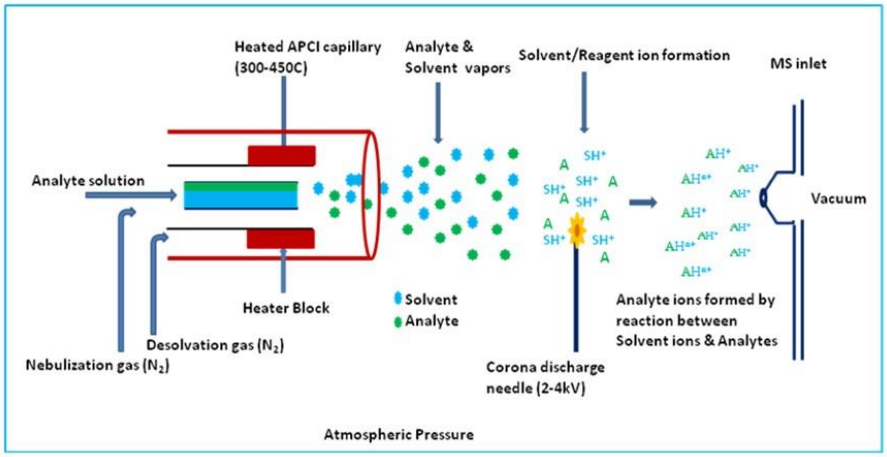


Figure 1.1 Diagram of APCI source

The ionisation process is similar to the process taking place in standard chemical ionisation but occurs under atmospheric pressure. In the positive mode, either proton transfer or adduction of reactant gas ions occurs to produce the ions of molecular species, depending on the relative proton affinities of the reactant ions and gaseous analyte molecule. In the negative mode, the ions of the molecular species are produced by either proton abstraction or adduct formation. However, APCI ionisation is rather complex owing to the presence of many possible ionisation agents. Other problems are the suppression of ionisation in the source, leading to non-quantitative results, difficulties in the unequivocal identification of compounds solely based on their *m/z* values and the relatively low ionisation efficiency. All these potential shortcomings were recently discussed from the point of view of flavour analysis [105]. These potential shortcoming can be in some cases, be resolved if experiments are run under carefully controlled conditions [105]. For several years, APCI has been the reference technique

in online monitoring of flavour compounds, especially for breath-to-breath analysis of in vivo release [105]. Because of its long history and its availability in most analytical laboratories, APCI remains a vital method, in spite of the development of other techniques, described below, with higher sensitivity (e.g. PTR-MS) or simpler ionisation (e.g. SIFT-MS) as demonstrated by its continuing development [106].

### 1.3.2 PTR-MS

Lindinger and co-workers in the mid-1990s first introduced the PTR-MS technique. The technique has been reviewed several times in literature, the first being by Lindinger [107] that presents a clear description of PTR-MS fundamentals and potential. Reviews that are more recent are presented in [105] and [108].

The main constituents of a PTR-MS apparatus are the ion source, a reaction region and a mass analyser. In most instruments, the $H3O^+$ primary-ion beam is produced by a hollow cathode ion source [107] or by a radioactive material [109, 110]. This setup presents some advantages as no mass selection is applied and all the reagent ions are directly fed into the reaction chamber. This setup simplifies the system and allows for better sensitivity. Alternative designs of the source have been tested to reduce impurities and back streaming from the reaction chamber [111] at the cost of sensitivity [112].

More recently, other reagent ions have been studied [106, 113, 114] and, a marketed commercial system from Ionicon Analytik allows for rapid switching between different ions such as $H3O^+$, $NO^+$ and $O2^+$. The literature on this recent development is limited, but it appears very promising, as there is a chance for a better compound identification by bracketing with different parent ions. The second part of a PTR-MS apparatus is the short drift region, where the parent ions driven by an electric field

and eventually interact with the neutral species to be detected. The process is controlled by drift-tube temperature (typically 50–100°C), pressure (~200 Pa) and electric potential. The PTR technique and the properties of drift tube and the reactions which occur therein are discussed in detail in Chapter 2 of this thesis.

The last part of a PTR-MS system is the mass analyser. Linear quadrupoles are one of the most widely used mass analyers in PTR-MS systems. They are robust and relatively cheap, but it is slow and typically provide only unit mass resolution. Two alternatives have been used and they are ion trap and time-of-flight analysers. Prazeller *et al.* [115] has used the ion-trap mass analyser with their PTR system. Ion trap mass analyser stores the ions for a given time, thus increasing the instrument sensitivity and allowing collision-induced dissociation experiments to identify isobaric compounds [116]. Coupling with time-of-flight mass detector [109, 110, 117, 118] has its strong points as in the very rapid spectra acquisition and high mass resolution. The Leicester PTR-TOF- MS instrument was used for the majority of work in this thesis as discussed in detail in Chapter 2.

### 1.3.3 SIFT-MS

SIFT-MS was developed by Španěl and Smith [119], who have reviewed the fundamentals and several applications [120]. Further applications and recent developments have also been reviewed [121]. In SIFT-MS, the parent ions are generated by microwave plasma or by an electron impact ion source, and the primary ions are then selected from the ion mixture by a quadrupole mass filter. Positive ions are created in a microwave discharge ion source containing a mixture of water vapour and air at a relatively high pressure. The ions then enter the low-pressure chamber through a small orifice. A current of ions of a given mass-to-charge ratio, m/z, is extracted from the mixture of ion species using a quadrupole mass filter. The current of primary ions is then injected into a fast flowing inert carrier gas stream by the pump. The ions are carried along the flow tube as a thermalised ion swarm and are sampled via a pinhole at the downstream end of the flow tube where the sampled ions pass into a differentially pumped, quadrupole mass spectrometer. The sample of air to be studied is introduced into the SIFT-MS instrument via a sample inlet port in the flow tube at a measured flow rate. Trace gases in the air sample can react with the primary ions and the product ions formed are detected and quantified by the downstream detector system.

Accurate quantification is possible with the SIFT-MS because the reaction time of the primary ions with the trace gas molecules is precisely defined in the SIFT, and the rate coefficients for the ion-molecule reactions can be measured accurately in separate SIFT experiments if they are not already known. From such SIFT experiments, a large database of rate co-efficient has been created, which is critical for the quantification [122]. The primary ions are chosen so that they do not react significantly with the major components of the air or breath sample ($N_2$, $O_2$, $H_2O$, or $CO_2$) which would result in them being rapidly lost from the ion swarm. However, they must react efficiently with the trace gases to produce identifiable product ions in order to be detected. In the case of VOC detection, the hydronium ion $H_3O^+$, $O_2^+$ and $NO^+$ fulfil these criteria. In SIFT-MS, the choice is for softer ionisation and higher flexibility at the cost of less sensitivity (due to a mass filter) and a more complex set-up when compared to PTR-MS. A clear advantage of SIFT-MS is that there is no need for regular calibration, in contrast to PTR-MS [107] as quantification is based on well-understood kinetic behaviour and ion chemistry (in-built kinetics library) [123].

PTR-MS and SIFT-MS are popular for the detection the VOCs at trace levels from a variety of matrices. The SIFT technique is also very effective in the investigation of ion-molecules reactions. Main technological differences between PTR-MS and SIFT-MS are: (1) PTR-MS, is based on the realisation of pure and intense ion sources that do not need further ion filtering; and, (2) SIFT-MS is more closely related to the ion-mobility reaction set-up, allowing a higher purity of the parent-ion beam and the possibility to select it easily, at the cost of a reduced sensitivity.

### 1.3.4 E-noses

An electronic nose has been described as “an instrument which comprises an array of electronic, chemical sensors with partial specificity and an appropriate pattern recognition system, capable of recognizing simple or complex odours” [124]. Its name stresses the fact that it mimics the behaviour of human olfaction and this is why its main fields of application are food and flavour sciences, where the substitution of proper sensory analysis, difficult and time consuming, with instrumental methods, is a

long hoped-for objective. The idea is that the output of a series of unspecific sensors can be used to build models to classify products or processes, by means of chemometrics or data mining [125, 126]. The headspace (HS) sample is the gas space above the sample in a container and volatile sample components diffuse into the gas phase forming the headspace. Headspace analysis is therefore the analysis of the volatile compounds present in that gas. The analysis of HS samples by a mass spectrometer operated in the electron ionisation mode without any GC separation. The feasibility of the method is demonstrated by various applications and this is often referred to as a "MS-based electronic nose" or an "MS e-nose". The mass pattern obtained, considered as fingerprint of the sample analysed.

E-noses, however, suffer from several drawbacks, including sensor poisoning, high sensitivity to moisture, poor linearity, poor reproducibility on different instruments and sensor drift. When an MS-e-nose is used with EI, it suffers from severe fragmentation, leading to complex spectra with many overlapping fragments, and little chemical information can be obtained. Besides EI, other ionisation methods are increasingly being introduced, some of which are promising in terms of both portability and robustness. They can be termed collectively "MS fingerprinting" approaches, and provide rapid, efficient tools for the characterization of samples. The fingerprint has been used to set up classification models [127], and for calibration models to predict the characteristics of food products [128]. In particular, coupling with ToF mass analysers further offers increases in speed and information entangled in rapid fingerprinting [122, 129].

## 1.4 Applications

### 1.4.1 Bacterial volatile organic compounds

VOCs have been used for bacterial identification since 1964 when Geldreich et.al. developed the Indole-Methyl Red-Voges-Proskauer and Citrate (IMVIC) test which was used for the detection of coliform based on the production of indole, acetoin, pyruvate and 2,3-butanediol in culture media [11]. Indole and 2-aminoacetophenone are

two examples of typical VOCs that have been used as common markers for *Escherichia coli* and *Pseudomonas aeruginosa* detection in culture media [12, 13]. VOCs from bacterial pathogen have been used to develop sensitive and accurate methods to prove the absence or presence of pathogens as well as phenotyping within bacterial species. Such information could be used to take the best action regarding prevention or antibiotic treatment [14]. Bacteria produce a wide range of VOCs as their primary or secondary metabolites in different physiochemical conditions [1]. VOCs from microorganisms are released mainly as metabolic products during growth, as secondary metabolites for protection against antagonists and competitors, or as signalling molecules in cell-to-cell communication [15-19].

Early investigations using gas chromatography-mass spectrometry (GC-MS) illustrate the capacity of bacteria to produce a wealth of volatile compounds [20-23]. One of the earliest papers that described the production of volatiles by bacteria demonstrated the release of formic and butyric acid [24]. Stotzky and Schenck [25] summarised the volatile organic compounds released from microorganisms and showed that fungi produce a wider variety of volatiles than bacteria, although this might have been attributable to the larger number of studies performed with fungi at that time. Stotzky and Schenck [25] described bacteria such as *Pseudomonas spp*. and *Streptomyces spp*. as being ethylene and hydrogen cyanide producers, *Clostridium spp*. as emitters of dimethyl disulfide, various short chain acids, 2,3-butanediol,isopentanol,and acetoin, and *Agrobacterium radiobacter*, *A. rhizogenes*, *Bacillus cereus*, *Enterobacter aerogenes, E. coli*, *Micrococcus luteus*, *Nocardia corallina*, *Proteus vulgaris*, *Sarcina lutea*, and *Serratia marcescens* as releasers of unidentified volatiles [25].

Furthermore, mixed bacterial cultures and microbes in soil under aerobic and anaerobic conditions have the potential to produce organic volatiles. In recent years, the technology has developed further, and the identification and quantification of volatile compounds has been mostly successful. More than 120 different compounds are emitted from *actinomycetes* [26], comprising alkanes, alkenes, alcohols, esters, ketones, and isoprenoids. *Myxococcus xanthus* has also turned out to be a rich source of volatile compounds, 42 compounds having been collected in a closed-loop stripping apparatus [27]. Two new natural products, (S)-9-methyl-decan-3-ol and 9-

methyldecan-3-one, have been identified. With an enormous effort and critical evaluation of the published data, Schulz and Dickschat [22] have summarised all known bacterial compounds so far detected by using the different methodologies, with 346 different compounds released from various bacteria being described. The classification of bacterial volatiles has revealed 75 fatty acid derivatives, 50 aromatic compounds, 74 nitrogen-containing compounds, 30 sulfur compounds, 96 terpenoids, and 18 halogenated, selenium, tellurium, or other metalloid compounds.

As discussed earlier, VOCs are emitted by bacteria as by-products of metabolic processes but also as a means of communication between bacteria. [17]. However, these emissions used for communication are not well understood. The counter action by bacteria when in response to stimulus is also poorly understood. Understanding such processes may assist in the development of drugs to fight bacterial infection. Given that different microbial cell cultures emit characteristic aromas, sampling of the headspace above microbes should lead to different PTR-MS mass spectral information according to the type of cell cultures, and hence provide a basis for assignment and discrimination. There have been a number of PTR-MS studies interrogating the headspace above bacterial cultures [11–18]. The greatest potential of PTR-MS in this field is to exploit its *in situ* real-time capabilities to undertake *in vivo* breath analysis or other alternative biological specimens in order to rapidly identify bacterial infection. Real-time non-invasive diagnosis of bacterial infection in the body would enable the correct antibiotic treatment to be administered more rapidly. This is crucial for the effective and early management of infections because appropriate early treatment results in lower mortality rates.

One of the first few studies of microbial VOC emissions using PTR-MS were undertaken by Critchley *et. al.*[28]. They reported the use of PTR-MS for *in vitro* headspace analysis of volatile emissions from specific microbial cultures. In their study, headspace of pure blood agar cultures of *P. aeruginosa* and *Streptococcus milleri* were sampled and two different spectral profiles were obtained. This shows that there is a possibility to discriminate between different types of bacterial infection using mass spectral 'fingerprint' analysis. Although only a pilot study, the work by Critchley *et al.* demonstrated the potential of PTR-MS for this type of online analysis and its use for

discriminating different bacterial species. Subsequent researchers have consistently shown that distinctive pattern of VOCs are emitted by different bacterial species. Profiling of VOC emissions from different strains of *Escherichia coli*, *Klebsiella*, *Citrobacter*, *P. aeruginosa*, *Staphylococcus aureus* and *H. pylori* was performed in an *in vitro*study by Lechner *et al.* [29]. The work by Lechner and co-workers showed substantial differences in the mass spectral patterns between the various microbial cultures. Furthermore, *m*/*z* ions were associated with specific bacteria that further supported the idea that the analysis of VOC emissions from bacteria using PTR-MS could be used to differentiate bacterial strains *in vitro*. Bunge *et al.* investigated the headspace above *E. coli*, *Shigella flexneri*, *Salmonella enterica* and *Candida tropicalis* bacterial cultures [15]. It was found that *C. tropicalis* could be uniquely identified by several characteristic marker ions, while *E. coli* and *S. enterica* could be discriminated from each other and from *S. milleri* by specific marker ions.

The limitation of all the above studies is that the identification of VOCs leading to the observed ions is often speculative. By combining GC–MS and PTR-MS, Kai *et al.* provided a comprehensive profile of VOCs emitted from the rhizobacterium (root associated bacterium) *Serratia odorifera* [30]. Sodorifen (C16H26, a bicyclic polymethylated diene) was found to be the dominant compound emitted, as determined from the GC–MS measurements. This compound was not detected in the PTR-MS measurements because the mass range was limited to 20–160 Da. Volatiles observed by PTR-MS included dimethyl disulfide, dimethyl trisulfide, methanethiol, terpenoids, 2-phenylethanol and various other aromatic compounds. Specific compound assignments were predominantly through the use of GC–MS but was also partly based on isotopic pattern analysis of the PTR-MS mass spectral profiles.

### 1.4.2 Breath analysis

#### 1.4.2.1 Exhaled breath volatile organic compounds

Assessment of metabolic and physiological processes by means of non-invasive breath biomarkers may complement current (invasive) diagnostic procedures and add important scientific value to already established markers. Metabolic monitoring by means of volatile target compounds in human breath, therefore, is a promising alternative to already established methods. Analysis of VOCs in exhaled air represents another non-invasive approach for observation of biochemical processes in the body.

Exhaled volatiles provide a non-invasive window to the metabolic processes occurring within the body [3]. Breath analysis is therefore attracting growing clinical and scientific attention as a highly novel means for delivering non-invasive, real-time rapid diagnosis, fast screening and monitoring of complex diseases, such as cancers and acute infections. To date, however, research has been limited to 'proof-of-principle' trials with poor breath sampling procedures, without any in-depth clinical follow-up to substantiate the effectiveness of VOCs as selective biological indicators of disease. Using novel technology with suitable breath sampling techniques, more detailed in-depth studies are required to determine whether there are unique patterns of VOCs, made up of specific and/or non-specific biomarkers, for a particular disease that can be discerned from the complex chemical environment of breath. Furthermore, robust algorithms for identifying and modelling these patterns need to be developed.

VOCs in exhaled breath were first analysed by Pauling *et.al*. in 1971 [31]. Hundreds of VOCs have been identified on the breath at concentrations ranging from parts-per-trillion to parts-per-million, many of which are known to be characteristic markers for disease. VOCs can therefore be clinically useful for the early detection and diagnosis of diseases, physiological disorders and therapeutic monitoring. To help unlock the messages provided by them requires the application of trace gas detection and careful analysis of VOC patterns (*m/z* values and reliable intensity measurements).

The online monitoring of the VOCs in the air exhaled during respiration, is attracting growing interest as a possible medical research and diagnostic tool [32-35]. Although there is not yet a clear answer regarding the most suitable approach for fast, non-invasive breath analysis, the instrumentations as discussed in Section 1.3 are certainly interesting in terms of time resolution and sensitivity when evaluated in parallel with a GC and spectroscopic methods that are the traditional alternative technologies. Monitoring VOCs in exhaled human breath has attracted considerable attention because of its potential as a technique that is non-invasive and painless to patients. It is already used in the diagnosis of several health disorders and pathologies (e.g. lung cancer and inflammatory lung diseases) [36-39].

PTR-MS and SIFT-MS have been applied in breath analysis. It is possible, with reduced effort, to screen a large population of healthy volunteers and to describe the distribution of major breath BVOCs (e.g. ammonia, acetone, methanol, ethanol and isoprene). A further step is to characterise population segments with specific characteristics (age) or habits (smokers and non- smokers) [40].

In the case of halitosis, SIFT-MS has been used to measure concentrations of both in mouth and in-nose VOCs to differentiate systemic and orally generated compounds [37]. For example, acetone, methanol and isoprene are mostly systemically produced, while ethanol is mostly produced in the mouth. SIFT-MS [41] has been used successfully for the measurement of breath isoprene. Breath isoprene has been shown to be related to blood cholesterol levels and it has been found to be enhanced in end-stage renal disease patient following dialysis. PTR-MS has been used for real time breath monitoring of the intravenous anaesthetic agent propofol and its volatile metabolites in patients undergoing surgery [42, 43].

The possibility of following metabolic processes in real time is a useful application to gain information in the field of health sciences. APCI is also a widely used method and extensive literature is available but it is not an ideal technique due to the complex ionisation processes caused by the presence of different precursor species (water clusters, in particular) [40].

Evidence in the literature suggests that breath analysis has considerable potential for medical applications [44]. Notable are the pioneering works of Phillips, which provide evidence for the presence of identifiable VOCs in the breath related to lung and breast cancers [45-47]. Using mass spectrometric techniques to analyse these VOCs, Phillips claims to have been able to discriminate between patients with and without lung cancer [46]. Changes of VOC production in cancer patients were believed to be related to oxygenation of cell membrane-based polyunsaturated fatty acids and the increased level of reactive oxygen species within cancer cells [45, 46]. Other than that, the identification of VOC biomarkers from exhaled breath in lung cancer [45–47], breast cancer [48, 49], colorectal and prostate cancer [49] have been reported showing the growing interest in the research of breath analysis. All of these reports suggest that exhaled breath analysis could be developed as a non-invasive diagnostic tool.

#### 1.4.2.2 Alternative headspace samples to breath analysis

The measurement of VOCs in urine is used extensively in medicine. Both urine and breath VOC analysis are useful for the measurement of the VOCs of metabolic status or for the early detection of the disease. The main advantages associated with breath and urine compared to blood, is that they are non-invasive and painless to extract. Collection of urine sample is possible also possible from the children. Urine is a very complex mixture of water, electrolytes, hormones, protein and enzymes and is thus considered a “dirty” matrix. On the other hand, breath consists of air with a large quantity of moisture and inert gases and is a relatively clean matrix compared to urine. This has an advantage when measuring VOCs in small quantities as any mass spectrometry signals or a signal from any measurement technique will be free from the background noise.

PTR-MS also has seen a number of medical applications that do not involve breath measurements. For example, it has been used to assess the concentration of acetonitrile in the urine of habitual cigarette smokers and in non-smokers as a quantitative marker of recent smoking behaviour [53]. The results showed a significant enhancement of acetonitrile concentration in the urine of the heavy smokers.

Headspace analyses of urine, faeces, and blood and skin volatiles have been attempted. Probert *et al.* [50] have reviewed the potential of faecal volatile compounds for diagnosis of gastrointestinal disease using GC-MS. Another example is the measurement of biological matrices such has a human breath or urine [10]. Urine analysis [51, 52], *in vivo* human skin studies [53] and occupational health exposure in medical/laboratory environments [54-56] are other examples where non-invasive diagnostic analysis may be beneficial.

### 1.4.3 Breath Components – Disease Diagnosis

Since the days of Hippocrates and the Greeks, the sense of smell has been used to diagnose diseases. Physicians have used their nose to learn more about their patient's condition. It was observed that patients with uncontrolled diabetes had the smell of rotten apples because of the acetone in their breath [57]. Patients with renal failure had urine like odour attributing to trimethylamine and lung abscesses gave off a sewer like odour due to the anaerobic bacteria.

Microbiologist has been able to identify bacteria in culture by their unique smell, rekindling the idea that microbial infections can be identified as such. For many years, physicians have known that the sense of smell can provide information about a person's physiological state. However, using one's sense of smell is an inaccurate method of diagnosis as this sense will vary from physician to physician, as well as with age and other factors. Hence, most current methods of diagnosis involve analysis of blood, urine and/or other bodily fluids.

Commonly, physicians analyse blood samples along with descriptions of symptoms to diagnose for illnesses. More specifically, blood resides within the body and is continually flowing throughout it, acting as a medium for providing oxygen and nutrients, and receiving waste products (i.e. carbon dioxide ($CO_2$)) to expel from the body.

Similarly, certain disease states upset the chemical balance within the body, also resulting in measurable changes to blood chemistry. Gas exchange between body tissues and blood cells occurs between red blood cells and the tissue walls [58].

During the period in the lungs where blood uptakes oxygen, it exchanges waste gases which are then exhaled out of the body. The waste gases exhaled also provide significant information about the person's physiological state. Therefore, analysis of these gases could be an ideal non-invasive method of disease diagnosis if compounds that are associated with disease states are detectable.

The human respiratory system can be broadly divided into two sections, the upper and lower airways. The upper airways consist of the nasal and oral cavities, trachea, and the necessary plumbing, the bronchi, required getting air to the lung tissues (the lower airway). It is in the lung tissues of the lower airway that gas exchange takes place. As discussed previously, the primary function of the lungs is rapid gas exchange between circulatory blood and inhaled air.

The human lung tissues can be broken down into smaller, individual components. After many airway branches, inhaled air reaches the lower lung tissue: the bronchioles and the alveoli. The alveolus consists of alveolar ducts, sacs and individual alveoli. Gas exchange occurs in the alveolus where a laminar flow of blood and inhaled air are separated by a thin tissue layer. Blood flow occurs in a network of capillaries that encase each alveolus. The source of this blood flow is the pulmonary artery and the arteriole network extending from it to the lung tissues [58].

The human body requires its entire blood volume (approximately 5 litres) to be re-oxygenated approximately every minute. Therefore, the gas exchange process needs to be fast, and the lungs must have many alveoli to supply a high surface area for gas exchange. To meet this demand humans have around 300 million alveoli. Because there is a high turnover of blood through the lungs, humans exhale breath containing these waste gas molecules and compounds into the atmosphere. The portion of exhaled air containing gasses that have exchanged with blood is referred to

as alveolar breath. Exhaled compounds within the alveolar breath have been the focus of respiratory researchers for many years in trying to detect and diagnose respiratory, and other diseases [59, 60].

Numerous compounds are found in the breath of healthy individuals at parts per billion (ppbv) or parts per million (ppmv) levels. These compounds are created through metabolic processes including energy production, cholesterol synthesis, glucose metabolism and lipid peroxidation of fatty acids [61]. Table 1.1 lists some common compounds found in breath and their reported concentration ranges from various studies [62, 63].

Table 1.1 - Typical concentrations of breath analytes [62, 63]

| **Compound & Levels** | **Concentration Range** | **Metabolic Processes** |
|---|---|---|
| Ammonia | ppbv - ppmv | Digestion |
| Acetone | ppbv - ppmv | Glucose metabolism |
| Isoprene | ppbv - ppmv | Cholesterol synthesis |
| Ethanol | ppbv | Gut bacteria |
| Pentane | ppbv | Lipid peroxidation |

### 1.4.4 Sources of compounds found in breath

Compounds like ammonia, isoprene and acetone are associated with metabolism and occur in varying concentrations throughout the day. For example, studies have shown that acetone levels in breath are significantly higher during fasting [64]. Similarly, high protein meals, such as red meat, increase ammonia concentrations after digestion [64].

These commonly occurring compounds found in breath, or changes in their levels could potentially be used to diagnose alterations in metabolic processes due to illness or other factors. For example, pentane is the product of oxygen free radical (OFR) meditated lipid peroxidation of n-6 polyunsaturated fatty acids, and has potential for the diagnosis of disorders such as breast cancer, heart transplant rejection, acute myocardial infarction, schizophrenia and rheumatoid arthritis [61].

### 1.4.5 Sample delivery

Before collecting breath samples, an understanding of respiratory physics and physiology is required. Breathing normally, in a state of rest, air inspired and expired in one breath, is typically around 0.5 litres (500 mL), commonly referred to as the tidal volume. However, the total lung capacity is approximately 6 litres. If a person were to take a deep breath and exhale as much as they could, their total exhalation would be approximately 4.8 litres, a value called the vital capacity. The remaining 1.2 litres, which remains in the lungs, is called the residual volume. Figure 1.2 provides a more complete description of the physics of breathing.

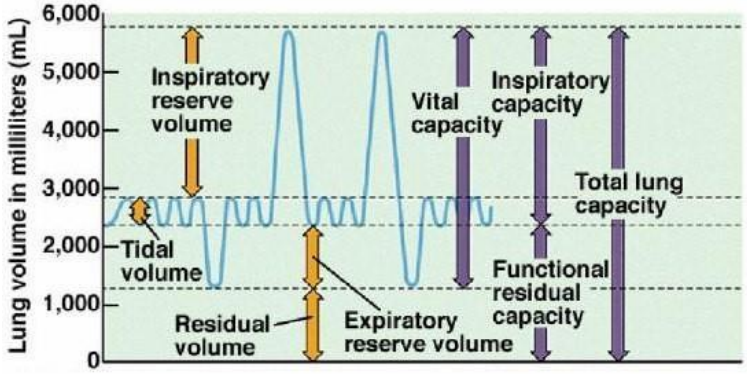


Figure 1.2 Respiratory Volumes and Capacities [65]

Because gas exchange occurs in the alveolus, exhaled breath that originates from the upper airways (dead-space) should be excluded from analysis. More specifically, excluding the dead-space from the analysis excludes chemicals from the analysis that originate from the atmosphere rather than from gas exchange with blood cells. Typically, the dead-space volume of an average human is 0.15 litres [65]. This value is 30% of a tidal volume or approximately 3.125% of a typical vital capacity.

To date, little research has examined the effect of breath delivery method on breath compound concentrations. Variations in delivery method may include collection of breath tidal volumes, vital capacities or set breath flow-rates. Additionally, the physical apparatus used to collect or analyse breath may vary in length, operating temperature, flow control/restriction and other design parameters. All of these parameters may affect the concentrations and/or interactions of compounds in the sample, thus affecting the resulting "true" value available to be measured by a given analysis system. For example, nitric oxide (NO) is a marker of asthma [5, 6] and the commercially available instrument NIOX (NIOX, 2004) is used as a tool solely for NO detection. However, patients providing a sample on the NIOX instrument need to provide breath at a set flow-rate, back pressure and resistance during exhalation. The developers of the NIOX instrument experimentally defined an exhalation flow-rate that would cause the soft palate in the back of the pharynx to lift, allowing the analysis of NO that originates only from the lungs and not the portion that resides in the nasal cavity as recommended in the guidelines for standardised testing of exhaled NO [7]. Similarly, variations in sample delivery may cause noticeable effects with other compounds, for example highly water-soluble molecules like ammonia may vary in measured concentration depending on the humidity of the sample.

### 1.4.6 Nasal vs. oral exhalation sampling

One of the challenges in breath analysis is the breath sampling, is oral or nasal breath sampling better? There are no internationally accepted standard sampling guidelines or protocol for the performance of sample collection to follow. Thus research groups tend to set up their own protocol on sampling breath according to their requirement

and infrastructure.

Mouth exhaled breath may contain VOCs produced in the airways, the oral cavity by bacterial infections, by bacteria in the gut; and saliva and mucus from the respiratory tract [66]. Smith *et. al*. studied the concentration of mouth and nasal exhaled breath using SIFT-MS for several compounds and they reported that ethanol and hydrogen cyanide are largely generated in the mouth compared to nasal breath [64]. Similarly, ammonia [64] was found at higher levels when measured in the mouth exhalations compared to nasal exhalations. Some compounds such as acetone, methanol and isoprene are said to be totally from the physiological state [64] as similar levels were detected in mouth and nasal exhalations. Other factors that can contribute to the total or partial exogenous VOCs in the mouth exhalation are food intake, drink [27], and poor oral hygiene [24]. In addition, VOCs produced in the gut by bacteria can be transported to and excreted by the lungs [25] for example *Helicobacter pylori* living in the human stomach releases VOCs that can be detected in mouth-exhaled air [37]. As such some research groups prefers nasal exhalation to breath exhalation to avoid the possibility of contamination of the exogenous VOCs.

On the other hand, most substances (e.g., nitrite) measured are present not only in the lower airway but throughout the respiratory tract including nasal passages [67]. As such nasal exhalations could be contaminated from the many factors as discussed above. Prevention of mouth exhaled breath from contamination remains an important step in sampling. The use of a two-way non-rebreathing valve allows for exhalation into the apparatus and preventing rebreathing of exhaled samples. If certain compounds (e.g., $H_2O_2$, nitrite) is high in the saliva, it may contaminate the sample data interpretation. Exclusion of salivary source can be achieved simply with a saliva trap, but rinsing of mouth prior to collection and/ or swallowing accumulated saliva to maintain a dry mouth during collection usually suffice to prevent contamination [67].

Nasal secretions would very well contaminate nasal exhaled breath VOCs especially for subjects with asthma, allergic rhinitis and hay fever [68]. Furthermore, some subjects with severe upper airway diseases may not be able to inhale or exhale through their noses. This also applies to the elderly and very ill patients. For mouth

breath exhalation, the use of nose clips is still questioned [68]. Mouth exhalation with inhalation through the nose (without using a nose clip) may have some advantages over collection wearing a nose clip since some resistance occurs when inhaling through the mouth due to the valve of the mouthpiece. This resistance is inconvenient for patients with airway obstruction. For these patients, inhalation through the nose is easier, less tiring, more natural and more convenient because it does not dry up the mouth [68]. The disadvantage of the removal of the nose clip (inhalation through the nose) is that patients must concentrate on exhaling through the mouth instead of the nose, which would be more natural. For experiments that need exhaled breath condensate (EBC), this could be obtained from these patients only by oral breathing [68].

### 1.4.7 Breath sampling

Ever since the early days of breath analysis, researchers have realised that the way that breath is sampled is a crucial issue [69, 70]. The current status of breath sampling and the matters that need to be addressed have been summarised in recent publications [71-73]. A valid breath sampling must include a suitable sampling apparatus for the collection and transport of exhaled breath between patient and instrument, along with a sampling protocol for either on-line or off-line collection of breath. 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 [74, 75]. 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 as in not to 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 [32].

Breath sampling can be carried out online or offline. Online collection requires a subject to breathe into apparatus that is connected directly to the analytical instrument, whereas offline collection requires the breath to first be collected in some form of container. Offline 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, although there is concern over contamination and sample loss [76, 77], which makes online sampling more favourable.

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 [72]. 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 [69], 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 [72]. 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 [78].

Until now the focus has been on the online, 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 offline 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 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, offline sampling has the added concern of how to preserve sample composition during collection and storage. Containers for offline breath collection include stainless steel canisters, adsorbent traps and polymer bags [79-81]. 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 [82]. It has been claimed that canister samples are stable for storage periods of 30 days [82], although others have shown that this is not the case for some compounds such as thiols and alcohols [83]. A wide variety of sorbents are available for the trapping of VOCs [80], 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 [84, 85].

Collection into polymer bags has become a popular method for offline 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 [76, 86-88]. Tedlar bags are the most commonly used with regard to breath analysis (see for example [89- 95]). 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 [76, 85-87, 89, 90, 92, 96]. 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 [76, 97]. 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 [98, 99], which are assumed to result from the production process [98]. Other lower intensity contaminants have also been observed [86, 99].

Unless a standardised protocol for breath sampling method is created, factors such as contamination, dead space, and loss of analyte would lead to huge variations in the determination of VOC concentrations. This raises grave concerns about the reproducibility and accuracy of published results, especially those that have been obtained using non-validated breath sampling procedures.

Breath samples for VOC analysis is collected in large volumes into containers free from contamination. Such samples then need to be concentrated, which usually involves passing the samples through a trap that captures the compounds of interest. This may be chemical interaction, resin adsorption or cryogenic distillation. The concentrated samples must then be transferred to the assay instrument. Currently, tedlar bags are widely used for the sample collection, in which subjects can blow the breath straight into the tedlar bag. After the collection, bags can be transferred to the laboratory. It is vitally important that at each stage of sample preparation and transfer that there is a minimal loss of the volatiles presents and no introduction of contamination to the samples.

Other than Tedlar bags, Nalophan bags are also becoming popular. The bags are low in price, inert, and have good durability. In a study of the use of Nalophan bags for storing tobacco samples, it was reported that samples remained relatively stable between 4 and 12 h after sampling. A study using Nalophan and Tedlar bags were investigated for the collection and storage of breath samples containing hydrogen cyanide [92]. Results show that samples were stable up to 24 h for all bag types. However, it depends on the research groups on the use of collection bags as Nalophan bags are much cheaper than Tedlar bags and because of this Nalophan bags can be discarded after single use. Unlike Tedlar bags where the need for bag cleaning procedure and infection control measures.

The half-lives of various VOCs in Tedlar bags, namely methanol, acetaldehyde, acetone, isoprene, benzene, toluene and styrene, were investigated by Harren and group [96], with the half-lives being found to be between 5 and 13 days. Although these half-lives are relatively long, they still highlight that it is best to analyse the breath samples as quickly as possible after collection for the most reliable, contaminant-free measurements.

Beauchamp *et al.* also investigated the use of Tedlar bags for breath sample collection, whereby a sample bag was filled with a mixture of VOCs (containing alcohol, nitrile, aldehyde, ketone, terpene and aromatic compounds) in known concentrations and air was then sampled regularly from that bag over a 70-hour period [100]. As expected, the concentrations of these compounds declined over time owing to the various loss processes mentioned above. The researchers concluded that accurate results can only be obtained providing measurements are made within 10 hours of sample collection.

## 1.5 Summary

This chapter has introduced the different techniques that are being used on the regular basis in the analysis of VOCs in breath and the headspace of bacteria. This chapter also discussed the various literature available in the field of breath analysis and headspace analysis for diagnostic analysis. This thesis documents the development of the analytical technique of PTR-ToF-MS for the analysis of small molecular weight VOCs in different biological specimens. This thesis aims to explore the challenges associated with sampling, analysis and metabolite profiling and identification in microbiology and clinical studies.

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# Chapter 2

# PROTON TRANSFER REACTION TIME-OF-FLIGHT MASS SPECTROMETRY: INSTRUMENTATION AND PERFORMANCE

## 2.1 Introduction

The analysis of metabolomics discussed in this thesis uses the PTR-TOF-MS. Two different PTR-TOF-MS instruments were used for the analyses described here within, the Leicester PTR-TOF-MS instrument which 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) [1-3]; and the Kore Technology commercially made PTR-TOF-MS called the Medi-PTR-TOF-MS [4] that was transferred to the hospital for the analysis discussed in Chapter 5. The Leicester instrument employed is able to use several different CI reagents [5, 6], although $H_3O^+$ remains the main CI reagent and the one used exclusively in the work in this thesis. The Leicester instrument has been well characterised, particularly with regard to atmospherics measurement [7, 8] and breath analysis [9]. This chapter describes the PTR-TOF-MS instrument and operating principles, together with the assessment of its performance and calibration under the conditions and settings used during the metabolite measurements.

## 2.2 Proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS)

### 2.2.1 Instrumentation

Samples which are in the form neutral molecules need to be ionised first in order to be analysed by a mass spectrometer. Most mass spectrometers use the traditional electron ionisation (EI) method but some researches uses the chemical ionisation (CI) method as an alternative to overcome problems met with EI such as matrix interference, and can offer better sensitivity and specificity. The chemical ionisation technique was first introduced by Munson and Field in 1966 [11] as an alternative to the traditional electron ionisation method. High energy electrons in the region of 70 eV in an EI source interacts with gaseous analyte to eject an electron to form a radical cation [10]. This process produces excess energy, which is typically around 10 eV for VOCs, that often results in fragmentation of the molecular ion. Fragmentation patterns of analyte of interest can be used to identify the analyte but extensive fragmentation can confound identification when complex mixtures are analysed. In comparison, the CI method, which is based on gas phase ion-molecule reactions between analyte and reagent ion produces ions with lower excess energy with either little or no fragmentation [10]. This is known as 'soft' ionization technique producing simple mass spectra which suitable for complex mixture analysis. The CI ion-molecule reaction occurs through one of four reactions: proton transfer, charge transfer, anion abstraction or association [10].

**Commented [CRL(2]:** Most GC-MS instruments do but not the majority of all… LC-MS is all ESI or APCI

In the PTR-MS technique exothermic proton transfer is used for ionisation, and occurs for compounds with a proton affinity greater than that of the reagent gas. Typical reagent gases and the corresponding reagent ions for proton transfer include methane/$CH_5^+$, ammonia/$NH_4^+$ and water vapour/$H_3O^+$ [11, 12]. As proton transfer occurs for a compound only if its proton affinity is higher than the reagent ion this must be taken into account when selecting a suitable reagent in the CI method.

PTR-MS was developed by Lindinger et al. and introduced in the 1990s [12, 13], which uses CI with flow-drift tube technique. Gas chromatographic techniques were used

prior to the development of PTR-MS for VOC analysis [14]. GC-MS methods are able to offer high sensitivity and the capability of identifying compounds with the help of mass spectral library, but it requires offline sample collection and pre-concentration, as well as time for chromatographic separation; limiting it from monitoring mixtures with relatively fast changing concentrations [15]. PTR-MS, however can offer online, real-time measurements of trace gases without the need for pre-concentration.

PTR-MS instruments consist 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 take place, and (3) a mass analyser/detection system.

PTR-MS systems either uses a hollow cathode discharge (HCD) ion source or radioactive ion sources ($^{241}Am$, $^{210}Po$) to discharge sources [16, 17]. Radioactive ion sources can work at higher pressures which translates to higher instrument sensitivity. Reagent ions from the ion source are continuously introduced into the drift tube, with analyte species acting as the buffer gas. The potential gradient over the drift tube directs the ions towards the exit and into the analyser.

In an HCD ion source containing water vapour the discharge process causes the 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 [13]. High concentrations of $H_3O^+$ ions are produced (often as high as > 99% purity) without the need for pre-selection of ions before entering the drift tube, as opposed to SIFT-MS, which employ a mass filer to select a single reagent. Some $O_2^+$ and $NO^+$ impurity ions are formed because of back diffusion of air from the drift tube into the ion source, an unwanted occurrence since these ions can also react with VOCs [15]. HCD sources normally operate between 2 – 3 mbar, and $H_3O^+$ count rates of $10^6$ counts $s^{-1}$ at the downstream mass spectrometer are routinely achieved [15]. Radioactive ion sources ($^{241}Am$, $^{210}Po$) have been employed as an alternative to discharge sources and can operate at higher pressures (up to 13 mbar) [16, 17]. Higher drift tube pressures can translate into higher instrument sensitivity [16, 18, 19]. 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 [2]. 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.

The majority of PTR-MS instruments uses quadrupole analysers, which act as mass filters only allowing 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 (assuming the same dwell time for each channel). 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 cycle times of 1 – 10 seconds [20, 21]. Another limitation of quadrupole analysers is the relatively low mass resolution [16].

Some instruments have been developed with alternative analysers in the form of ion trap (IT) or TOF systems, both of which offer the simultaneous measurement of ions of all masses. The first PTR-IT-MS 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 been reported subsequently [1]. One specific advantage of IT-MS over quadrupole analysers is a higher duty cycle, up to 95 – 99% compared to 1 – 10% with a quadrupole in a typical operating mode. In addition, the IT-MS is ideally suited to MS/MS experiments, whereby collision-induced dissociation is performed on ions of a single mass after the ejection of all others from the trap [2]. This tandem mass spectrometry can be exploited in several ways, *e.g.* to help to distinguish isobaric compounds. The detection limit of PTR-IT-MS systems are not yet as good as that of quadrupole instruments, but sub-ppbv levels have been achieved [32, 33]. Since the first publication of the Leicester time-of-flight instrument, a few other PTR-TOF-MS systems have been reported [31]. 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 [24]. Tanimoto *et al.* designed a linear-TOF system that had limited mass

resolution, but achieved sub-ppbv detection limits for 1 minute integration times [17]. A limitation of TOF-systems is the duty cycle, which is typically less than 3% [16, 18].

### 2.2.2 Proton transfer reaction

Exothermic proton transfer reactions between $H_3O^+$ and 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).

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

M represents the neutral analyte compound, $MH^+$ its protonated product ion and $k$ is the proton transfer reaction rate coefficient. The reaction rate coefficient indicates the intrinsic speed of the reaction, and exothermic proton transfer will proceed at or near the collision rate, *i.e.* proton transfer occurs on every collision [12].

The measurement of ion signals by the mass spectrometer in PTR-MS provides a means for determining the absolute concentration of a specific constituent of a gas mixture. With $H_3O^+$ as the proton source and assuming reaction with only a single organic gas, designated M, the proton transfer reaction is shown in equation 2.1. This reaction is a second-order elementary reaction and satisfy the rate equation.

$$-\frac{d[H_3O^+]}{dt} = k\,[H_3O^+][M] \qquad (2.2)$$

Assuming that $[M] >> [H_3O^+]$, which is reasonable since M is a neutral gas (even if present at trace levels), then [M] is constant (the reaction is pseudo first order) and equation 2.2 can be integrated to yield

$$[H_3O^+]_t = [H_3O^+]_0\, e^{-k[M]t} \qquad (2.3)$$

The reaction time *t* is the time it takes for the reagent ion, in this case $H_3O^+$, to travel from the point where it is first mixed with the analyte to the end of the drift tube (beyond which reaction essentially stops). The concentration of $H_3O^+$ can be related to that of $MH^+$ by

$$[MH^+]_t = [H_3O^+]_0 - [H_3O^+]_t \quad (2.4)$$

Substituting equation 2.3 into equation 2.4 gives:

$$[MH^+]_t = [H_3O^+]_0 [1- e^{-k[M]t}] \quad (2.5)$$

The final assumptions yield a particularly simple expression. First, we assume that conditions are chosen such that only a small proportion of $H_3O^+$ is consumed by the reaction, that is, $[H_3O^+]0 \approx [H_3O^+]t$, or equivalently $[MH^+]t << [H_3O^+]_0$. If $k[M]t << 1$, which is usually true providing M is present at well below the parts per million level, then a Taylor expansion of the exponential term in equation 2.5 and retaining only the first term in the Taylor expansion of $e^{-k[M]t}$ leads to the following:

$$[MH^+]_t / [H_3O^+]_0 = i(MH^+) / i(H_3O^+) = k[M]t \quad (2.6)$$

In equation 2.6, $i(MH^+)$ 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 drift tube. 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 compound concentrations determined in this way can be limited for several reasons. These reasons includes the uncertainties associated with the rate coefficient, uncertainty in the reaction time, variation in ion transmission through the mass spectrometer, differing mobility of reagent and analyte ions, and the fact that equation (2.6) does not account for additional processes such as fragmentation [3, 25]. Equation (2.6) modified to take some of these factors into account, but for determining reliable analyte concentrations, calibration with a specific gas standard is preferred.

### 2.2.3 Cluster ion chemistry

As a result of unreacted neutral water vapour molecules in the drift tube, $H_3O^+$ ions can react to form $H_3O^+(H_2O)n$ cluster ions via the process:

$$H_3O^+(H_2O)_{n-1} + H_2O + M \rightarrow H_3O^+(H_2O)_n + M \qquad (2.7)$$

Where, M is a third body. Water vapour in the analyte gas can also enhance the formation of $H_3O^+(H_2O)_n$. In PTR-MS, attempts are made to minimize the proportion of $H_3O^+(H_2O)_n$ ions ($n \geq 1$) relative to $H_3O^+$ in the drift tube (for reasons explained below) through the use of collision-induced dissociation. Nevertheless, despite these efforts, hydrated hydronium ions still frequently observed in mass spectra from the PTR-MS and so it is important to beware of the impact they may have on the ion chemistry. The extent of water cluster ion formation increases as the humidity of the sample increases.

The water cluster ion can complicate interpretation of mass spectra. Hydrated hydronium cluster ions also possess higher proton affinity than the bare water molecule ($691 \pm 3$ kJ $mol^{-1}$). For example, the water dimer, $(H_2O)_2$, has a proton affinity of $808 \pm 6$ kJ $mol^{-1}$ [26]. The higher proton affinity is the result of the added stability of the $H_3O^+$ brought about by sharing the positive charge with additional water molecule. As more water molecule added, the proton affinity increases, but the incremental effect decline in magnitude as the cluster grows.

There are two important consequences of water cluster ion formation. First, the increased proton affinity means that some reactions that occur with $H_3O^+$ do not occur with $H_3O^+(H_2O)_n$. An example is acetaldehyde, whose proton affinity lies between those of $H_2O$ and $(H_2O)_2$. Secondly, with hydrated hydronium ions proton transfer is not the only possible reaction channel. In the case of acetaldehyde, it is known that reaction with $H_3O^+$ does indeed proceed by proton transfer and occurs at the collision limiting rate. However, as shown by flowing afterglow and SIFT studies [27], reaction of $H_3O^+(H_2O)$ with acetaldehyde also occurs at the collision-limiting rate, but in this case proceeds via so-called ligand switching:

$H_3O^+ (H_2O) + CH_3CHO \dashrightarrow H_3O + (CH_3CHO) + H_2O$ (2.8)

If the ligand-switching reaction proceeds at the collision limited rate, the presence of $H_3O^+(H_2O)_n$ ions in PTR-MS is not necessarily a problem, since product ions containing the analyte molecule will still be formed. However, the presence of additional product channels of hydrated hydronium ions increases the complexity of data analysis and is best avoided if possible [3].

### 2.2.4 Time-of-Flight Mass Spectrometry

Following its generation within the drift cell, the ion beam is passed into the Kore MS-200 ToF-MS for mass analysis. In this section the basic principles and components of ToF-MS is discussed, as this is the type of mass analyser used in all of the work presented in this thesis. Many substantial reviews on the ToF-MS technique and its underlying theoretical principals have been conducted [28], and so only, a basic overview is provided here.

Stephenson [29] first introduced ToF mass analysers in 1946 for use in experiments in nuclear physics. Wiley and McLaren [30] have developed a two-stage acceleration zone to improve the resolution. The main principle at the heart of the ToF-MS technique is the separation of ions in time rather than in space, where the latter is the norm for most other mass spectrometers.

For example, consider a distribution of ions covering a range of masses. If each ion within this pool is given an equal amount of kinetic energy by accelerating the ion 'packet' over a finite distance, the ions will travel through a field free region with a terminal velocity proportional to their mass-to-charge ratio (*m/z*). This is demonstrated in Figure 2.1, where ions A and B have equal charges and masses *mA* and *mB*, respectively (*mB* > *mA*), are accelerated over a distance $P_L$; Ion A will acquire a greater velocity because of its lower mass relative to ion B and hence the former will have the shorter time-of-flight to detector. If the distance that the ions travel in the field free region, $F_L$, is fixed and well known, each ion of specific mass-to-charge ratio will have

its own characteristic time-of-flight (ToF). Consequently, the measurement of an ion's ToF can be employed to determine its *m/z*.

A ToF-MS instrument is composed of four distinct regions which are (i) an ion extraction/acceleration region (or so called pulser), (ii) x and y steering plates, (iii) a field free flight tube and (iv) a detector. All components of the ToF-MS reside under high vacuum (< $10^{-6}$ mbar), as collisions with gas molecules will cause scattering of the ion beam and perturbation of the ion flight. The arrangement of some of these components in a ToF-MS system is shown diagrammatically in Figure 2.1.

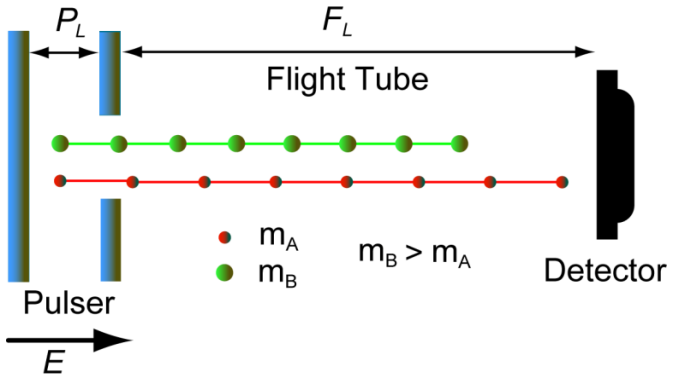



Figure 2.1 Diagram to show the basic components and principles of a linear ToF-MS system (adapted from Wyche (2008) [8].

In continuous beam systems, once the ion beam exits the ion source it often undergoes some form of focusing on a transfer region within the ToF-MS before being passed to the pulsed ion extraction region. In its most simple form, the pulse extraction region, or the pulser, consists of two electrodes separated by a distance, $P_L$, over which a pulsed potential difference is applied.

Independent of the ionisation method, the electric charge $q$ of an ion of mass $m_i$ is equal to an integer number $z$ of electron charges $e$, and thus $q = ez$. The energy uptake $E_{el}$ by moving through a voltage $U$ is given

$E_{el} = qU = ezU$ (2.9)

Thereby, the former potential energy of a charged particle in an electric field is converted into kinetic energy $E_{kin}$, i.e. into translational motion

$E_{el} = ezU = 12\ m_i\ v^2 = E_{kin}$ (2.10)

Assuming the ion was at rest before, which is correct in a first approximation, the velocity ($v$) attained is obtained by rearranging equation 2.10 into

$v = \sqrt{2ezU\ /\ m_i}$ (2.11)

*i.e. v* is inversely proportional to the square root of mass.

Having acquired their terminal velocity, the ions enter a field free flight tube, in which they are isolated from any external forces. The ions will travel the length of the flight tube distance ($s$) after having been accelerated by a voltage $U$. The relationship between velocity and drift time $t_d$ needed to travel the distance $s$ is

$t_d = s\ /\ v$ (2.12)

Which upon substitution of $v$ with equation 2.11 becomes

$t_d = s\ /\ \sqrt{2ezU\ /\ m_i}$ (2.13)

Equation 2.13 delivers the time needed for the ion to travel the distance s at contact velocity, *i.e.* in a field-free environment after the process of acceleration is completed. It is also obvious from equation 2.13 that the time to drift tube through a fixed length of field-free space is proportional to the square root of $m_i/z$.

$t_d = s\ /\ \sqrt{2eU}\ \sqrt{m_I\ /\ z}$ (2.14)

Thus, the time interval $\Delta t$ between the arrival times of ions of different $m_i/z$ is proportional to $s\ x\ (m_i/z_1^{1/2} - m_i/z_2^{1/2})$.

If there were no other factors to consider, equation 2.10 would give the final flight time of the ions. Other factor that might also require consideration is the time taken for the ions to undergo acceleration.

The ion acceleration time equated by,

$F=Eq$ (2.15)

Where, *E* is electric field and *q* is charge

$F=m_i a$ (2.16)

Where, $m_i$ is mass and *a* is acceleration

$a=Eq/m$ (2.17)

The velocity and time require it reach it is calculated by,

$a=dv/dt$ (2.18)

After substituting equation 2.17 and 2.18, gives

$v = (\int Eq \,/\, m)\; t$ (2.19)

When initial velocity ($V_0$) is added to equation 2.19,

$v = v_0 + (Eq \,/\, m) t_a$ (2.20)

Re-arranging the equation 2.20, will give the time that ion is accelerating from initial velocity ($V_0$) to drift velocity ($V$)

$t_a = (v - v_0 \,/\, E)\; (m \,/\, q)$ (2.21)

The drift time $t_d$ as calculated by means of equation 2.13 is not fully identical to the total time-of-flight. The time needed for acceleration of ions *ta* calculated by means of equation 2.21 has to be added. Furthermore, a short period of time $t_0$ in which ion begins to accelerate is typically in the order of few nanoseconds also has to be added. Thus, total time-of-flight ($t_{total}$) is given by [28],

$t_{total} = t_0 + t_a + t_d$ (2.22)

From the basic principles of ToF-MS described above, it is clear that only one 'packet' of ions may be extracted from the continuous source beam for analysis at any one instant. This means that whilst each ion packet is 'in flight' the continuous ion beam

not under analysis is simply unused. That fraction of ions which are extracted for analysis is known as the duty cycle of the instrument ($D_C$). The duty cycle of a ToF-MS is defined by three parameters: (i) the size of the extraction aperture of the extraction electrode ($X_1$), (ii) the speed of ions within the continuous ion beam ($u_c$) and (iii) the repetition frequency of the scan ($f$) [19]:

$$D_c = X_1 f \,/\, u_c \qquad (2.23)$$

In typical modern ToF mass spectrometers, the duty cycle rarely exceeds ~ 2 %, meaning 98 % of the generated ion beam is simply lost. Consequently, the low instrument duty cycle of ToF-MS results in its major drawback, i.e. limited sensitivity.

### 2.2.5 The Leicester PTR-ToF-MS

Full details of the development and characterisation of the PRT-ToF-MS instrument can be found in the PhD theses of R. S. Blake [7] and K. P. Wyche [8], and so here, details are kept to a minimum. The PTR-TOF-MS instrument (shown in Figures 2.2 and 2.3) consists of a radioactive ion source connected to a drift tube (built in-house) and a commercial orthogonal acceleration reflectron time-of-flight mass spectrometer (Kore Technology Limited, Ely, UK).

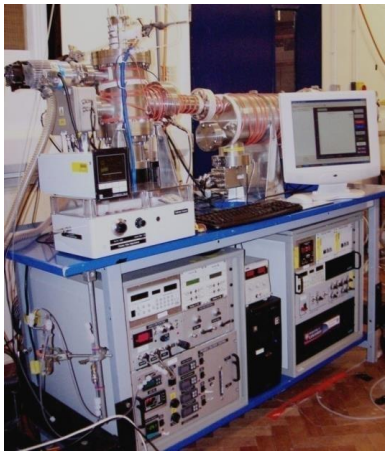


Figure 2.2: The Leicester PTR-TOF-MS instrument

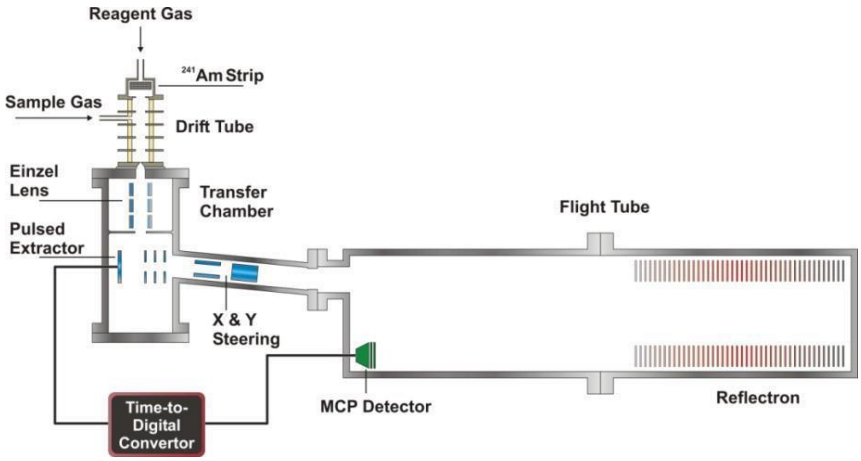



Figure 2.3: The schematic diagram of PTR-TOF-MS instrument

#### 2.2.5.1 Gas inlet system

PTR-TOF-MS uses $H_3O^+$ reagent ions for ionisation of analyte gases. 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 line between the MFCs and the ion source/drift tube is maintained at 40°C with heating wire. This prevents condensation in the lines when analysing a humid sample. Water vapour is introduced by bubbling high-purity nitrogen gas (grade N6.0, BOC Special Gases) in deionised water contained in a glass vessel containing high-purity deionised water (15 MΩ). The nitrogen flow through the vessel is regulated by a needle valve.

### 2.2.5.2 Ion source and drift tube

The radioactive ion source and drift tube (Figure 2.3) are based on a design by Hanson *et al.* [16]. The ion source consists of a radioactive strip of $^{241}Am$ (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 standard cubic centimetres per minute (sccm). Water molecules are ionised by the α-particles to form $H_3O^+$ ions, which are assumed to proceed via the mechanism shown in the following equations [32].

$$H_2O + \alpha \longrightarrow H_2O^+ \quad (2.22)$$

$$H_2O^{\bullet+} + H_2O \longrightarrow H_3O^+ + HO \quad (2.23)$$

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 thickness) separated by 2 cm thick insulating Semitron spacers. Viton O-rings sit in a groove in the upper and lower surface of the spacers to enable a vacuum-tight seal to be made. 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. Figure 2.4 shows the schematic diagram of the drift tube installed in the Leicester PTR-ToF-MS instrument (Diagram was adapted from Wyche [8]).

Commented [CRL(3]: Refer to in text.

The analyte gas 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 $m^3\ h^{-1}$, 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 kept constant using a heating wire, which is coiled around the outside and maintains a temperature of 40°C.

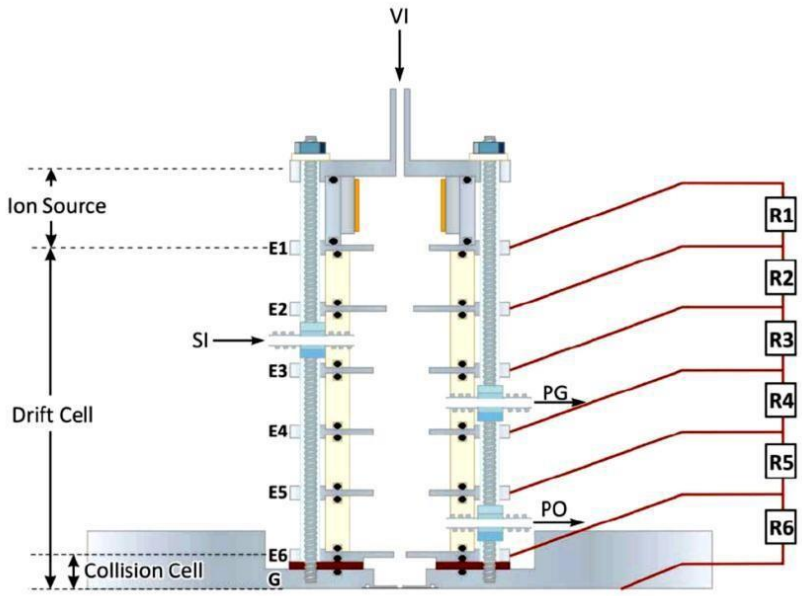



Figure 2.4 A schematic diagram of the drift tube. E1 – E6 represents electrodes 1 – 6 and R1 – R6 represents resistors 1 – 6, of which R1 – R5 are fixed a 1 MΩ and R6 is variable between 0 – 1 MΩ. S1, V1, PG and PO refer to the sample and vapour inlets, pressure gauge and pump outlet respectively. The 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.

The electrodes in the ion source/drift tube arrangement are connected to a resistor chain consisting of five 1 MΩ resistors and one variable resistor (maximum 1 MΩ, usually set to 860600 Ω) over the CDC. The top electrode 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 dissociate cluster ions prior to detection. Using the operating conditions described, the number density within the drift tube has been calculated to be $1.388 \times 10^{17}$ $cm^{-3}$. The above values of drift tube voltage and variable resistor setting provides the desired *E/N* value of 80/170 Td, where 1 Td = 1

Townsend = $10^{-7}$ $cm^2$ $V^{-1}$ (where the first value refers to the main drift cell and the second value refers to the CDC).

The sample ions exit the drift tube via a 200 $\mu$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 [21]. The 200 $\mu$m aperture provided a compromise between the ion transmission and the drift tube pressure.

#### 2.2.5.3. Mass analyser and detection

After passing through the aperture, ions enter the transfer chamber containing an Einzel lens for focussing the ion beam. Two turbo pumps (70 $Ls^{-1}$, Varian V70) evacuated this chamber. A rotary pump backed each turbo pumps. The ion beam than passes into a secondary chamber containing the pulsed extraction system for injecting small packets of ions into the orthogonally positioned reflectron ToF-MS. This chamber is evacuated by a 255 $Ls^{-1}$ large turbo pump (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 under typical operating conditions is close to $1.0 \times 10^{-6}$ mbar as measured by a cold cathode pressure gauge (MKS Series 943).

Inside the flight tube of the ToF-MS, 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.

#### 2.2.5.4 Data collection, processing and normalisation

The mass spectral 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 2.24, displaying the raw spectra as a plot of recorded signal in counts against *m/z*.

$$m/z = (t - t_0 / C_b) \quad (2.24)$$

In the above 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 mass spectrum is about 80 µs for a mass range up to 200 amu, so around 104 scans $s^{-1}$ can be achieved. Many scans are necessary to obtain meaningful data since the ions are detected using a pulse counting system. Each successive scan was added to the previous one to build up a satisfactory signal-to-noise (*S/N*) ratio. Most of the results employed in this thesis involve continuous data collection of one minute, with the exception of some of the whiskey and urine experiments where data were acquired for several minutes. Experimental parameters such as mass range, experiment length (integration time) and the number of experiments are set in the collect options of GRAMS/AI.

For the data to be processed and analysed outside of the GRAMS/AI software, two separate programs are used, MaxiSum and MaxiGroup. 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 (± 0.3 amu). 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 the larger integration times. Using the convention described by Warneke *et al.*, data are normalised to 106 reagent ion counts per second, whereby 'reagent ion' are classed as both $H_3O^+$ and $H_3O^+(H_2O)$, using the following equation.

$$i(MH+)_{ncps} = . i(MH^+)_{cps} \; X \; (10^6 / (i(H_3O^+)_{cps} + i(H_3O^+(H_2O)_{cps}) \quad (2.25)$$

$i(MH+)_{ncps}$ is normalised analyte signal. $i(MH^+)_{cps}$, $i(H_3O^+)_{cps}$ and $i(H_3O^+(H_2O)_{cps}$ are the raw signals of protonated analyte, $H_3O^+$ ion and $H_3O^+(H_2O)$ ion respectively. The normalisation process tries to account for any changes in the hydronium ion signal [31].

#### 2.2.5.5 PTR-ToF-MS mass spectrum and mass resolution

Figure 2.5 shows an example of a raw PTR-MS mass spectrum, displaying the signal intensity against *m/z*, in comparison to that obtained after data processing, *i.e.* after peak integration. The dominant peak at *m/z* 19 represents the $H_3O^+$ reagent ion signal and the secondary peak is derived from $H_3O^+(H_2O)$. As PTR-ToF-MS instruments produce only singly charged ions, the *m/z* scale treated as a mass scale. Raw integrated $H_3O^+$ count rates of $2 – 4 \times 10^3$ counts $s^{-1}$ (cps) are typically acquired while scanning at 12 – 200 amu mass range.

In mass spectrometry, the term mass resolution defines the ability of the instrument to distinguish between two ions of similar mass. Because of instrument limitations, ultimately there will always be some degree of 'spread' in the energy distribution of ions within a single ion packet. Owing to this energy spread, a peak in the TOF mass spectrum will not comprise a single discrete signal; instead, it will be composed of a range of signals with a roughly Gaussian distribution.

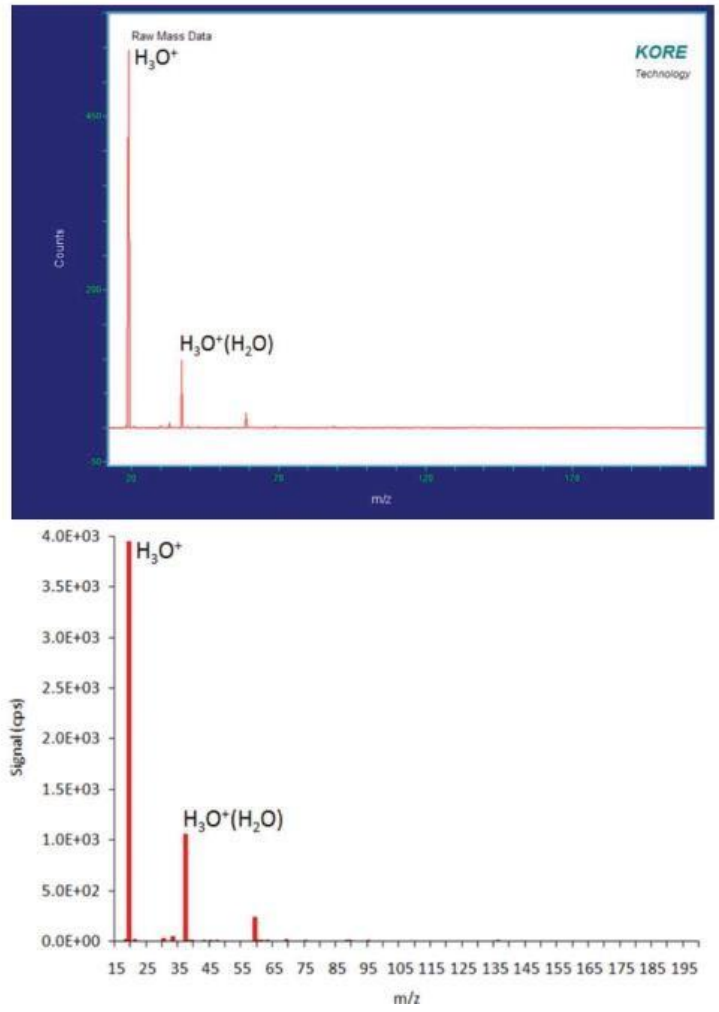



Figure 2.5 Comparison of raw (top) and processed (bottom) PTR-TOF-MS mass spectrum [9].

For a single peak with no spectral interference, the resolution, *R*, of a mass spectrometer can be quantified by taking the ratio of the peak mass (*m*) to the peak width (*Δm*) at half the height of the peak, *i.e.* the full width half maximum (*FWHM*) mass:

$R = m / \Delta m$ (2.26)

The mass resolution ($m/\Delta m$) of the PTR-ToF-MS instrument under standard operating conditions was determined to be in excess of 1000 over the majority of the mass range [30]. This is an order of magnitude greater than that reported for quadrupole PTR-MS instruments [16] and a linear-PTR-ToF system [17], both of which quoted ~ 100. The sensitivities of this first generation instrument was well below those of existing quadrupole-based PTR-MS instruments. A major limitation here is the duty cycle of the ToF-MS, which was estimated to be as low as 1–2%. On the positive side, the mass resolving power was found to be better than 1000, although this is still not sufficient to resolve most compounds with the same nominal mass.

PTR-MS has several advantages for analysing trace volatile compounds in air. First, proton transfer does not deliver a large excess of energy and therefore it is a fairly soft ionization process [6]; can directly analyse gas mixtures without sample pre-concentration [8]; the instrument has high sensitivity; and measurements can be taken in-situ and analysed in real-time [30].

### 2.2.6 The Medi-PTR-ToF-MS

The PTR-MS [4] instrument consists of a hollow cathode discharge ion source, a drift tube and ion transfer lens assembly, and a reflectron ToF-MS. $H_3O^+$ ions are generated via a dc electrical discharge through water vapor. After passing through a source drift region, which maximizes the production of $H_3O^+$, the ions then pass through a 2 mm aperture into the drift tube, where they encounter the analyte. The drift tube is pumped by a mechanical pump with an effective speed of $\sim$3 L $s^{-1}$, and a pressure of $\sim$1 mbar is maintained in this region under normal operating conditions.

In the standard PTR-MS version of this instrument the drift tube consists of a series of equally spaced disk electrodes with a central aperture of 40 mm diameter. This arrangement delivers a uniform electric field along the drift tube. In the current study the electrode structure is modified to allow part of the drift tube to act as an ion funnel. At the end of the drift tube is a 400 μm orifice, which allows ions to enter the ion transfer

zone for the TOF-MS. This ion transfer assembly consists of collection/collimating optics and feeds ions into the source region of a reflectron ToF-MS.

The reflectron, which is pumped by a 70 L $s^{-1}$ turbomolecular pump, has a total effective ion flight path of ~1.2 m. Ions are detected at the end of their trajectory by a large entrance (8 mm × 32 mm) discrete dynode detector. The ion signal is then amplified and fed into a proprietary pulse counting system for data acquisition, which consists of a time-to-digital converter interfaced to a PC. Data display and processing was carried out using the GRAMS software package.

The ion funnel consists of 29 stainless steel plates of 0.2 mm thickness, mounted on precision-machined ceramic rods at an even spacing of 3.2 mm per plate. The orifice diameters of the plates through the first half of the stack is 40 mm, as used in the standard drift tube reactor. In the second half of the drift tube the orifice diameter steadily decreases to 6 mm at the final plate before the exit orifice. A resistive divider consisting of a ceramic substrate patterned with thin-film resistors is used to deliver the static voltages to individual plates, with vacuum feed throughs providing connections to the ends of the divider. The dc voltage across the reactor may be varied between 30 and 450 V. When operating in rf mode, the lower end of this scale gave the best results in terms of detection sensitivity. Capacitor chains are used to feed a balanced rf input voltage to the plates in the downstream half of the drift tube.

The rf voltage is provided by a simple proprietary ac generator whose output transformer is arranged to be in resonance with the load capacitance. This resonance is used to achieve a roughly sinusoidal waveform at around 800 kHz, with adjustable amplitude up to about 200 V peak to peak.

The ion funnel delivered a large increase in the $H_3O^+$ ion count rate detected at m/z = 19. For the optimized ion funnel a reading of $6 \times 10^6$ Hz was observed, whereas in the optimized dc-only mode using exactly the same electrode configuration an $H_3O^+$ count rate of $5 \times 10^5$ Hz was observed. The far higher ion count rate in the rf mode is exactly what we are seeking, but it also has the disadvantage of contributing to rapid degradation of the ion detector if allowed to persist, as well as overloading the time-

to-digital converter. The TOF-MS detector was therefore equipped with a purpose built gating system to prevent ions with m/z = 19 from generating a cascade of electrons and hence wear in the detector. Instead, the $H_3O^+$ count rate was deduced by monitoring the $H_3{}^{18}O^+$ signal at m/z = 21 and using the known $^{16}O/^{18}O$ natural abundance ratio (499:1) to determine the count rate of $H_3O^+$. The detector gating was achieved by applying a negative going voltage pulse at dynode number two of the detector, rejecting electrons generated by the ion impact and thus eliminating wear from all dynodes except the first, the conversion dynode.

Full details of the development and characterisation of the Medi-PTR-ToF-MS instrument can be found in the thesis of S.B.Barber [14].

### 2.2.7 Calibration and instrument characterization

#### 2.2.7.1 The Leicester PTR-ToF-MS

Throughout this thesis the PTR-ToF-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 (± 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, allowing for 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

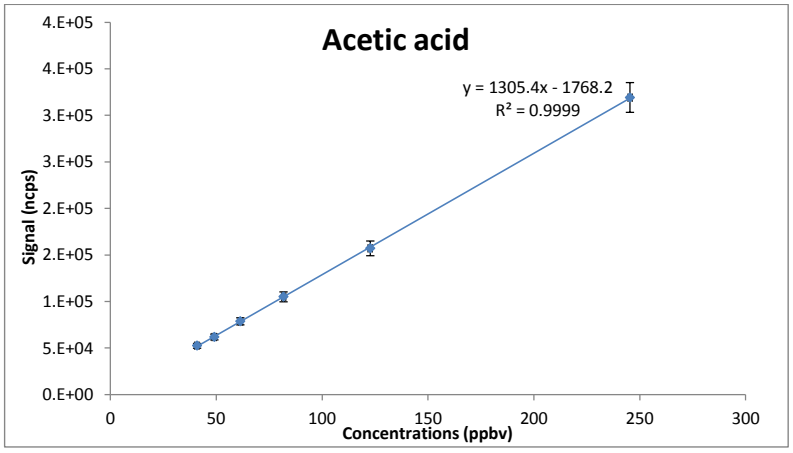
flow required over the two ovens, although the minimum experimental dilution flow also needed to be greater than the sample inlet flow to the PTR-ToF-MS instrument.

Measuring VOCs in real-time on-line sampling requires high instrument sensitivity and low detection limits. Calibration experiments were performed to assess the PTR-TOF-MS sensitivity and detection limits. Calibrations were carried out using permeation tubes (KIN-TEK, supplied through Eco-Scientific, Stroud, UK) as listed in Table 2.1 and a laboratory gas standards generator, and standard gas mixture from a standard gas cylinder, at 0% relative humidity (RH). 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). The standard gas mixture contained a mixture of methanol, acetaldehyde, trans-2-butene, acetone, methacrolein, cyclohexanone and β–pinene, all gases concentration at approximately 1 ppmv. The measurements were made for 1-minute integration times and *E/N* of 90/190 Td (Instrument operating setting for bacterial analysis – Chapter 3).

Table 2.1: Permeation tube details.

| Permeation Tube | Tube Type | Emission Rate (ng min-1) | Certification Temperature (°C) |
|---|---|---|---|
| Acetic Acid | High Emission | 329 | 30 |
| Formic Acid | High Emission | 463 | 40 |
| Isoprene | Standard | 685 | 40 |
| Formaldehyde | High Emission | 116 | 60 |

The calibration curve for acetic acid, formic acid, isoprene and formaldehyde are presented in Figure 2.6 and 2.7. As standard practise, all calibration data were background subtracted.



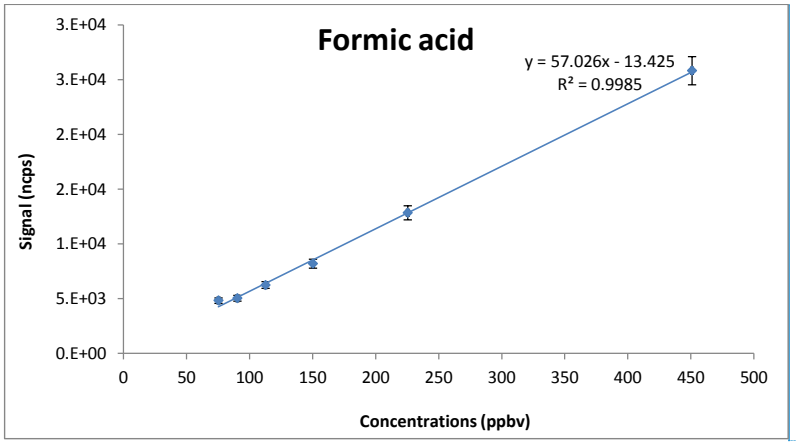




Figure 2.6: Calibration plots signal vs. concentration for protonated acetic acid and formic acid at 1-minute integration times and *E/N* of 90/190 Td (0% RH).

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**Commented [RC5]:** You really need to have explain what this way of expressing E/N means.

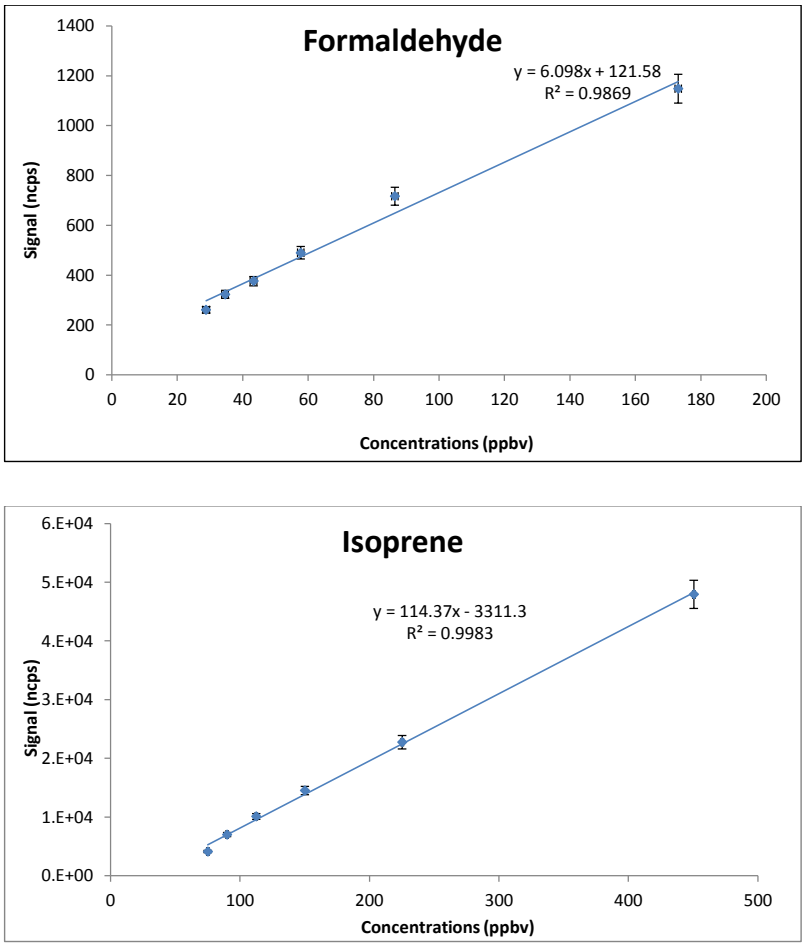





Figure 2.7: Calibration plots signal vs. concentration for protonated formaldehyde and isoprene at 1-minute integration times and *E/N* of 90/190 Td (0% RH).

For the *E/N* of 90/190 Td at 0% RH setup, linear response observed for all the compounds analysed in the experiment. The correlation coefficient, denoted by *r*, is a measure of the strength and direction of a linear response between two variables. The value of *r* can range between +1 and -1, with a *r* value of 0 indicating no linear relationship while 1 indicates a perfect linear relationship. The values between 0 and

0.3 indicate a weak linear relationship, 0.3 to 0.7 indicate a moderate linear relationship and 0.7 to 1.0 indicates a strong linear relationship. The *r* values observed from the calibration curves presented in Figure 2.6 and 2.7 for acetic acid, formic acid, formaldehyde and isoprene (permeation tubes) were within 0.98 to 0.99. These values indicate that a strong linear relationship was observed.

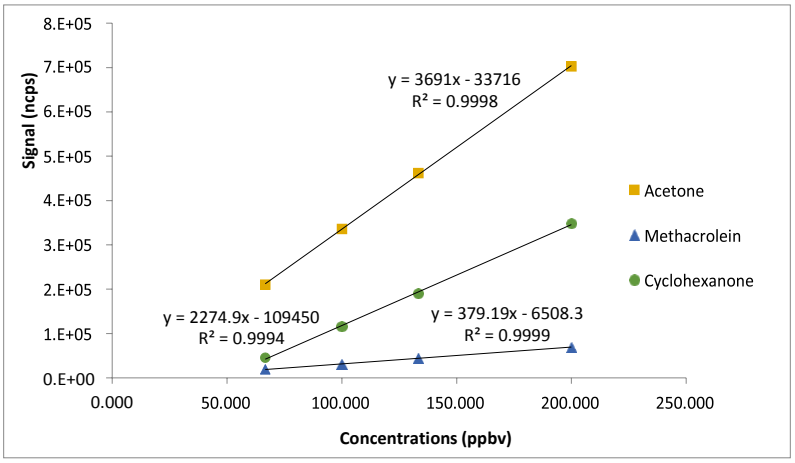


Figure 2.8 Calibration plots signal vs. concentration for protonated acetone, methacrolein, and cyclohexanone at 1-minute integration times and *E/N* of 90/190 Td (0% RH).

The *r* values observed from the calibration curves presented in Figure 2.8 and 2.9 were 0.99 for all the compounds calibrated using the standard gas mixture that were acetone, methacrolein, cyclohexanone, methanol, acetaldehyde, trans-2-butene and β-pinene. As discussed earlier in this section, the *r* value ranging from 0.7 to 1.0 indicates a strong linear relationship. Therefore, the observed *r* values indicate that a strong linear relationship was observed. The calibration curves demonstrated good linear response over the concentration range tested, achieving $R^2 > 0.99$ for most of the calibration curves except for formaldehyde.

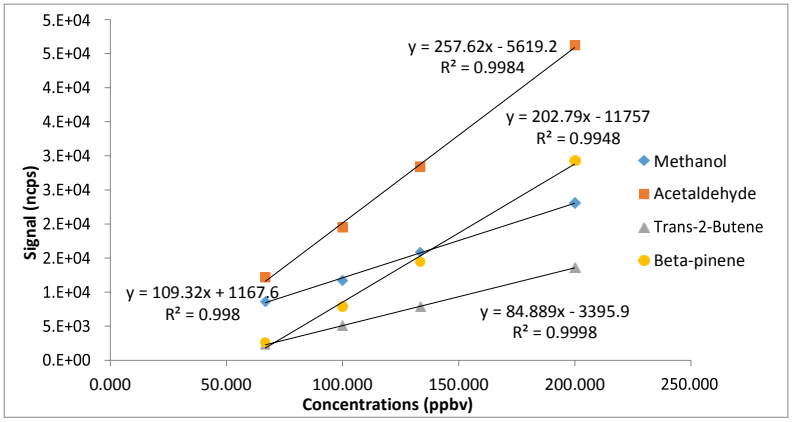


Figure 2.9 Calibration plots signal vs. concentration for protonated methanol, acetaldehyde, trans-2-butene and β-pinene at 1-minute integration times and *E/N* of 90/190 Td (0% RH).

The Limit of Detection (LoD) for the different compound was also calculated. The LoD is the lowest amount of the investigated compounds in a sample that can be detected but not necessarily quantified. The limit of detection can be calculate by [17],

1). Signal-to-noise ratio method: Signal-to-noise ratio method is usually used for the calculation of LoD in separation and spectrometric method. The minimum signal/noise ratio of ≥ 3 is used in all the different experiments documented in this thesis.

2). Based on the calculation using the standard deviation of the response and the slope method: By using this method, the LoD can be calculated by using the following equation,

$$C_{LoD} = 3\,s\,/\,m \qquad (2.26)$$

Where *s* is the standard deviation and *m* is the slope of related calibration line.

Table 2.2: A summary of the PTR-ToF-MS sensitivities and limits of detection at 1-minute integration times and *E/N* of 90/190 Td (0% RH).

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| Compound | Concentration Range (ppbv) | Sensitivities, *S* (ncps ppbv$^{-1}$) | Limit of detection, LOD (ppbv) |
|---|---|---|---|
| Acetic Acid | 41 – 245 | 1299.6 | 0.4 |
| Formic Acid | 75 – 451 | 54.0 | 3 |
| Isoprene | 75 - 451 | 6.5 | 34 |
| Formaldehyde | 29 - 173 | 110.0$^{\infty}$ | 3 |
| Methanol | 64 – 194 | 94.1 | 6 |
| Acetaldehyde | 66 – 198 | 219.4 | 4 |
| Trans-2-butene | 68 – 206 | 66.7 | 7 |
| Acetone | 68 – 204 | 3522.9 | 0.7 |
| Methacrolein | 65 – 196 | 349.2 | 0.5 |
| Cyclohexanone | 86 – 258 | 1778.8 | 0.4 |
| β-pinene | 70 - 210 | 149.0 | 2 |

$^{\infty}$ $R^2$ = 0.98

Table 2.2 summarises the sensitivities and limits of detection of compounds used in the calibration. Overall, the PTR-ToF-MS instrument measured sensitivities ranging from 6.5 – 3522.9 ncps ppbv$^{-1}$ and low detection limit ranging from 0.4 – 34 ppbv for all compounds analysed. The detection limits for the majority of the compounds were found to be less than 10 ppbv. As shown in Table 2.2, the instrument showed poor sensitivity, and therefore limit of detection, for isoprene when compared with other standards in the same table. The PTR-ToF-MS showed good performance with respect to other compounds, for example, acetone showed one minute detection limits of 0.7 ppbv with the highest sensitivity.

The calibration measurement was repeated for the exhaled breath analysis instrumental settings (Chapter 5) at *E/N* of 80/170 Td. Calibrations were carried out using permeation tubes (Table 2.1) and standard gas mixture from a standard gas cylinder, at 0% relative humidity (RH). The measurements were made for 1-minute integration times.

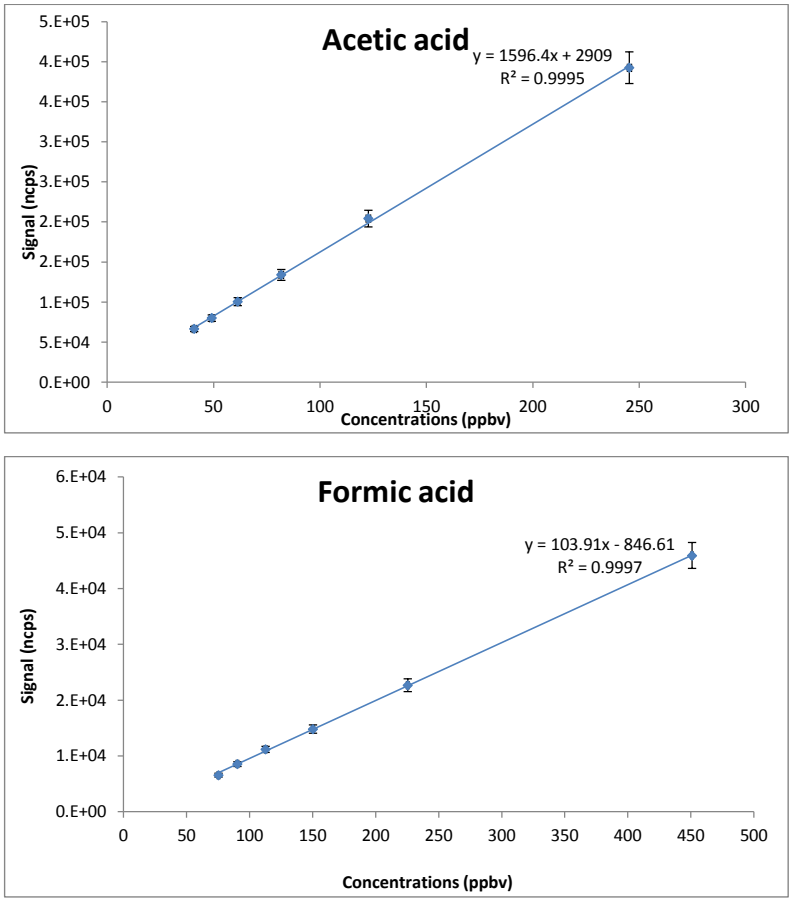


Figure 2.10 Calibration plots signal vs. concentration for protonated acetic acid and formic acid at 1-minute integration times and *E/N* of 80/170 Td (0% RH).

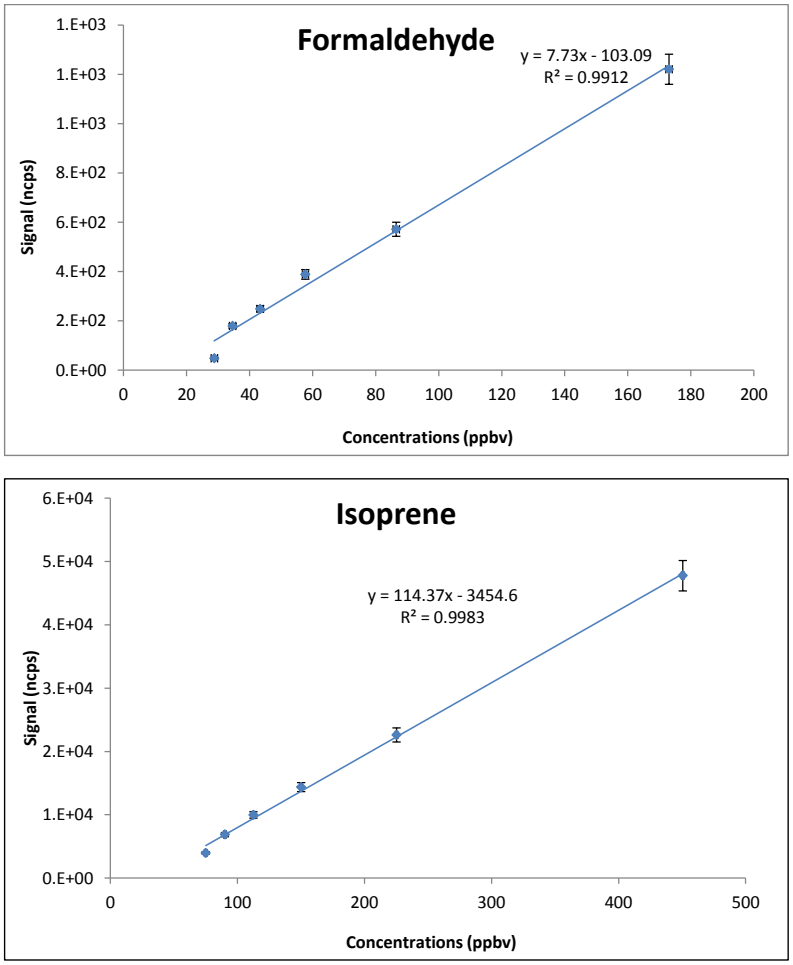





Figure 2.11 Calibration plots signal vs. concentration for protonated formaldehyde and isoprene at 1-minute integration times and *E/N* of 80/170 Td (0% RH).

The calibration curve for acetic acid, formic acid, isoprene and formaldehyde are presented in Figure 2.10 and 2.11. As standard practise, all calibration data were background subtracted.

For the *E/N* of 80/170 Td at 0% RH setup, linear response observed for all the compounds analysed in the experiment. As discussed earlier, when the value of correlation coefficient, *r*, is 0.7 to 1.0, it indicates a strong linear relationship. The *r* values observed from the calibration curves presented in Figure 2.10 and 2.11 for acetic acid, formic acid, formaldehyde and isoprene (permeation tubes) were more than 0.99. These values indicate that a strong linear relationship was observed.

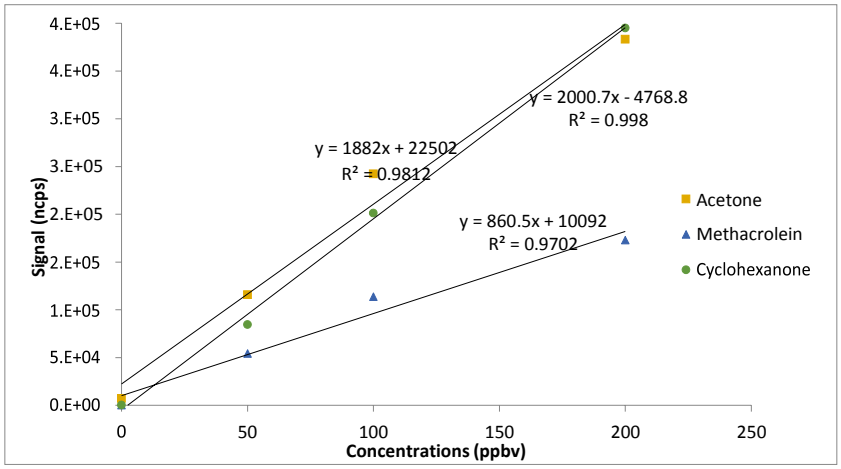


Figure 2.12 Calibration plots signal vs. concentration for protonated acetone, methacrolein, and cyclohexanone at 1-minute integration times and *E/N* of 80/170 Td (0% RH).

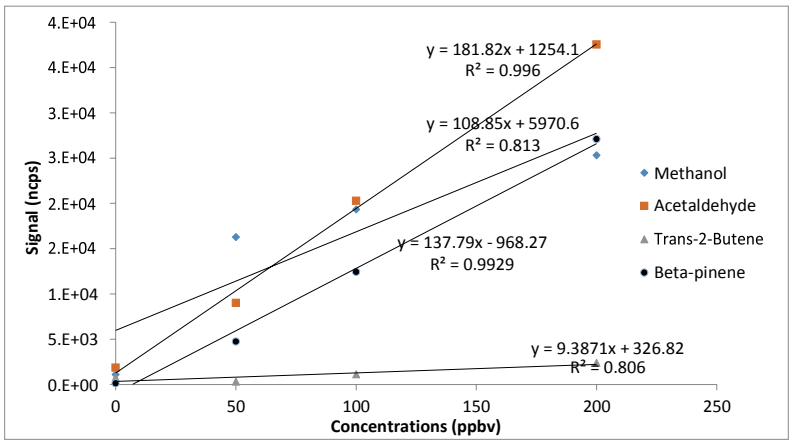


Figure 2.13 Calibration plots signal vs. concentration for protonated methanol, acetaldehyde, trans-2-butene and β-pinene at 1-minute integration times and *E/N* of 80/170 Td (0% RH).

The *r* values observed from the calibration curves presented in Figure 2.12 and 2.13 were 0.99 for cyclohexnone, acetaldehyde and methanol, 0.98 for acetone, 0.97 for methacrolein and 0.80 – 0.81 for trans-2-butene and β-pinene. As discussed earlier in this section, the *r* value ranging from 0.7 to 1.0 indicates a strong linear relationship. Therefore, the observed *r* values indicate that a strong linear relationship was observed although trans-2-butene and β-pinene correlation coefficient value were much lower than the other compounds. This may be caused by the different *E/N* settings applied compared to the bacterial analysis setting as discussed above. However, these two compounds has never been reported as biomarkers or reported detected in exhaled breath, as these two compounds were generally reported in atmospheric analysis.

Table 2.3: A summary of the PTR-TOF-MS sensitivities and limits of detection. The calibration was performed over the concentration ranges shown below at 70% humidity.

| Compound | Concentration Range (ppbv) | Sensitivities, *S* (ncps $ppbv^{-1}$) | Limit of detection, LOD (ppbv) |
|---|---|---|---|
| Acetic Acid | 41 – 245 | 1602.4 | 0.4 |
| Formic Acid | 75 – 451 | 102.7 | 4 |
| Isoprene | 75 - 451 | 8.1 | 7 |
| Formaldehyde | 29 – 173 | 110.1 | 2 |
| Methanol | 48 – 194 | 108.9 | 2 |
| Acetaldehyde | 49 – 198 | 181.8 | 2 |
| Trans-2-butene | 51 – 206 | $9.4^{\alpha}$ | 32 |
| Acetone | 51 – 204 | $1881.9^{\infty}$ | 0.7 |
| Methacrolein | 49 – 196 | $860.5^{*}$ | 0.2 |
| Cyclohexanone | 64 – 258 | 2000.7 | 0.1 |
| β-pinene | 52 - 210 | $137.8^{\alpha}$ | 1 |

$^{\infty}$ $R^2 = 0.98$

$^{*}$ $R^2 = 0.97$

$^{\alpha}$ $R^2 = \sim 0.81$

Table 2.3 summarises the sensitivities and limits of detection of compounds used in the calibration. Overall, the PTR-ToF-MS instrument measured sensitivities ranging from 8.1 – 2000.7 ncps $ppbv^{-1}$ and low detection limit ranging from 0.1 – 32 ppbv for all compounds analyzed. The detection limits for the compounds were found to be less than 10 ppbv. As shown in Table 2.3, the instrument showed poor sensitivity for trans-2-butene, and therefore high limit of detection, when compared with other compounds in the same table. Isoprene has better sensitivity and lower limit of detection when compared to the *E/N* of 90/190 Td setting. This would not be a problem since the concentrations of isoprene in breath is typically in excess of 100 ppbv. The PTR-ToF-MS showed good performance with respect to other breath compounds, for example, acetone showed one minute detection limits of 0.7 ppbv with a high sensitivity.

Breath samples are humid samples. High instrument sensitivity and low detection limits were required to analyse these humid breath samples. Calibration experiments were performed to assess the PTR-TOF-MS sensitivity and detection limits. Acetic acid and formic acid standards from permeation tubes were used for calibration at varying relative humidity from 0% - 70% humidity level. The measurements were made at 1 minute integration times and at an *E/N* of 80/170 Td (Instrument operating setting for exhaled breath analysis-Chapter 5).

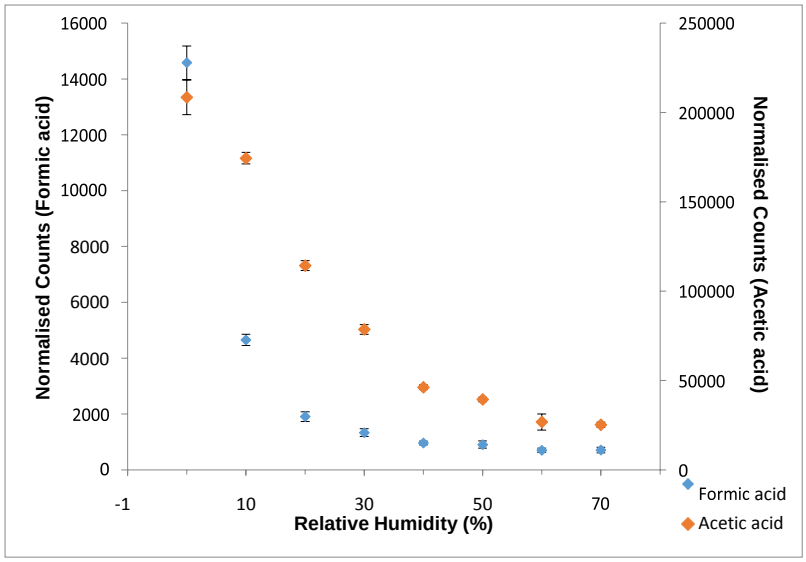


Figure 2.14 The change in the abundance of acetic acid and formic acid in normalised counts as a function of sample humidity at an E/N of 80/170 Td. The error bars denote the standard deviation calculated over the analysis time of 1 minutes.

Two calibration experiments at varying relative humidity (0% - 70%) were carried out using acetic acid and formic acid certified permeation tubes at concentrations of 121 ppbv and 223 ppbv, respectively. Figure 2.14 shows that the higher the sample

humidity, the lower the amount of the compound detected. It appears that these two compounds were highly dependent on the humidity of the sample.

#### 2.2.7.2 The Medi-PTR-ToF-MS

Full details of the calibration of the Medi-PTR-ToF-MS instrument can be found in the thesis of S.B.Barber [14]. The findings are summarised below.

To assess the analytical performance, a variety of VOCs at known concentrations were delivered in both the dc-only and rf funnel modes of operation [4]. The source of gases was a calibrated gas mixture containing methanol, acetaldehyde, trans-2-butene, acetone, methacrolein, cyclohexanone, and β-pinene at known concentrations (BOC Special Gases; nominal mixing ratio 1 ppmv for each compound, with an estimated uncertainty of ±10%) in a balance gas of nitrogen. For these experiments this mixture was subjected to a 10-fold static dilution to generate a new mixture with roughly 100 ppbv of each component, with the balance gas being nitrogen. Subsequent dilutions were then performed dynamically by feeding the cylinder gas into a Kintek 491 M and diluting in nitrogen.

The instrument was calibrated by delivering gas from a commercially prepared standard gas mixture, as detailed earlier. This contained seven principal components detectable by PTR-MS: methanol, acetaldehyde, trans-2-butene, acetone, methacrolein, cyclohexanone, and β-pinene. The gas mixture was dynamically diluted to deliver known concentrations of each compound ranging from the very low through to several hundred parts-per-billion by volume. For each compound the protonated parent species was monitored to generate the data shown in Figure 2.15. Of the seven compounds in the gas mixture, six show excellent linear relationships between the number of ion counts and delivered concentration, with linear correlation coefficients ($r^2$) close to unity. The exception is methanol, but even here $r^2 \approx 0.90$. It seems likely that there is some interference with the protonated methanol signal caused by contributions from the neighbouring $O_2^+$ peak at m/z = 32, which is observed because of some diffusion of analyte gas into the ion source region. Although small relative to $H_3O^+$ the $O_2^+$ signal still dwarves that from protonated methanol.

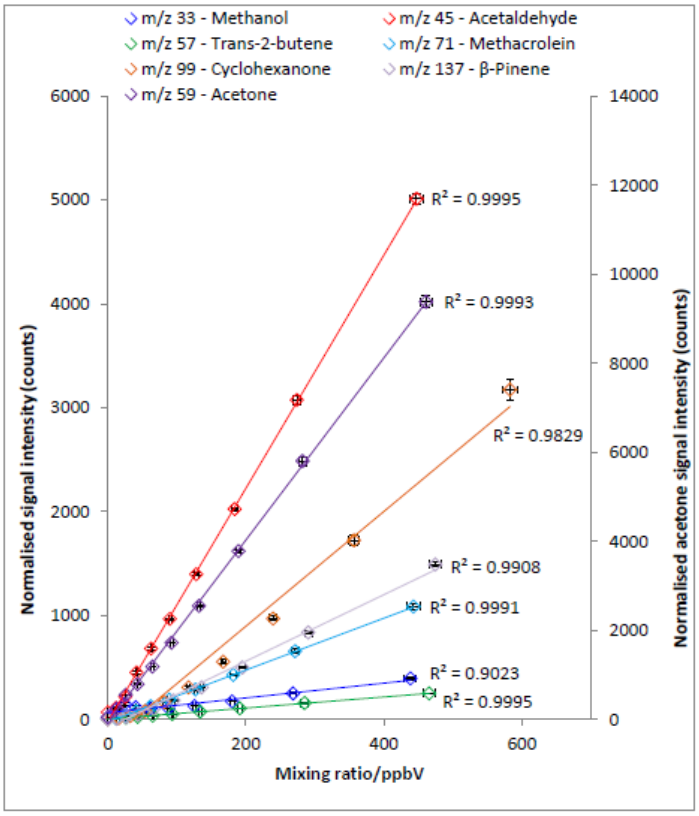


Figure 2.15 Multi-point calibration showing the normalised sensitivities for a seven-component VOC gas standard. The data was collected in the RF mode. The sensitivity is linear over dynamic range of at least three orders of magnitude [14].

The sensitivities extracted from these plots are summarized in Table 2.4. Values are shown for both dc and rf modes of operation, along with the rf/dc sensitivity ratio. For the majority of compounds, the switch from dc to rf mode improves the sensitivity by between 1 and 2 orders of magnitude, although this is exceeded for acetone, methacrolein, and cyclohexanone.

Ground signal can be mass-specific and will include contributions from traces of compounds present in the instrument (e.g., from components used in the instrument construction, degassing, etc.), as well as in the gas employed for background measurements. A mass-dependent background noise level was determined without any added analyte by making repeat measurements of S for a fixed integration time and then associating the standard deviation with N.

Table 2.4. Comparison of sensitivities and Limits of Detection (LOD) for several VOCs[a] [14].

| | Raw sensitivity/Hz ppbv$^{-1}$ | | | LOD/pptv[b] | | |
|---|---|---|---|---|---|---|
| Compound (m/z for $MH^+$) | dc mode | rf mode | rf/dc | dc mode | rf mode | rf/dc |
| methanol (33) | 1.8 | 15 | 8 | 6125 | 1054 | 6 |
| acetaldehyde (45) | 4.8 | 218 | 45 | 486 | 161 | 3 |
| trans-2-butene (57) | 0.7 | 38 | 54 | 930 | 346 | 3 |
| acetone (59) | 5.8 | 1162 | 200 | 445 | 30 | 15 |
| methacrolein (71) | 2.4 | 387 | 161 | 351 | 26 | 13 |
| cyclohexanone (99) | 3.1 | 686 | 221 | 271 | 15 | 18 |
| β-pinene (137) | 1.1 | 164 | 149 | 425 | 123 | 3 |

[a]The dc data was collected at an E/N of 120 Td, and the rf data was likewise obtained at an effective E/N of 120 Td. [b]LOD determined for 20 s of data collection.

Table 2.4 compares the LODs for the dc and rf modes of operation obtained for 20 s of data accumulation. There is a substantial improvement in LOD for all compounds in moving from dc-only operation to the rf mode. In almost all cases the LOD is reduced from several hundred parts-per-trillion by volume in the dc mode to ≤200 pptv in the rf mode, and in some cases considerably better. The most marked exception is methanol, for reasons already identified above (contamination from $O_2^+$). Also, trans-2-butene does not fare too well, with an LOD near 350 pptv in the rf mode for 20 s of data accumulation. This relatively poor LOD may partly be due to the comparatively low polarizabilty and the lack of a permanent electric dipole moment for this molecule,

which will reduce the proton transfer rate when compared to many other molecules in Table 1. Furthermore, trans-2-butene has the lowest proton affinity of all of the molecules in Table 2.4 and therefore will be more susceptible to back-reaction with water vapor.

### 2.3 Statistical Analysis

As a standard practice, throughout the different experiments performed for this thesis, background noise of the PTR-ToF-MS instruments was subtracted from the data. All data collected were normalized to $10^6$ counts per second (ncps) of the sum of $H_3O^+$ and $H_3O^+(H_2O)$ (*m/z* 19 and *m/z* 37, respectively) signals. To avoid the carrying over of artefacts between individual samples, data recorded for the first 5 minutes were not included in the data analysis. First, the significance of the VOCs measured in the experiments were compared to the measured background using a two-sided Mann-Whitney test (Minitab). This test was used to assess whether the VOCs detected from each sample were significantly different from the background, where a *p*-value < 0.05 was considered as statistically significant. The VOCs selected that satisfy the Mann-Whitney test and that had signals more than 100 normalised counts per second (ncps) after subtracting background measurements, were then used in multivariate analysis. Specifically, the data were subjected to principal component analysis (PCA), partial least square discriminant analysis (PLSDA) and cluster analysis. PCA is a statistical tool used to visualize patterns of classification from the data sets analysed. It simplifies high dimensionality data sets by converting observations into principal components that emphasise the variances in the data sets [22]. A leave-one-out cross validation PCA method was applied. A dendrogram was produced using hierarchical cluster analysis with Mahalanobis distance coefficients, which is a visualizing tool for classifying *C. difficile* ribotypes. The aim here was to show similarities and differences found in the samples investigated. PLSDA, a multivariate regression method, was used for classification purposes by the leave-one-out cross-validation. PLSDA assesses the relationship between a *m/z* measurement (descriptor matrix) and sample class (response matrix) to predict whether a class belonged to its own class or to other classes investigated. PCA, PLSDA and cluster analysis was performed using PLS Toolbox (Eigenvector Research Inc., USA) operated in Matlab.

Generally, two measures of accuracy named reproducibility and reproducibility is required when the accuracy of a measurement method is to be known. Repeatability is measurement results under repeatability conditions where independent measurement results are obtained with the same method on the identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. Reproducibility is measurement results under reproducibility conditions where measurement results are obtained with the same method on identical test items in different laboratories with different operators using different equipment [34].

In Chapter 4 and 5, the studies were unable to engage a large enough sample to analyse the significant m/z whether the m/z were due to contamination or a possible marker in the studies. To further aid the discussion, reproducibility of the experimental procedure was calculated to observe the sampling and measurement consistency using PTR-ToF-MS. Repeatability was not calculated as different operators or equipment or methods were not used to measure the similar sample items.

### 2.4 Summary

The PTR-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. It is, therefore, 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 PTR-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 PTR-TOF-MS instrument should prove to be a capable means to actively monitor low weight metabolic volatile organic compounds.

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# Chapter 3

# HEADSPACE ANALYSIS OF VOCS EMITTED BY *CLOSTRIDIUM DIFFICILE*: RIBOTYPE IDENTIFICATION THROUGH METABOLITE PROFILING

## 3.1 Introduction

*Clostridium difficile* is a spore-forming anaerobic bacterium that causes infectious diarrhoea ranging from a mild disturbance to severe illness with ulceration and bleeding from the colon. In extreme cases, perforation of the intestine is possible which can be fatal [1,2]. Patients treated with broad spectrum antibiotics are at the greatest risk of contracting *C. difficile* infection. Suspected diarrhoea cases are empirically treated with broad spectrum antibiotics because definitive diagnosis requires time consuming tests. These delays significantly increase patient mortality because treatment with broad spectrum antibiotics subdues normal, healthy intestinal bacteria which can lead to an overgrowth of *C. difficile*, which flourishes under these conditions and produces a toxin that causes diarrhoea. Appropriate, early antibiotic treatment results in lower mortality rates than when given once tests results are known. In addition to antibiotic exposure, elderly patients with compromised immune systems, or who have recently undergone surgery and have had lengthy stay in hospital are also at higher risk [3]. If *C. difficile* infection is not diagnosed promptly and treated appropriately, it could progress to colitis and possible death [3].

There are many diverse *C. difficile* ribotypes that frequently cause infection or outbreaks. The Health Protection Agency (HPA) created the *Clostridium difficile* Ribotyping Network (CDRN) for England and Northern Ireland, as part of an enhanced surveillance program for *C. difficile* in 2007 [4]. According to CDRN 2011-2013 report, after processing 10 974 feacal samples from 264 healthcare facilities in England and

Northern Ireland, fourteen *C. difficile* ribotypes were reported as the top most prevalent strains in causing *C.difficile* infections, i.e. those with >2% prevalence between 2007 to 2013 [5]. Wilcox et al. have identified a large number of ribotypes from hospital samples [6], which also includes hypervirulent ribotypes that can cause large outbreaks of infection: a good example is PCR ribotype 027 and 078 [7,8].

At present, cytotoxicity assay, enzyme immunoassay (EIA), glutamate dehydrogenase (GDH) enzyme immunoassay, stool cultures and Polymerase Chain Reaction (PCR) tests are used to identify faecal toxins produced by *C. difficile* [9]. Cytotoxicity assay has high sensitivity in detecting *C. difficile* toxin but it requires tissue culturing, technically demanding and takes about 24 – 48 hours before results are obtained [10]. EIA tests are rapid; takes 2-6 hours, but it is not as sensitive as cytotoxicity assay tests and its sensitivity has been in question [11] and it was recommended that this test should be combined with another method before reporting the results [12]. GDH tests can be performed less than an hour but it must be combined with another toxin detecting method to verify the diagnosis. Stool culture test is seldom used as it has high turnaround time between 2 – 5 days for a diagnosis reporting. PCR is a rapid test with high sensitivity; however it is labour intensive, expensive and usually analysed in batches.

Furthermore, the problematic ribotypes are an evolving target and it is difficult for the existing tests to respond in appropriate timeframes in order to detect these changes in epidemiology. Molecular PCR-based tests depend on *a priori* knowledge of the gene sequences of strains being detected and thus new or different strains may be missed. In contrast, a test that is dependent on metabolic VOCs would be free from such constraints.

Microorganisms produce metabolic VOCs for various reasons, such as (1) during growth [15], (2) as info-chemicals for inter- and intra-organism communication [28, 29], (3) for cell-to-cell communication signals [15], or (4) as growth-promoting or inhibiting agents to their own populations, or to other species [15,28].There is a growing interest in the detection and identification of bacteria by measuring their release of volatile organic compounds (VOCs). Among many VOCs produced by bacterial metabolism,

includes fatty acids, aliphatic alcohols, ketones, dimethyl polysulfides, alkenes, nitrogen-containing compounds and volatile sulfur-containing compounds [13, 14, 15]. Presently, the biological functions of many bacterial volatiles are not understood in detail.

Metabolite VOCs of *Clostridium* have been measured in the past using gas-liquid chromatography and gas chromatography-mass spectrometry from microbial headspace *in vitro*. One paper by Stotzky and Schenck described that bacteria has the potential to produce VOCs [16]. They summarised the VOCs produced by *Clostridium* species as being emitters of dimethyl disulfide, various short chain acids, 2,3-butanediol, isopentanol and acetoin [16]. Pons et al., using gas chromatography- mass spectrometry measured volatile amines such as dimethylamine, trimethylamine, isobutylamine and 3-methylbutylamine and showed that these could be used as markers to differentiate between *Clostridium* species [17]. In the 1980s many studies concentrated on rapid identification by gas-liquid chromatography and gas chromatography and reported that this was possible using *p*-cresol and caproic acid as markers of *C. difficile* [18, 19, 20]. Nunez-Montiel et al. noted that no other *Clostridium* species or other microorganisms tested in their analysis produce p-cresol and caproic acid when inoculated in norleucine-tyrosine broth [18]. Species-specific based VOC differentiation between *C. difficile* and other bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* were attempted by Bruins et al using an electronic nose called MonoNose [21]. The study concluded that real-time VOC analysis could be performed to identify and discriminate between *C. difficile* strains from other bacteria. However, the VOCs released were greatly dependent on factors such as growth media, growth phase, growth conditions (pH, temperature, humidity, oxygen content) and species investigated.

*C. difficile* continues to be a major healthcare problem and new strains continually emerge and circulate [34]. Although there has been an increased effort in sequencing the genome of *C. difficile* strains, less work has been carried out on downstream analysis of the sequence data. For this study, ten ribotypes of *C. difficile* were selected which were isolated from local sources but which represent clinically relevant strains of various degrees of prevalence. The ribotype 027 is a known hypervirulent strain that

is associated with many outbreaks [35]. R014/020 is historically the most prevalent ribotype in Europe and it still accounts for around 15% of all cases [36, 37]. The ribotypes 002 and 078 are both abundant in Europe, with 078 being increasingly a cause for concern by its association with a high prevalence of infection [38, 39]. In contrast, ribotypes 013, 05, 107, 026, 087 are less prevalent. The environmental strain 076 was also used as a comparison [40]. The motivation behind this investigation was twofold; the first was a fundamental exploration of multiple VOC "fingerprints" as a metabolome for *C. difficile* ribotypes, the second was to assess the potential in outline of the application of emitted VOCs as a rapid and non-invasive method to diagnose *C. difficile* infections without the need for sample preparation. As a first step towards these goals, the profiles of gas-phase VOC metabolites for ten distinct ribotypes of *C. difficile* were analysed. An attempt was also made to metabolically profile *C. difficile* at the ribotype by analysing volatile organic compounds released by the bacteria using PTR-ToF-MS, and the findings are presented here.

## 3.2 Initial *Clostridium difficile* analysis

### 3.2.1 *Clostridium difficile* headspace measurement

#### 3.2.1.1 Experimental methods

Initial bacterial experiments were conducted using the Leicester-PTR-ToF-MS [22-26]. The first bacterial headspace experiments were carried out in order to set suitable PTR-ToF-MS instrumental operating arrangement and to determine the amount of bacterial culture needed for VOC measurement. Five different ribotypes of *C.difficile*: R027, R014, R015, R078 and R076 (T6) cultures were analysed to determine the differences in volatile organic compounds emitted by these ribotypes/strains. Five individual inoculated plates were prepared for each ribotype. The R027, R014, R015 and R078 strains were the main ribotypes identified to cause *C.difficile* infection in hospitals. The R076 was an environment strain and non-pathogenic. The strains were cultured at room temperature in standard petri dishes containing blood agar (Brain Heart Infusion, BHI agar supplement with 7% defibrinated horse blood). All cultures were supplied by the Department of Immunity, Infection and Inflammation at the University of Leicester.

A custom glass container was used to accommodate a single culture plate. Prior to the addition of any sample, background measurement of the empty container was performed. An inoculated culture plate with the lid removed was placed into the sample container with the upper and lower sections of the container secured using rubber bands. The container was flushed for 5 minutes with zero nitrogen gas (BOC, UK) and then the headspace was analysed. The upper section of the sample container has two outlets, one connected to the PTR-ToF-MS instrument and the other acts as an inlet for the zero nitrogen gas flow into the sample container.

The nitrogen gas was set at a flow rate of 60 ml $min^{-1}$ and the sample and water vapour flow rate into the drift tube were set to 150 and 20 sccm, respectively. The removal of bacterial headspace remained constant throughout the analysis performed on each plate. The drift tube was operated at a pressure of 6 mbar and an *E/N* of 90/190 Td. The culture plate with the lid removed was sealed inside the container and flushed for 5 minutes with zero nitrogen gas (BOC, UK) to remove any traces of laboratory air. The contents were then analyzed for 30 minutes at 1 minute integration times over a mass range of 15 – 300 u. Emission of VOCs from a single uninoculated plate of BHI was similarly analyzed with the rest of the samples. Five individual inoculated plates were prepared for each ribotypes: R027, R014, R015, R078 and R076 (T6) cultures making the total number of plates analysed of 25. The headspace VOCs of the *C. difficile* strains were measured at different times but at the same growth phase (after 48 hours of incubation) to monitor the consistency of VOCs emitted by each individual inoculated culture plate.

#### 3.2.1.2 Preliminary results

All of the *C.difficile* strains analysed were found to produce high intensity signals at *m/z* 18, which can be assigned to protonated ammonia. The ammonia concentration was high enough to deplete the number of $H_3O^+$ reagent ions. The signal at *m/z* 18 was found to increase over time with some of them recording signals as high as signals

at *m/z* 19 ($H_3O^+$). *C. difficile* R076 and R027 strains in particular were found to produce ammonia signals greater than the reagent ion. The standard normalisation process cannot be applied here as it would result in a large normalisation factor which causes the data of all mass channels to scale to artificially high levels in comparison to the other samples. Therefore, the data of all the samples were normalized to $10^6$ counts according to the sum of $H_3O^+$, $H_3O^+(H_2O)$ and $NH_4^+$(*m/z* 19 + *m/z* 37 + *m/z* 18). This assumes that the total number of reagent ions (m/z 19 + m/z 37) would have been approximately equal to this value prior to proton transfer to ammonia.

The measurements of the blank culture media was subtracted from the bacterial measurement to obtain the VOCs emitted by the bacteria. To avoid equilibration effects and carrying over artifacts between individual samples, data recorded for the first 5 minutes were not included in the analysis.

Multivariate analysis techniques such as principal component analysis were used to explore whether the pattern of signals detected could potentially differentiate the strains. Principal component analysis (PCA) was performed using PLS Toolbox (Eigenvector Research Inc., USA) operated in MATLAB. Principal component analysis is a statistical tool that converts observations (observed variables) into artificial variables called principal components, which can account for most of the variance found in the observed variables. It is a common method to find patterns or trends in high dimensionality data sets. PCA was used in this preliminary data processing solely for pattern visualisation and not for extracting significant masses for identification purposes. It was used to observe whether there are differences in the VOC emission by the different ribotypes.

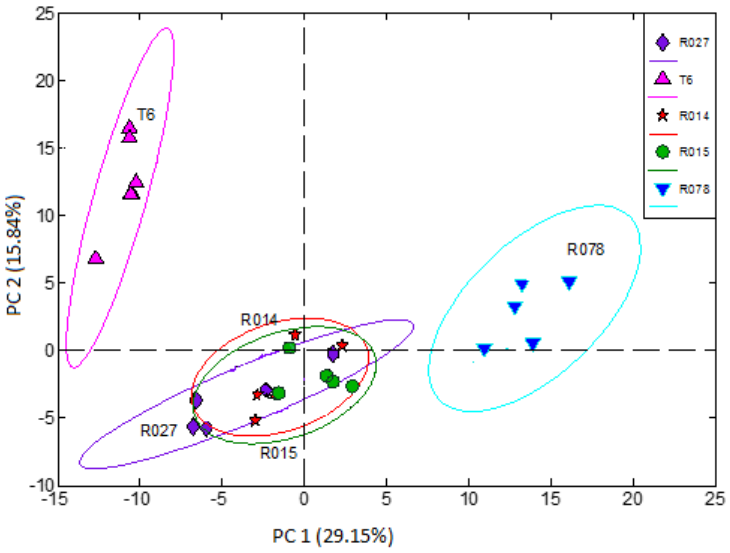


Figure 3.1 A principal component analysis biplot showing the grouping of *Clostridium difficile* strains analysed. The plot displays the first principal component against the second principal component. The principal component analysis was performed using m/z values of 15 – 200 and the data was pre-processed by autoscaling. The eclipse line depicts 95% confidence limit. Five individual inoculated plates were prepared for each ribotypes: R027, R014, R015, R078 and R076 (T6) cultures making the total number of plates analysed of 25.

The preliminary result was quite surprising as the PCA biplot (Figure 3.1) reveals very distinctive clusters for the pathogenic and non-pathogenic *C.difficile* strains. This was unexpected as VOC emission by the same species was thought to be similar. The 014, 015 and 027 ribotypes were found to be grouped together and were not distinguishable. However, the 078 strain was separated from the other strains, which may indicate that the metabolic process of this particular strain is different from the other ribotypes. Thus, it is believed that the VOC analysis is able to identify and distinguish the different ribotypes of *C. difficile* as long as the sampling was performed

at the same growth phase for each ribotype, i.e. day 1 after incubation. Further analysis of identifying the different ribotypes of *C. difficile* would be undertaken.

The headspace inlet flow was decided to be increased to 150 ml $min^{-1}$ as the initial setting of 60 ml $min^{-1}$ was found to be insufficient to maintain the flow of headspace VOCs into the PTR-ToF-MS and there was an overflow of zero nitrogen gas into the sample container. Other instrumental operating arrangements were found suitable for the measurements and were maintained for the further *C. difficile* headspace analysis. The preliminary experiment was conducted under zero nitrogen gas condition which was changed to anaerobic gas in the next analysis to simulate the optimum condition needed to maintain *C. difficile* growth. The changes also included the maintenance of experimental temperature at 37°C by thermally heating the sample container.

## 3.3 *Clostridium difficile* ribotypes: metabolite profiling [43]

### 3.3.1 Experimental methods

#### 3.3.1.1 *Clostridium difficile*: growth and maintenance

The following *C. difficile* ribotypes/strains were used in this study: R027, R014/R020, R002, R013, R005, R107, R026, R087, R078, and R076. The ribotypes except R076 were clinical samples isolated from stool specimens collected from patients infected with *C. difficile* at the UHL Leicester hospitals (in descending order of number of cases detected) during the period of May to November 2009 as shown in Figure 3.2. Among these ribotypes, R027 and R078 are classed as hypervirulent. The R076 was an environment strain and non-pathogenic which was isolated from environmental samples. Each isolated ribotype were cultured in standard agar plates containing blood agar (Brain Heart Infusion agar supplemented with 7% defibrinated horse blood) and incubated at 37 °C anaerobically for 48 hours when required. All cultures were supplied

by the Department of Infection, Immunity, and Inflammation at the University of Leicester using standard procedure [41]. Ribotypes were determined according to standard protocols with reference to the strains categorized as part of the *C. difficile* ribotyping network [42].

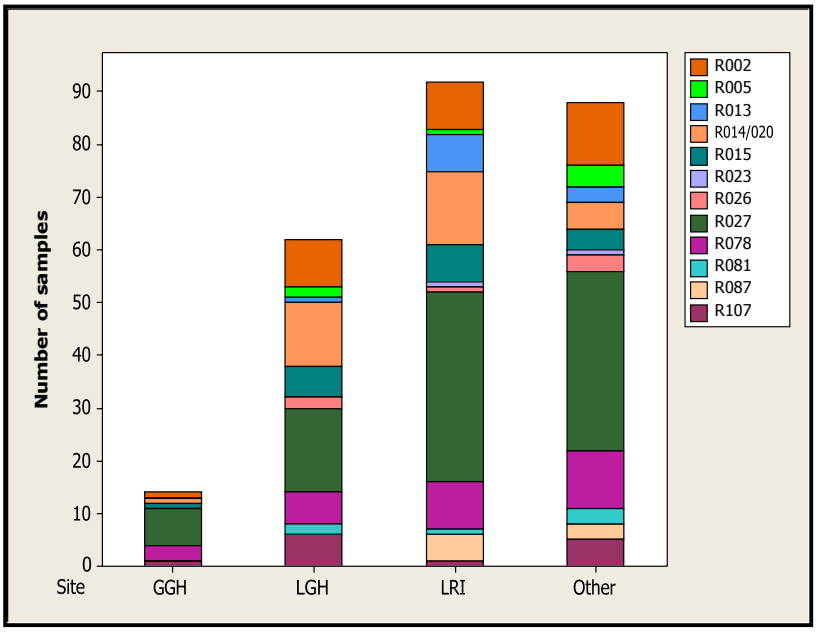


Figure 3.2 Number and diversity of *C. difficile* ribotype isolated from UHL Leicester Hospitals from May-November 2009. GGH-Glenfield General Hospital; LGH-Leicester General Hospital; LRI-Leicester Royal Infirmary. (Courtesy: Department of Infection, Immunity, and Inflammation at the University of Leicester).

#### 3.3.1.2 Bacterial culture experimental design

All bacterial culture headspace analysis was performed under anaerobic condition and at 37°C, the optimum condition needed to maintain *C. difficile* growth. A custom made glass container of approximately 570 mL volume which can accommodate a single

culture plate was used (Figure 3.3). The upper section of the container has two outlets, one connected to the PTR-ToF-MS and the other acts as an inlet for anaerobic gas flow into the sample container. Prior to the addition of any bacteria-laden culture plate, anaerobic gas (80% $N_2$, 10% $H_2$ and 10% $CO_2$) (BOC, UK) was supplied into the glass container and a background measurement of the empty container was taken.

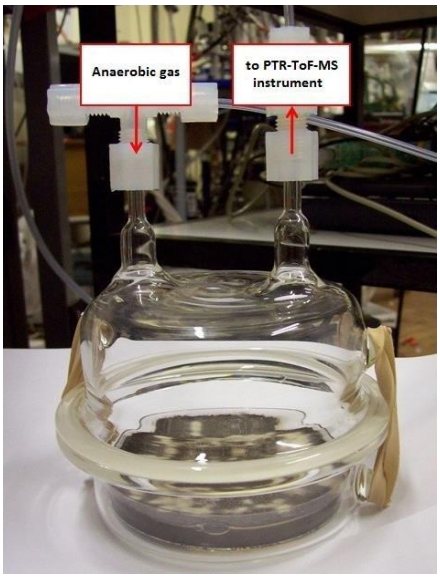


Figure 3.3 The glass container used for the headspace analysis of bacteria cultures.

For the analysis of *C. difficile* cultures, a culture plate with the lid removed was placed into the glass container with the upper and lower sections of the container secured using rubber bands to form an air-tight chamber. The glass chamber was heated to 37°C by wrapping the chamber with a thermal blanket. An agar plate inoculated with *C. difficile* was then placed into the glass container and analysed for 10 minutes (1 minute integration times, 15 – 300 amu) at an *E/N* of 90/190 Td with an headspace inlet flow rate of 150 ml $min^{-1}$ into the PTR-ToF-MS instrument. Other PTR-ToF-MS operating conditions were as follows: drift tube voltage, 2190 V; drift tube pressure, 6

mbar and drift tube temperature, 23°C. After each headspace analysis a corresponding uninoculated blood agar plate (blank) was similarly analysed as culture medium control. Five distinct cultures from each *C. difficile* ribotype were analysed on different days in a period spread over two months in order to monitor the consistency of VOCs emitted by the cultures, resulting in a total of 50 plates of cultures for analysis. Furthermore, a test group (blind test) consisting of four unknown *C. difficile* ribotypes of five distinct samples each (total 20 individual unknown culture plates), also supplied by the Department of Infection, Immunity, and Inflammation at the University of Leicester were analysed in the same manner as per other cultures previously measured. This analysis took place one month after the initial analysis ended.

#### 3.3.1.3 Statistical Analysis

For all of the culture samples the raw PTR-ToF-MS data collected were normalized to $10^6$ counts per second of the sum of $H_3O^+$ and $H_3O^+(H_2O)$ (*m/z* 19 and *m/z* 37, respectively) signals. To avoid the carrying over of artefacts between individual samples, data recorded for the first 5 minutes were not included in the data analysis. The next 5 minutes of data recorded were used for statistical analysis. First, the significance of the VOCs measured in the bacterial cultures were compared to the measured headspace of the glass chamber containing the uninoculated blood agar plate using a two-sided Mann-Whitney test (Minitab). This test was used to assess whether the VOCs detected from each of the ribotype cultures were significantly different from the uninoculated agar, where a $p$-value $< 0.05$ was considered as statistically significant. The VOCs emitted from all ribotypes selected that satisfy the Mann-Whitney test and that had signals more than 100 normalised counts per second (ncps) after subtracting blank medium measurements, were then used in multivariate analysis. Specifically, the data were subjected to a leave-one-out cross validation principal component analysis (PCA) to show similarities and differences found in the ribotypes investigated, partial least square discriminant analysis (PLSDA) was used for ribotypes classification by the leave-one-out cross-validation and cluster analysis; a dendrogram was produced using hierarchical cluster analysis with Mahalanobis distance coefficients, which is a visualizing tool for classifying *C. difficile* ribotypes.

### 3.3.2 Results and discussions

#### 3.3.2.1 *Clostridium difficile* ribotype analysis

Figure 3.4 compares the mass spectra accumulated over one minute (after excluding the initial 5 minutes at the start of the analysis and blank subtracted) obtained from the headspace of the analysed cultures of *C. difficile* ribotypes. The measurements reveal complex mass spectra within the investigated range between 15 and 200 amu for six of the ribotypes: R002, R005, R0013, R014/R020, R027 and R107; and less complex mass spectra between the range of 15 and 150 amu for R026, R076, R078 and R087. The mass spectra show that low signal counts were detected in the 200 to 300 amu mass region after subtracting with blank medium measurements (uninoculated culture plate), most of the signals were eliminated. After the significance test of Mann-Whitney were performed, 69 signals in the mass range between 15 and 120 amu were found to be significantly different ($p< 0.05$) compared to those of the blank medium samples were identified as likely markers of *C. difficile*. Each signal was produced at different relative abundances by the *C. difficile* ribotype cultures, some ranging over more than 6 orders of magnitude and some VOCs were not detected from some ribotypes. These were then employed in PCA to generate a visual representation of the discrimination between the ribotypes by their metabolite profiles using the 69 mass peaks identified as being significant, as shown in Figure 3.4.

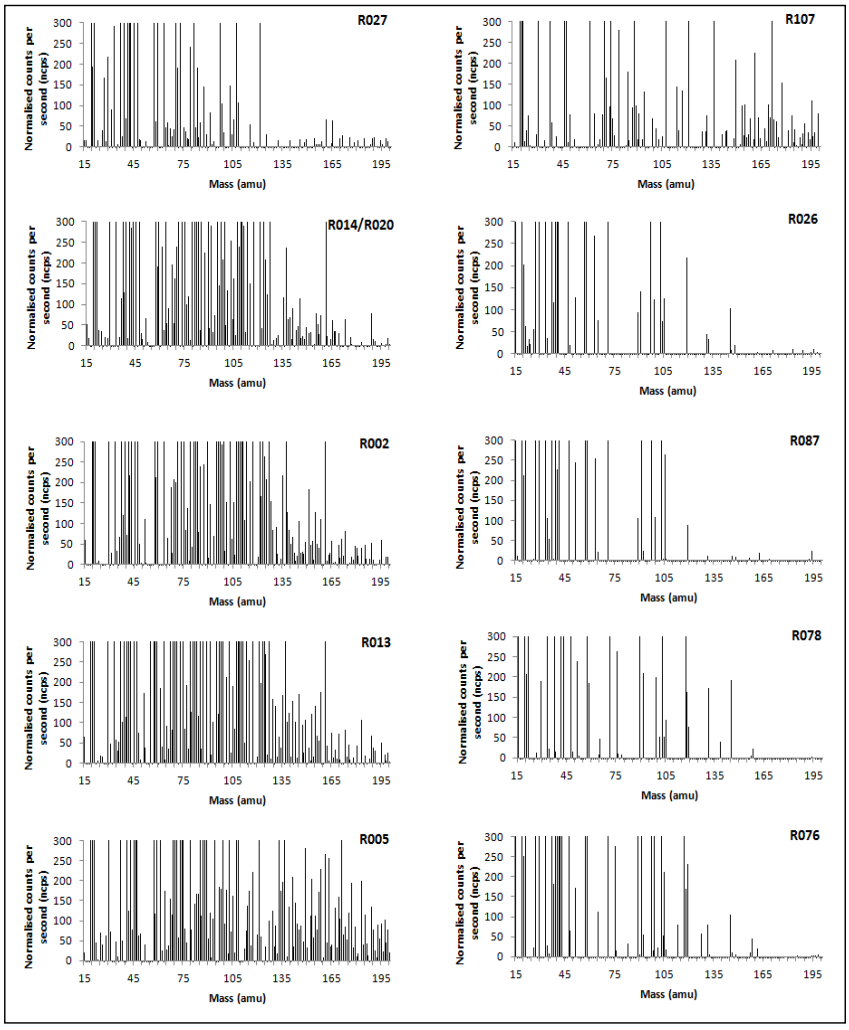


Figure 3.4 Mass spectra (after subtraction of blank) from the VOC headspace analysis of different ribotypes of *C. difficile* cultures.

The first two principal components (PCs) accounted for 68.97% of the variance and Figure 3.5 shows that most of the ribotypes can be separated from each other, with the exceptions of ribotypes R014/R020, R002 and R013, which almost overlapped each other. Ribotype R107 and R005 were clustered very closely but there is clear

separation between them. All the other ribotypes can be clearly distinguished from each other.

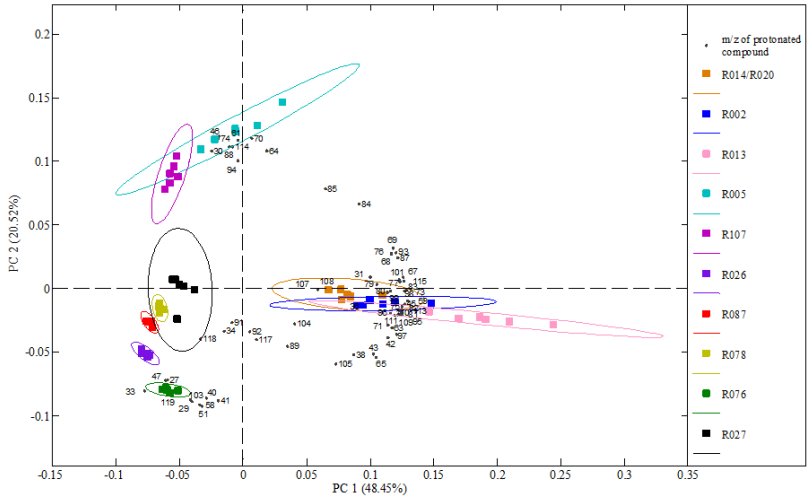


Figure 3.5 A PCA biplot for the different *C. difficile* ribotypes. The first principal component has been plotted against the second principal component. The PCA was performed using peaks of 66 distinct *m/z* values. The oval lines surrounding each class depict a 95% confidence level.

In order to move this to a more quantitative description a partial least square discriminant analysis was performed. PLSDA predicts how good the statistical model built using the data sets is in terms of sensitivity and specificity values. Sensitivity (true positive) evaluates how good the model is at classifying a ribotype into its correct class. Specificity (true negative) indicates how likely a ribotype is not classified into a wrong class. The sensitivity and specificity values of the classification by PLSDA are shown in Table 3.1. The PLSDA model predicts all ribotype except R027 classification with 100% sensitivity. R027 was classified with 80% sensitivity because of larger variances in VOC abundances found in the five samples analysed when compared with the other ribotypes. The specificity exceeded 70% for all ribotypes and for R027, R005 and R076 the specificity was 100%. As some of the ribotypes have similar VOC emission

patterns, a particular ribotype, for an example R013, could be wrongly identified as R014/R020 or R002, thus reducing its specificity value.

Table 3.1: Sensitivity and specificity values of the *C. difficile* ribotype classification by PLSDA

| Cross Validation: leave-one-out | Sensitivity % | Specificity % |
|---|---|---|
| R027 | 80.0 | 100 |
| R014/ R020 | 100 | 73.3 |
| R002 | 100 | 86.7 |
| R013 | 100 | 93.3 |
| R005 | 100 | 100 |
| R107 | 100 | 88.9 |
| R026 | 100 | 88.9 |
| R087 | 100 | 77.8 |
| R078 | 80.0 | 91.1 |
| R076 | 100 | 100 |

The similarities and dissimilarities between the ribotypes can also be represented in a dendrogram produced using cluster analysis, as shown in Figure 3.6. The resulting dendrogram shows that each ribotype was grouped together and overall ribotype relatedness was observed at <30 on the variance weighted distance between clusters centers (according to the Malanobis distance used for analysis). According to the dendrogram in Figure 3.6, R013, R014/R020 and R002 show the largest differences in terms of VOC emissions from the other ribotypes. R107 and R005 ribotypes were closely related to each other. R027, R026, R078, and R087 are more similar when compared to other the ribotypes. This form of analysis provides evidence for how closely these ribotypes are related to each other and may ultimately help to explain why there are differences of metabolic activity in the ribotypes, although this is beyond

the scope of the current study. It could also reveal additional metabolic signatures associated with the hypervirulence in strains such as ribotypes R027 and R078.

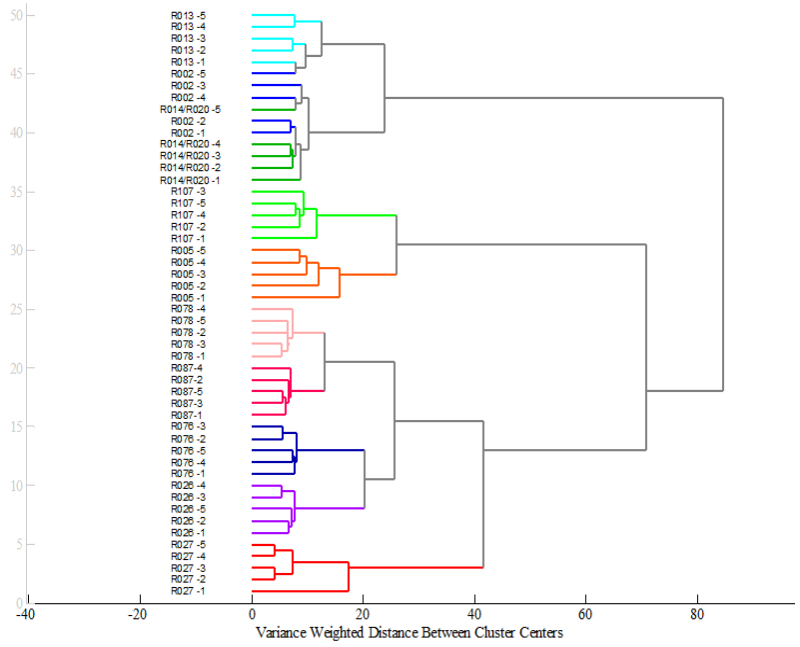


Figure 3.6 Dendrogram of *C. difficile* ribotypes produced by cluster analysis (using Mahalanobis distance) according to the 66 mass peaks selected from a Mann-Whitney test (5 culture sampels per ribotype).

### 3.3.2.2 *Clostridium difficile* ribotype 'blind test'

A 'blind test' was undertaken to validate the use of PTR-ToF-MS as a marker for *C. difficile* ribotypes. The data from the first analysis (Figure 3.5) were used as a training set to build the statistical model and then four unknown ribotypes, of which there were

five individual culture samples for each, were used as test set. Figure 3.7 shows the resulting PCA plot produced by the model.

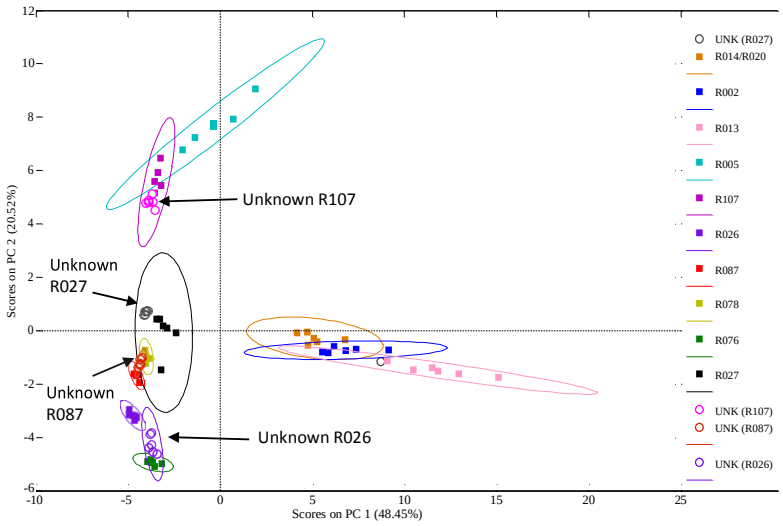


Figure 3.7 A PCA biplot for the blind test of *C. difficile* ribotypes. The first principal component has been plotted against the second principal component. The previous group of ribotype analysis was loaded as validation and the 'blind test' group as a test group. Cross validation using the leave-one-out procedure was used. *UNK-unknown. Four unknown ribotypes, with five individual culture samples for each.

A PLSDA analysis was carried out and the model correctly predicted R107 as being one of the unknowns. The three other ribotypes were more difficult to identify. One was positioned very close to R027 and was tentatively identified as R027 ribotype. Another ribotype was closely positioned to R087 and R078 and the final one had characteristics similar to R026 and R076. The laboratory that supplied the samples later confirmed the ribotypes as R027, R107, R087 and R026.

Even though the 'blind' R107 was clustered near R005, the relative intensities of VOCs released by R107 had clear differences that made it possible to easily differentiate it from R005. R027 data from the 'blind' test clustered near the R027 of test set, and the relative intensities of VOCs produced closely resembled the intensities of test set R027. The unknown R026 sample was between the R026 and R076 in the test set, making it impossible to firmly identify the ribotype, as it could only be said to be either R087 or R078 in the test set. The 'blind test' results show that the metabolite VOCs produced by the ribotypes were consistent and in several cases allowed the clear identification of the ribotypes or a prediction of ribotype to be performed. Even in the least well performing cases, where the ribotype could not be identified with 100% certainty, they could be classified into a group of strongly related ribotypes from the PTR-ToF-MS measurements.

#### 3.3.2.3 Metabolite identification

Metabolite identification by PTR-ToF-MS is based on the detection and attribution of protonated product ions (neutral analyte molecule plus 1 amu). The resolution in our PTR-ToF-MS instrument is insufficient to distinguish ions with the same nominal (integer) mass but different accurate masses. Consequently, it is not easy to identify the specific VOC(s) responsible for a specific peak in the mass spectrum and it is impossible when two or more compounds have the same nominal mass. Identification of compounds can further be complicated by fragmentation and clustering of product ions.

From Figure 3.7, we can see the influence of the masses used as loadings or vectors in PCA analysis, towards the clustering pattern of the different ribotypes. Masses, *m/z* 30, 46, 61, 64, 70, 74, 88, 94 and 114 had higher influences over the clustering of R005 and R107 compared to other ribotypes. These masses may be found at higher intensities in R005 and R107 compared to the rest. The combination masses of *m/z* 27, 33, 34, 47, 91, 92, 117, 118 determined the grouping pattern of R027, R078, R087 and R026. The R076, the non-pathogenic ribotype had a combination of masses (*m/z* 27, 29, 33, 40, 41, 47, 51, 58, 103 and 119) that influenced the clustering of this ribotype and the position of these masses away from the other ribotypes could imply

that the VOCs responsible may be produced in pathogenic ribotypes at lower concentrations, or indeed may not be produced at all when compared to the non-pathogenic R076.

The presence of a characteristic volatile metabolite or a combination of metabolites is attributable to specific metabolic pathways that are active in the bacteria [28]. The compounds released could vary in concentrations depending on the growth media and growth conditions. Identifying the chemical information of these volatiles will allow insight into the underlying metabolic pathways that are active during bacterial growth. However, the number of compounds underlying the statistical analysis was large and therefore an attempt to identify them all was not made. To do this further work would be required, such as a GC-MS analysis. Instead here a tentative identification is provided for some of the more significant peaks seen in the mass spectra.

To aid the discussion, Figure 3.8 shows the signal intensities of peaks arising from several *m/z* values for the various ribotypes and for which a tentative compound assignment was provided. One of the most prominent peaks was observed at *m/z* 33 and it seems likely that this derives from protonated methanol. Methanol was observed to be highest in R026, R078, R087 and R076 and was detected at significantly lower levels in R027, R014/R020 and R013. No methanol signals were found for R005 and R107. Little is known about methanol production by *C. difficile*, although Garner et al. reported detecting the compound in 27% of stool samples of patients with *C. difficile* infection compared to 40% and 36% in healthy donors in cohort and longitudinal studies, respectively [30].

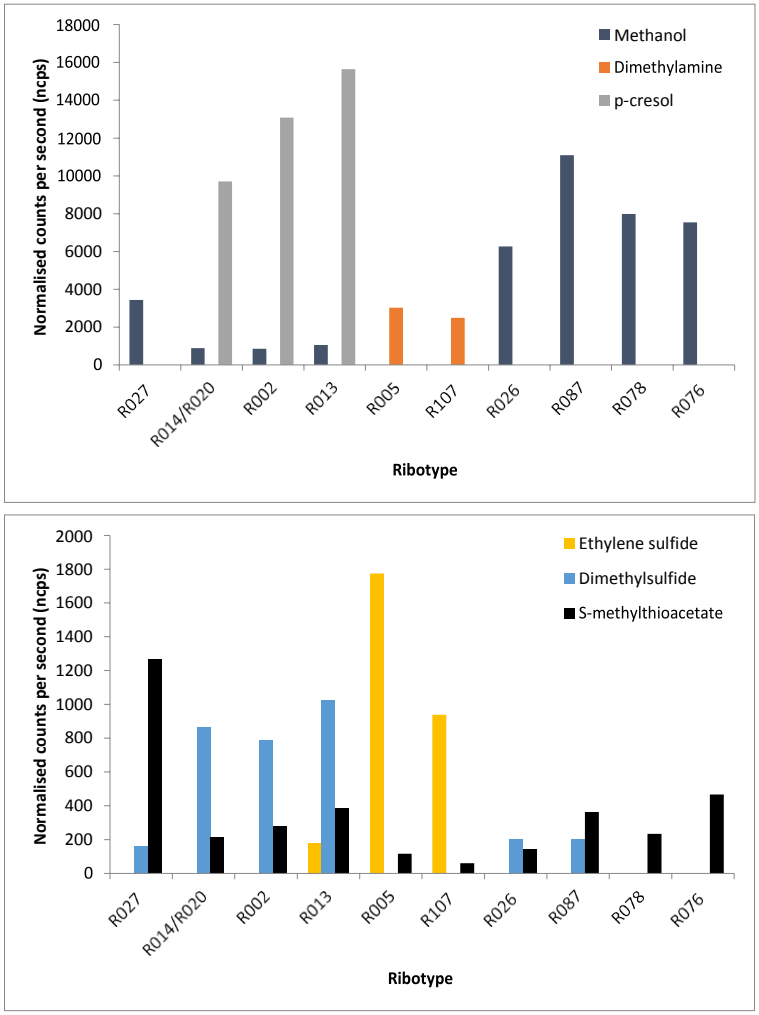


Figure 3.8 Signal intensity chart of *C. difficile* ribotypes for selected mass peaks. The measured signal levels have been subjected to subtraction from the blank spectrum. Tentative assignments of the selected peaks are for protontated versions of the following compounds: *m*/*z* 33 = methanol; *m*/*z* 46 = dimethylamine; *m*/*z* 61 = ethylene sulfide; *m*/*z* 63 = dimethyl sulfide; *m*/*z* 91 = *S*-methyl thioacetate; *m*/*z* 109 = *p*-cresol.

Another significant peak at *m/z* 46 can be assigned to dimethylamine. Evidence for this is twofold. First, the even mass is consistent with a nitrogen-containing molecule. Second, a previous study has detected dimethylamine in the headspace of *C. difficile* [31]. In the current study significant quantities of dimethylamine were only produced by R005 and R107, as can be seen in Figure 3.8. It was observed to be emitted by other ribotypes in the raw data but the intensity in the blank medium was much higher than the detected intensity from the ribotypes.

Earlier studies have reported *p*-cresol emission from *C. difficile* [18,19, 20]. Another study, which concentrated on the production of end products from the metabolism of aromatic acids of phenylalanine, tyrosine and tryptophan by growing *Clostridia* cultures, found that only *C. difficile* emitted p-cresol and this compound was not detected in 22 other *Clostridia* bacteria [31]. Consequently, a signal at *m*/*z* 109 was tentatively assigned to *p*-cresol and was detected in R014/R020, R002 and R013. Again a substantial signal at *m*/*z* 109 from the blank medium made it difficult to identify *p*-cresol from the other ribotype emissions.

Signal at *m/z* 61 was assigned to ethylene sulfide and was found in five of the ribotypes, registering particularly high intensities for ribotypes R107 and R005. Ethylene sulfide was previously reported to be released by *Clostridium* species [32]. As discussed earlier, the resolution in the PTR-ToF-MS instrument used is insufficient to distinguish ions with the same nominal (integer) mass but different accurate masses. For that reason, signal at *m/z* 61 was assigned to ethylene sulphide based on previous literature as it is only a tentative assignment. *m/z* 61 could be argued as acetic acid or 1-propanol as these have been reported as VOC emission of bacteria such as *E.coli,* Since the PTR-ToF-MS does not have the resolution to differentiate ions with same nominal mass, *m/z* 61 will be tentatively assigned as ethylene sulfide based on literature. Past researches also has not yet reported any detection of acetic acid or 1-propanol from *C.difficile*.

Signal at *m/z* 63 was tentatively assigned to dimethyl sulfide and was measured with high intensities for R014/R020, R013 and R002. It was found to be about 2 to 3 orders

of magnitude lower in R027, R026, R087 and R076. This finding is supported by a previous study that detected dimethyl sulfide released by *Clostridium* species, [16] although it should be noted that Garner et al. did not detect dimethyl sulfide in any stool samples infected with *C. difficile* [30]. This may suggest that dimethyl sulfide is not emitted by *C. difficile* when growth conditions are changed. The same group also reported that methanethiol was not detected in the stool samples studied [30] and indeed our own observations seem to support this, since no signal was observed at *m*/*z* 49 in any of the ribotypes analysed in this study. Another tentative assignment is that signal at *m/z* 91 derives from methyl thioacetate. This was detected in R027 with a signal intensity over three times larger than for other ribotypes, and indeed the compound was not detected at all in the non-pathogenic ribotype R076. (S)-methylthioacetate was reported to be emitted by *Clostridium* species in headspace microbial using gas chromatography [32,33]

### 3.4 Summary

The work described here has two potential impacts. First, it suggests that the detection of emitted VOCs by PTR-ToF-MS may have utility as a rapid means of identifying *C. difficile* infection. Second, the VOCs may be markers for different active metabolic pathways in specific ribotypes.

The presence or absence of VOCs in both pathogenic and non-pathogenic ribotype could provide information on the different active metabolic pathways existing in various *C. difficile* ribotypes. The different intensities of the metabolic VOCs released by the *C. difficile* ribotypes may explain the biological functions that occur in each ribotype and how this would affect infectious behaviour. For example, PCR ribotype R027 has found to be the cause of many hospital-related *C. difficile* infections and outbreaks so why is R027 far more aggressive than the other ribotypes? It is non-trivial to go from VOC emissions to answering this question but data of this type may ultimately provide useful clues.

The rapid detection and identification of *C. difficile* is a primary concern of healthcare facilities and clinical microbiology laboratories. Rapid and accurate diagnoses are important to reduce cases associated with *C. difficile* infections as to provide the right

treatment to infected patients. Delayed treatment and inappropriate antibiotic regimens would not only cause high morbidity and mortality, but increases extra annual NHS cost and loss of bed days [33]. Current detection method generally takes two to five days to detect and identify C. difficile. This study demonstrates that PTR-ToF-MS analysis is capable of detecting VOCs of *C. difficile* metabolites in the headspace of cultures within minutes. The ten *C. difficile* ribotypes were successfully distinguished from one another and the scanned VOC patterns were consistent to identify each of the ribotypes tested. The profiles of detected metabolites from the different ribotypes strongly suggested that VOC pattern profiling may provide a useful indication as to the identification of the ribotypes investigated. The results presented here based on metabolic VOCs distinguished these ribotypes and would provide a foundation for a *C. difficile* biomarker library that could one day serve as an information base and diagnostic tool in identifying *C. difficile* infections. The approach detailed here may lead to a clinical diagnostic test based on the VOCs released from faecal samples of patients infected with *C. difficile*. Such an approach would have many obvious advantages when coupled with PTR-ToF-MS as a rapid detection method.

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# Chapter 4

# THE APPLICATION OF PTR-TOF-MS IN THE DIAGNOSIS OF FEMALE GENITOURINARY (GU) INFECTIONS

## 4.1 Sexually transmitted infections (STIs)

Sexually transmitted infections (STIs) have a great impact on sexual and reproductive health worldwide. More than 1 million people are infected with a STI every day, most common infections include chlamydia, gonorrhea and trichomoniasis [1]. STIs are caused by more than 30 different bacteria, viruses and parasites and are passed from one person to another by sexual contact [1]. Some STIs may be spread via skin-to-skin contact and through blood products and tissue transfer. Many STIs can also be transmitted from mother to child during pregnancy and childbirth.

Diagnostics tests for STIs includes microscopy, isolation of culture, gram-stained smear test and Nucleic acid amplification tests (NAATs) are performed in the laboratory on the samples collected with cotton-tip swabs. Patients may need to wait up to two weeks to receive the results, and this causes delay in accurate and complete care and treatment to be given to the infected patients.

### 4.1.1 Chlamydia

Chlamydia is a sexually transmitted infection (STI) caused by a bacterium called *Chlamydia trachomatis*. Chlamydia is the most common STI in the UK. In 2012, 206,912 people were tested positive for chlamydia in England with 64% of those infected people being under 25 years old [3]. NAATs are routinely used in the diagnosis

of *C. trachomatis* infections mainly using the polymerase chain reaction (PCR) assays. Currently, NAATs are the best tests for screening used in GU clinics, however, the tests sensitivity are lowered with problems such as inhibitors, contamination, reproducibility and hormonal factors. Chlamydia is asymptomatic in up to 70 per cent of infected women and is one of the main causes of pelvic inflammatory disease (PID) [15]. PID can lead to infertility, ectopic pregnancy and miscarriage [16,17,18]

### 4.1.2 Gonorrhoea

Gonorrhoea is a STI caused by bacteria called *Neisseria gonorrhoea* or gonococcus. *Neisseria gonorrhoea* is a highly infectious, bacterial sexually transmitted pathogen that is frequently identified and treated in GU Medicine clinics in the UK [2]. Gonorrhoea is the second most common bacterial STI in the UK after chlamydia [3]. It is associated with significant morbidity. The bacteria are mainly found in penis discharge and vaginal fluid discharge from infected men and women. *Neisseria gonorrhea* is isolated using culture prepared from collected swab specimens. This culture method remains the preferred test for routine screening in GU clinics.

### 4.1.3 Bacterial vaginosis

Bacterial Vaginosis (BV) is a very common condition causing vaginal symptoms such as malodourous discharge often described as fishy odour. BV is caused by overgrowth of certain bacteria such as *Gardnerella vaginalis* and anaerobes replacing the lactobacillus-dominated flora of the normal vagina [3]. It is a poorly understood condition as what causes these changes in the levels of bacteria remains unclear. The bacteria associated with BV can be treated but recurrence is common [4]. Gram-stained vaginal smear test have been used extensively in GU clinics for screening BV and has remained as a test of choice. The method has been simplified into different grading or scoring schemes [5-10] resulting in a lack of consistency in diagnosis and reporting [3].

Brand *et.al.* [12] reported that the fishy odour caused by BV infection could be due to trimethylamine. Samples collected from 11 women with vaginal discharge analysed by headspace gas chromatography-mass spectrometry confirmed the presence of trimethylamine and they concluded that it is the main cause of the fishy odour associated with BV. Hill *et.al.* [13] investigated the association between BV, vaginal odour and trimethylamine concentrations using gas chromatography on 94 alkalinised specimens from vaginal fluid. The results suggested that trimethylamine is not a useful biomarker of BV infection unless it is detected at high concentrations. The clinical threshold for detection was around 200 µg/L.

A gram-stained smear test using a device called Osmetech Microbial Analyzer – Bacterial Vaginosis (OMA-BV) by headspace analysis with conducting polymer sensors determined a patients BV status based on acetic acid measurement present in a vaginal swab specimen [14]. The study concluded that the measurement of vaginal acetic acid can be used as an alternative screening method to present tests available for BV diagnosis.

#### 4.1.4 Trichomoniasis

*Trichomonas vaginalis* is a sexually transmissible protozoal parasite [3] also known as trichomoniasis. It is the commonest curable STI with WHO estimating that about 170 million new cases occur annually [11]. Microscopy of a wet mount preparation is the most commonly used diagnostic test for *T. vaginalis* infection [3]. Microscopy for *T. vaginalis* should be performed as soon as possible after the sample is taken as motility of the parasite diminishes with time.

#### 4.1.5 Candidiasis

Vaginal thrush is a yeast infection commonly experienced by most women. It is caused by a yeast-like fungus called *Candida albicans*. Candidiasis infection is harmless but can be uncomfortable and is a common recurrent thrush.

### 4.1.6 Clinical study objective

BV is known to be associated with release of amines detectable on vaginal discharge specimens by mass spectrometry but VOCs from vaginal secretions associated with other GU infections such as candida, chlamydia, gonorrhea and trichomoniasis are not known. If detectable VOC changes are found PTR-ToF-MS may offer the possibility of point of care testing for immediate diagnosis of STIs. Time delay in current laboratory diagnostics techniques for STIs carries the risk of delayed treatment and onward spread of infection in the community. The advantage of PTR-ToF-MS, if this technique works, is that patients in the future would be able to get an instant diagnosis and be treated straight away. A feasibility study to determine whether PTR-ToF-MS can detect differing patterns of VOCs emission in common GU infections compared to healthy controls was performed. The purpose of this study is (1) to develop a PTR-ToF-MS application in detecting and identifying metabolic volatile organic compounds emitted by gonorrhoea, candida, trichomonas, chlamydia and bacterial vaginosis from vagina, cervix and throat swabs obtained from infected patients; (2) to develop a novel method of diagnosing genitourinary infections and sexually transmitted infections (STI) as a complementary screening method together with the current laboratory practice or as a diagnosing method of its own; and (3) to determine if PTR-ToF-MS technique could identify infections in throat swabs.

## 4.2 Preliminary STI culture headspace measurement

### 4.2.1 Experimental methods

Initial STI culture experiments were conducted using Leicester-PTR-ToF-MS [20-23]. The preliminary culture headspace experiments were carried out in order to set suitable PTR-ToF-MS instrumental operating arrangement and to determine whether VOC profile of the culture could be used to differentiate the cultures from each other. The preliminary VOC patterns and protonated compounds detected also could be used as information and clues in further experiments involving vaginal, cervical and throat swabs for infection identification.

Gonorrhoea and candida specimens were provided as inoculated cultures prepared by the testing laboratory in the Genito-Urinary (GU) Medicine clinic at the Leicester Royal Infirmary. However, chlamydia and trichomonas was not provided as cultures at the time of analysis. Both chlamydia and trichomonas specimens were supplied as vaginal swabs. Vaginal swabs were taken from infected patients using cotton-tipped swab at the GU clinic. All specimens were supplied by the GU Medicine clinic at the Leicester Royal Infirmary and preliminary analysis was conducted in the laboratory at the University of Leicester.

For the culture analysis, a custom glass container was used to accommodate a single culture plate. Prior to the addition of any sample, background measurement of the empty container was performed. An inoculated culture plate with the lid removed was placed into the sample container with the upper and lower sections of the container secured using rubber bands. The container was flushed for 5 minutes with zero nitrogen gas (BOC, UK) and then the headspace was analysed. The upper section of the sample container has two outlets, one connected to the PTR-ToF-MS instrument and the other acts as an inlet for the zero nitrogen gas flow into the sample container.

The nitrogen gas was set at a flow rate of 60 ml $min^{-1}$ and the sample and water vapour flow rate into the drift tube were set to 150 and 20 sccm, respectively. The removal of culture headspace remained constant throughout the analysis performed on each plate. The drift tube was operated at a pressure of 6 mbar and an *E/N* of 90/190 Td. The culture plate with the lid removed was sealed inside the container and flushed for 5 minutes with zero nitrogen gas to remove any traces of laboratory air. The contents were then analyzed for 10 minutes at 1 minute integration times over a mass range of 15 – 200 u. Emission of VOCs from a single uninoculated media plate was similarly analyzed with the rest of the samples.

For the swab analysis, a plastic receptacle was used to accommodate the cotton-tipped swab. Background measurement were first taken using empty plastic receptacle. Next, the headspace of the collected vaginal swab in the plastic receptacle was analysed. The same instrumental operating settings detailed above were used.

The measurements of the uninoculated culture media were subtracted from the bacterial measurements to obtain the VOCs emitted by the gonorrhoea and candida specimens. Similarly, the background VOC measurement of empty plastic receptacle were subtracted from the swab headspace measurement to obtain VOCs emitted by chlamydia and trichomonas specimens. To avoid equilibration effects and carrying over artefacts between individual samples, data recorded for the first 5 minutes were not included in the analysis. 83 mass peaks were selected based on measurements of more than 100 counts.

### 4.2.2 Preliminary STI results

Figure 4.1 compares the mass spectra accumulated over one minute (after excluding the initial 5 minutes at the start of the analysis and blank subtracted) obtained from the headspace of the analysed cultures of gonorrhoea and candida cultures. The mass measurements reveals a complex mass spectra for candida compared to gonorrhea within the investigated range between 15 and 200 amu. Gonorrhoea culture exhibited a less complex mass spectra between the range of 15 and 150 amu. In the mass region of 200 and 300 amu showed low signal counts and after subtracting with blank medium measurements (uninoculated culture plate), most of the signals were eliminated.

Figure 4.2 shows chlamydia and trichomonas swab analysis mass spectra accumulated over one minute (after excluding the initial 5 minutes at the start of the analysis and blank subtracted). Here it can be seen that chlamydia swab presents a much more complex mass spectra compared to trichomonas swab headspace mass spectra. Again, after subtraction with background, most signals in the mass region of 200 – 300 amu is eliminated. Therefore, the following experiments shall be conducted in the region of 15 – 200 amu as most signals can be observed in this region.

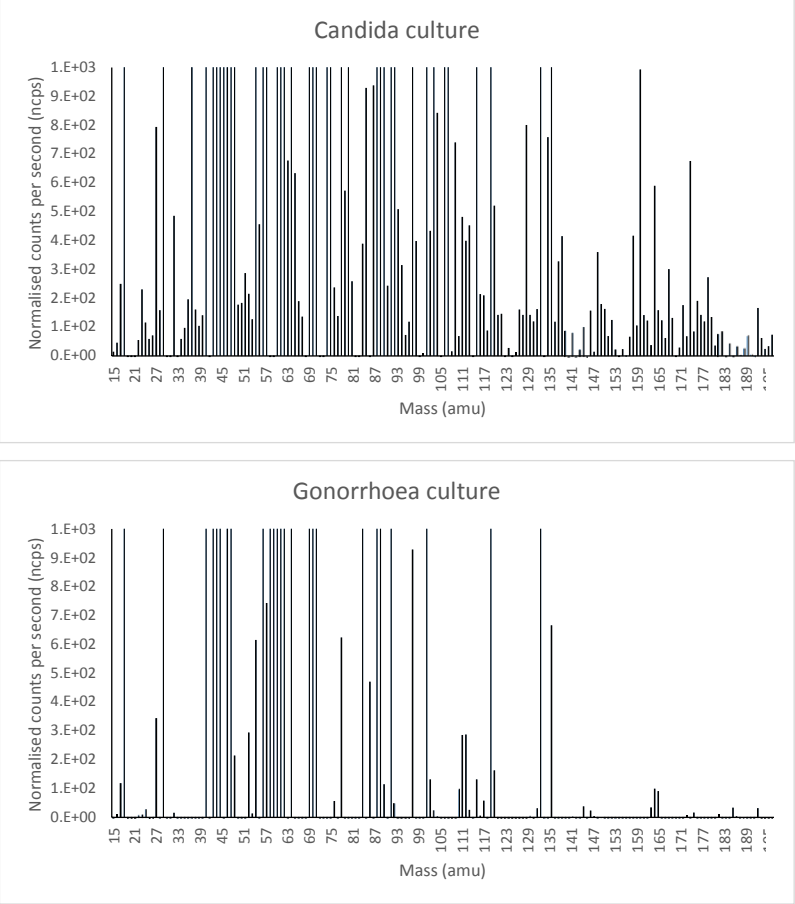



Figure 4.1 Mass spectra (after subtraction of blank) from the VOC headspace analysis of candida and gonorrhoea cultures.

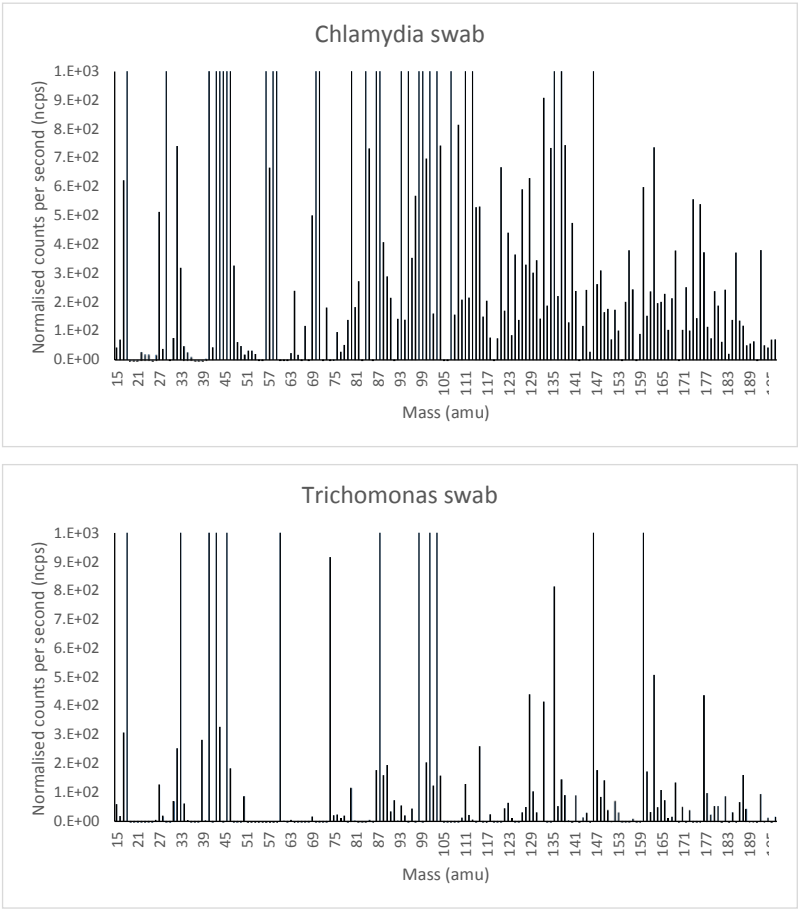



Figure 4.2 Mass spectra (after subtraction of blank) from the VOC headspace analysis of chlamydia and trichomonas swabs.

The preliminary result (Figure 4.1 and 4.2) shows that PTR-ToF-MS system was able to detect different VOC patterns in the headspace emission of gonorrhoea, candida, trichomonas and chlamydia specimens. Candida and chlamydia produced more VOCs with higher concentrations compared to gonorrhoea and trichomonas enabling the differentiation of the specimens analysed. Further analysis using positive vaginal, cervical and throat swabs were performed using the Medi-PTR-ToF-MS located at the

Diagnostic Development Unit (DDU). This unit is located in the Accident and Emergency Department at the Leicester Royal Infirmary (LRI). To analyse the STI specimens using the Medi-PTR-ToF-MS, some operating settings were changed. The headspace inlet flow was decided to be increased to 200 ml $min^{-1}$. Other instrumental operating arrangements that were changed are detailed in section 4.3.1.3.

## 4.3 Genitourinary clinical trial

### 4.3.1 Methods

#### 4.3.1.1 Study participant details

The study was carried out between October 2012 and July 2013 at the Genitourinary Department, Leicester Royal Infirmary. Forty-two female volunteers (median age = 26) participated in this study. Each of the female participants were required to give vaginal, cervical and throat swabs. However, ten female participants opted out from giving cervical and throat swabs; they agreed to provide vaginal swabs only. Replicate swabs were also taken for routine laboratory testing currently practised by the Genitourinary Department to identify the infections or non-infections. These infections are currently diagnosed by means of swab-taking during speculum examination at the GU Medicine Clinic. Bacterial vaginosis is diagnosed by microscopy in clinic. Candidiasis and trichomoniasis are diagnosed by microscopy in clinic along with laboratory culture. Chlamydia and gonorrhoea infections are tested by Nucleic Acid Amplification Tests (NAATs) in the laboratory, with an average turnaround time of two weeks. The clinical study participants were grouped as healthy controls, single infection or multiple infections based on the results obtained from the GU laboratory testing. Even though this clinical study focused on female GU infections, two male participants (median age = 31) were recruited to give throat swabs as they were found to be infected with either one of the infections studied.

A total of 42 vaginal swabs were collected from the study. 22 of the swabs were negative for infections and were grouped as healthy controls. 11 swabs were found positive for candida infection, 4 swabs positive for bacterial vaginosis infection and 1

swab positive for trichomoniasis. Three participants had multiple infections – two participants had gonorrhoea and chlamydia infections and one participant with candidiasis and bacterial vaginosis infections.

For the cervical swabs study, 32 swabs were taken in total with 23 found to not have any infections (healthy controls). Seven participants had single infections and two participants with multiple infections. Five participants were infected with chlamydia and two participants suffered from gonorrhoea infection. The two participants with multiple infections were tested positive for gonorrhoea and chlamydia.

The throat swabs were collected from 31 female and 2 male subjects. There were 30 throat swabs tested negative for infections and were categorised as healthy controls. The healthy control group were obtained from the female participants. The two male participants were found to be infected with gonorrhoea, and chlamydia and gonorrhoea infections, respectively. Another female participant was also found to be infected with gonorrhoea. Table 4.1 summarises the results obtained from the GU laboratory testing of the swabs collected in the genitourinary clinical study.

Table 4.1: Results from GU laboratory testing of swabs obtained from the clinical study participants.

| Participants | Vagina swabs | Cervical swabs | Throat swabs |
|---|---|---|---|
| Healthy controls | 2 | 23 | 30 |
| Gonorrhea | 0 | 2 | 2 |
| Gonorrhea & Chlamydia | 2 | 2 | 1 |
| Chlamydia | 1 | 5 | 0 |
| Candida | 11 | 0 | 0 |
| Bacterial vaginosis | 4 | 0 | 0 |
| Candida & Bacterial vaginosis | 1 | 0 | 0 |
| Trichomonas | 1 | 0 | 0 |
| *Total samples* | 42 | 32 | 33 |
| *Gender* | | | |
| Female | 42 | 32 | 31 |
| Male | 0 | 0 | 2 |

All participants were between 18 and 45 years old. Women over the age of 45 or peri-/post-menopausal women were excluded because of the possibility of altered vaginal flora. There were no other criteria used to exclude any of the participants from the clinical study such as diet, alcohol consumption, smoking, medications taken or multiple diseases. The study was approved by the National Research Ethics Service (NRES) Committee East Midlands-Leicester, and participants gave written informed consent. All of the paper works related to this study can be found in Appendix 2.

#### 4.3.1.2 Off-line sampling protocol

All vaginal, cervical and throat swabs were collected using cotton-tipped swab and the collected swab were inserted into a plastic receptacle for PTR-ToF-MS analysis. Specimens were collected at the Genito-Urinary (GU) Medicine Clinic at Leicester Royal Infirmary. The collected swabs were then brought to the DDU for analysis. After analysis all plastic receptacle containing the swabs were discarded into biological waste bin.

For PTR-ToF-MS analysis, the plastic receptacle was connected to the instrument using a rubber stopper with an inlet and an outlet tubing. The inlet allows zero nitrogen gas (BOC, UK) to flow in the receptacle and the outlet is connected to the instrument via tubing that was heated to 32°C to prevent condensation.

#### 4.3.1.3 Instrumental operating settings

The Medi-PTR-ToF-MS was used in this analysis [19]. The plastic receptacle contents were analysed for 180 seconds (30 seconds integration times, 15 – 200 amu) at an effective *E/N* of 120 Td with radio funnel (rf) voltage of 170 V. The headspace sampling flow rate was set at 200 ml $min^{-1}$. As discussed in Section 2.2.2, the ion funnel voltage increases the $H_3O^+$ ion count rate detected at $m/z$ = 19. The far higher ion count rate in the rf mode has the disadvantage of contributing to rapid degradation of the ion detector and overloading the time-to-digital converter. Therefore, the ToF-MS detector was equipped with a gating system to prevent ions with $m/z$ = 19 from generating a cascade of electrons and hence wear in the detector. Thus, the $H_3O^+$ count rate was

deduced by monitoring the $H_3{}^{16}O^+$ signal at *m/z* = 21 and using the known $^{16}O/^{18}O$ natural abundance ratio (499:1) to determine the count rate of $H_3O^+$.

#### 4.3.1.4 Data collection and processing

Each plastic receptacle containing swabs were analysed for 3 minutes at 0.5 minute integration times. Initial statistical analysis involved the use of two-sided Mann-Whitney test, in order to assess whether the mass peaks (*m/z*) identified from swab headspace were significantly different ($p<0.05$) from those of background of empty plastic receptacle of which might arise from the PTR-ToF-MS instrument. Swab specimens from all of the participants were compared to receptacle background measurement first and mass peaks that were found to be significantly different by Mann-Whitney were used for further comparison to exclude any possible exogenous VOCs present in the headspace analysis. The swab specimens from STI infected patients were compared to healthy controls and only VOCs significantly different were reported.

### 4.3.2 Results and Discussion

#### 4.3.2.1 Vaginal swab specimens

Using the Mann-Whitney tests, for vaginal swab STI analysis, 17 different mass-to charge ratio (in the range of $15 \leq m/z \geq 150$) were identified as significantly different ($p<0.05$) from healthy controls and from background measurement in the study. The significant peaks of interest were detected at *m/z* 17, 33, 41, 43, 45, 53, 59, 61, 79, 80, 90, 92, 96, 98, 104, 106 and 108 (see Table 4.2).

Figure 4.3 shows plots of sample swabs vs. signal intensities for *m/z* 33, 43 and 59. *m/z* 33, usually identified as methanol can be detected in all samples at varying intensities. Not all controls emitting m/z 33 at high intensities. There is notably high variances in the healthy control vaginal swab samples. *m/z* 33 can also be observed in all infected samples as well. Swabs of gonorrhea + chlamydia release m/z 33 in the similar amount.

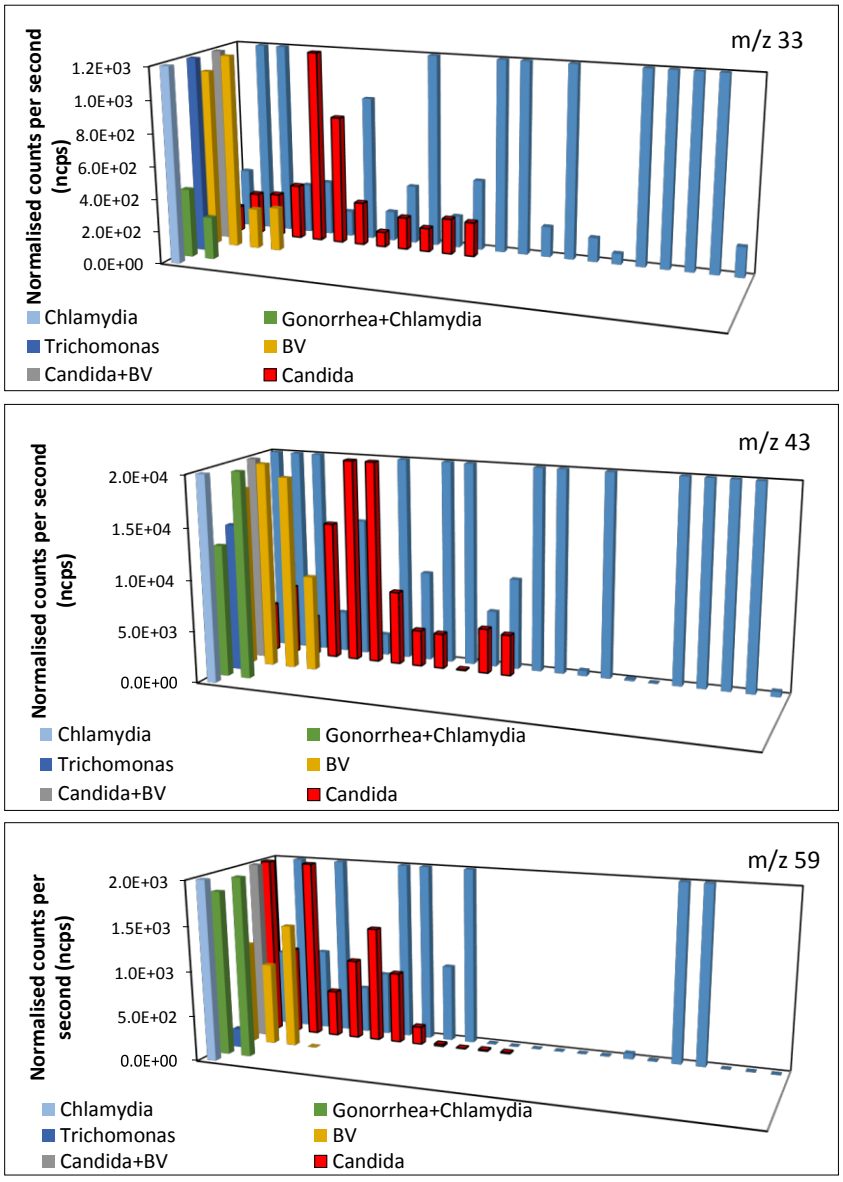



Figure 4.3 Plots displaying the measured signals (y-axis, ncps) in all of the vaginal swab headspace samples for *m/z* 33, 43 and 59.

Infected swab samples can be observed to be emitting consistently high level of peak mass of *m/z* 43 especially for gonorrhoea + chlamydia, chlamydia, BV and candida + BV. It may show that this particular mass peak of 43 is released by samples with infections except for candida only swab as the levels varies a lot within the candida group. However, *m/z* 43 can also be seen in controls, mostly in high intensities.

About 50% of the control swabs does not emit VOC with the mass of m/z 59. Usually this mass peak is assigned to acetone or propanal. It may well be acetone as many papers have reported detecting acetone in microbial samples. Again, all infected samples released m/z 59 except for candida, whereby about 1/3 of the sample did not emit a VOC with the mass peak of 59. It can be deduced that infected samples do emit VOC of m/z 33, 43 and 59, although at varying concentrations.

Table 4.2: Selected peak of significance, VOCs *m/z* (M+1), identified using Mann-Whitney test of significance for each swab groups

| Vaginal Swab | Cervical Swab | Throat Swab |
|---|---|---|
| 17 | 30 | 15 |
| 33 | 46 | 17 |
| 41 | 59 | 30 |
| 43 | 61 | 31 |
| 45 | 74 | 45 |
| 53 | 79 | 53 |
| 59 | 80 | 59 |
| 61 | 90 | 62 |
| 79 | 92 | 64 |
| 80 | 96 | 74 |
| 90 | 98 | 76 |
| 92 | 104 | 80 |
| 96 | 106 | 92 |
| 98 | 108 | 96 |
| 104 | 116 | 98 |
| 106 | 122 | 104 |
| 108 | | 106 |
| | | 113 |
| | | 122 |

#### 4.3.2.2 Cervical swab spesimens

In the cervical swab analysis, 23 controls were analysed to compare with infected swabs of gonorrhoea (2 swabs), gonorrhoea and chlamydia (2 swabs) and chlamydia (5 swabs). From the Mann-Whitney test of significance, the significant peaks of interest were identified as *m/z* 30, 46, 59, 61, 74, 79, 80, 90, 92, 96, 98, 104, 106, 108, 116 and 122 (Table 4.2).

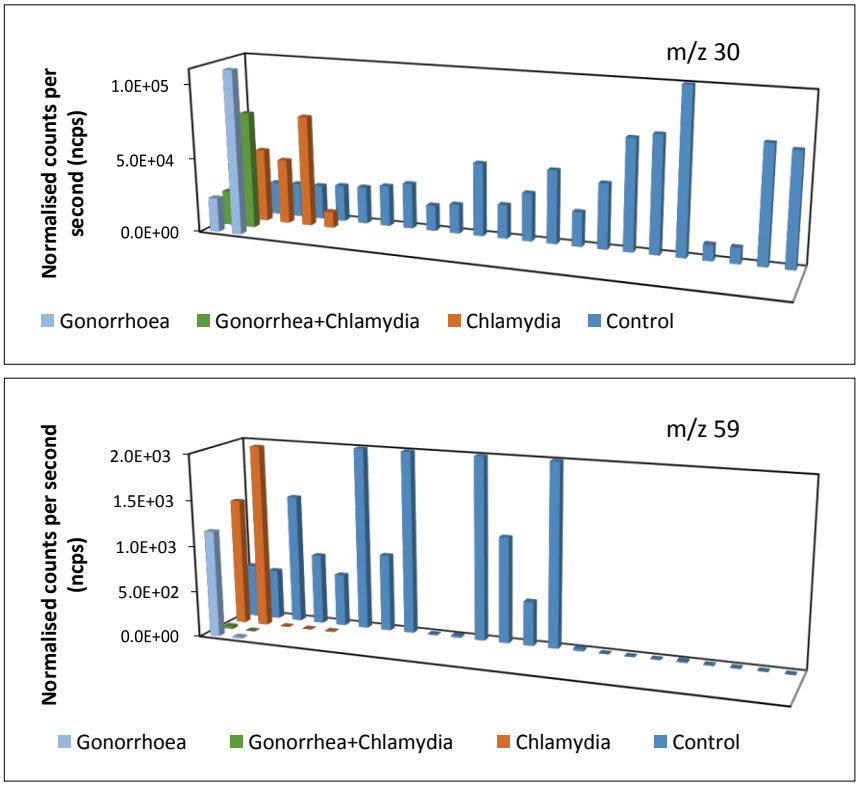
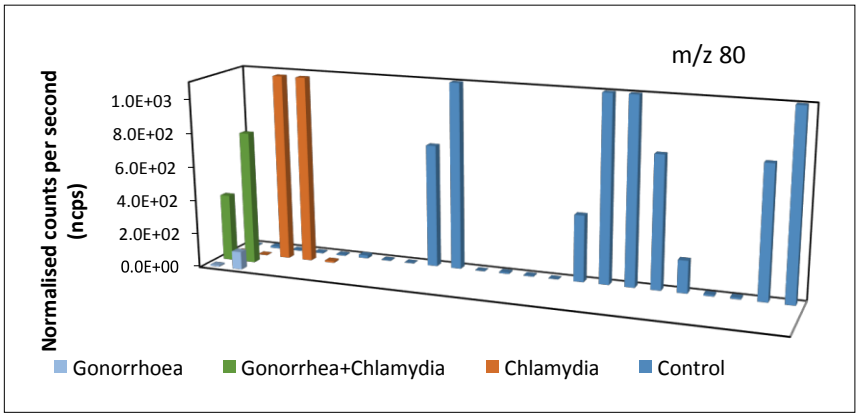



Figure 4.4 Plots displaying the measured signals (y-axis, ncps) in all of the cervical swab headspace samples for *m/z* 30 and 59.

Figure 4.4 shows that for m/z 30, the infected swabs were emitting higher levels of the VOC of m/z 30 compared to the majority of healthy controls swab samples. Gonorrhoea emitted the highest concentration followed by gonorrhoea + chlamydia and chlamydia swabs. m/z 59 can detected in two chlamydia and one gonorrhoea swab. About half of the healthy control swabs did not emit or emitted very low level of VOC of 59. m/z 59 can tentatively be assigned to acetone as many has reported detecting acetone in microbial studies. Both gonorrhoea and chlamydia swabs emitted very low level of m/z 59.



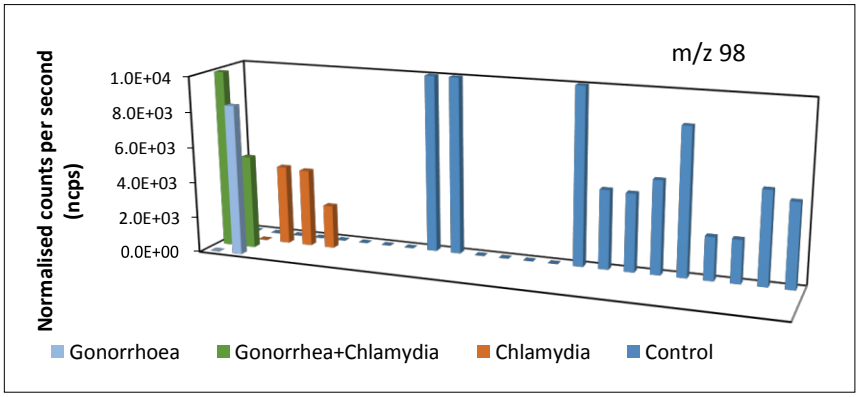




Figure 4.5 Plots displaying the measured signals (y-axis, ncps) in all of the cervical swab headspace samples for *m/z* 80 and 98.

In Figure 4.5, two of the chlamydia swab released high amounts of m/z 80. Both swabs of gonorrhoea + chlamydia emitted the VOC as well this may suggest that chlamydia is more prone to release m/z 80 but it suppressed in gonorrhoea presence. Only some of the healthy control swabs emitted this particular VOC. This may be of the presence of flora in the cervical region emitting VOCs at varying levels depending on individuals. Gonorrhoea and gonorrhoea + chlamydia infected swabs exhibits high intensities of m/z 98 VOC compared to controls and chlamydia swab. This mass peak of 98 could be exhibited mostly by gonorrhea infection. The chlamydia swab too releases the mass peak of m/z 98. Only some of the healthy controls emitting this VOC at varying levels of intensities.

#### 4.3.2.3 Throat swab specimens

Three categories were analysed in the throat swab experiment consisting of 30 healthy controls versus 2 gonorrhoea infected subjects and 1 infected with gonorrhoea and chlamydia. The number of infected specimens were too small that it was difficult to identify any differences so none of the group could be differentiated confidently. All three groups on average showed similar signal intensities disabling any identification. From the Mann-Whitney significance test, the significant peaks of interest were identified as *m/z* 15, 17, 30, 31, 45, 53, 59, 62, 64, 74, 76, 80, 92, 96, 98, 104, 106, 113, and 122.

Looking at *m/z* 30, 45 and 59 (Figure 4.6) for example, the signal for the 30 healthy controls varies greatly. This may be caused by the throat cavity containing VOCs produced in the airways, the oral cavity by bacterial infections, by bacteria in the gut; and saliva and mucus from the respiratory tract. Other factors such food intake, drink and oral hygiene could bring about the high variances of throat VOCs within the control subjects. The infected throat samples whether it is gonorrhea or gonorrhea and chlamydia, the signal of these infected samples are about the same for *m/z* 30, 45 and 59.

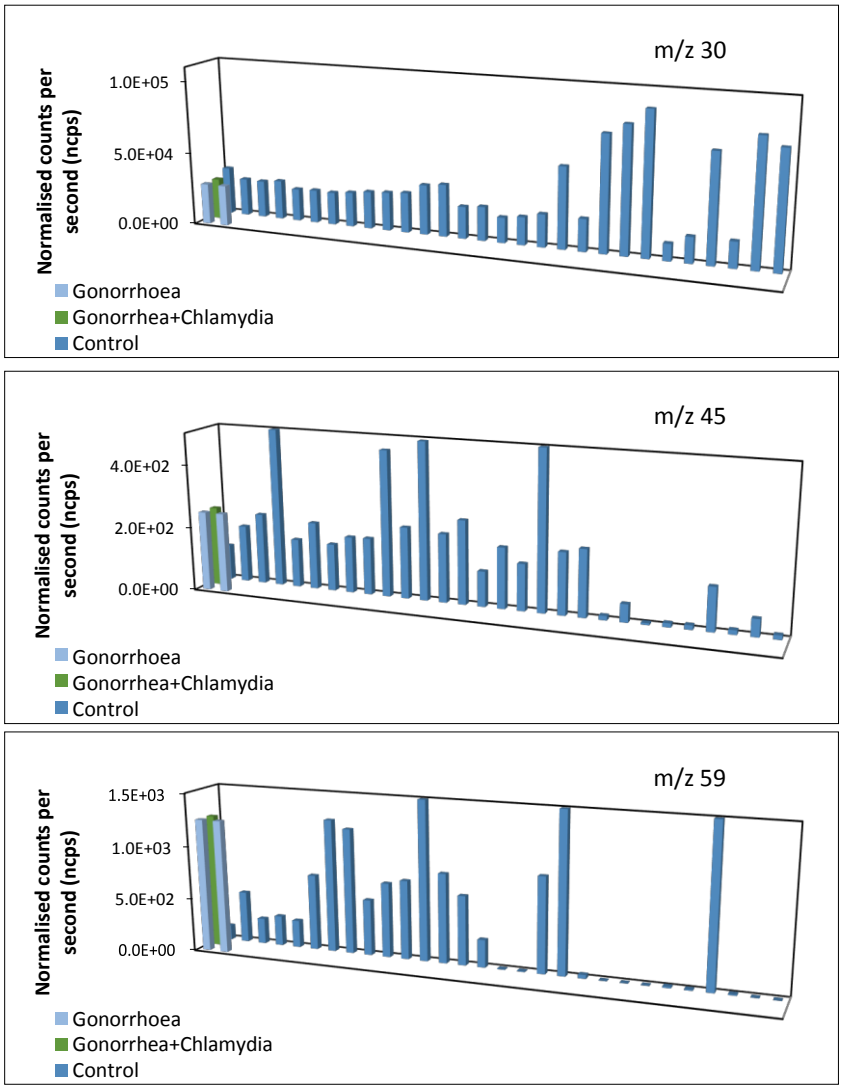



Figure 4.6 Plots displaying the measured signals (y-axis, ncps) in all of the throat swab headspace samples for *m/z* 30, 45 and 59. The first row bars represent infected gonorrhea samples, second row represents infected gonorrhea+chlamydia samples and third row represents healthy controls.

Specifically at *m/z* 59, the infected samples registered higher signal than compared to most of the control subjects. It can be summarized that in this study group, samples of infected gonorrhoea and gonorrhoea + chlamydia may not be differentiated from each other. It would be interesting to see if the same trend is followed in a larger size group with infected gonorrhoea and gonorrhoea + chlamydia samples. For the time being, it can be said that chlamydia does not give out strong VOC emission than gonorrhoea. VOCs from gonorrhoea are more prominent in the infected samples.

#### 4.3.2.4 Reproducibility Study

Each plastic receptacle containing swabs were analysed for 3 minutes at 0.5 minute integration times. To eliminate error in statistics caused by the limited number of samples analysed in this study, each analysis was divided into ten portions, creating ten data points for each specimen analysed.

The identified mass peaks as in Table 4.2 were assessed for variations in the overall pattern of exhaled compounds in the swab specimens using a multivariate statistical analysis technique, principal component analysis (PCA) by the leave-one-out method for vaginal, cervical and throat swab specimens. Figure 4.7, 4.8 and 4.9 shows the PCA plot for vaginal swabs, cervical swabs and throat swabs, respectively.

Since the study was unable to recruit large number of volunteers with different set of infections, the result have been analysed toward a discussion of the reproducibility of the measurement technique. A number of m/z values have been selected for the reproducibility analysis. Ions at m/z 53, 80, 92, 96 and 104 were selected from the selected mass peaks for all three groups as most of the m/z can be observed as significant in all the groups. These ions were chosen to reflect the reproducibility of the technique over a range of intensity.

For a comparison of reproducibility it is useful to have a standard method to calculate a value by which the reproducibility may be judged. The method suggested by Bland and Altman [24] is used for this purpose. For a series of repeat measurements of the same subject, it is generally assumed that the measurements are not same caused by changes

in the subject, the sample as a reflection of the test sample, or the accuracy of the measurement procedure itself. For a set of repeat measurements an average value can be calculated. If the distribution of measurements is assumed to be normal, the mean should reflect a true value for the measurement. The standard deviation of these measurements will give an understanding of the reproducibility of the technique; 68 % of measurements should lie within one standard deviation of the mean. This study can examine two sources of error affecting the reproducibility; the measurement accuracy and the sample consistency. The reproducibility of measurements taken from the same sample should be affected only by the measurement accuracy. Whereas, repeat samples will be affected by the accuracy of the measurements, the sample's consistency and the patient's natural variability.

The analysis of repeat measurements from the same sample has been performed by finding the mean and variance for each set of repeats. The average variance across all samples was used to find the average standard deviation. The average of the mean was also taken for each m/z value. These values are shown in Table 4.3. In accordance with work by Bland and Altman [24], the samples reproducibility and reproducibility coefficient was also calculated and is shown in Table 4.3 for the investigated m/z values.

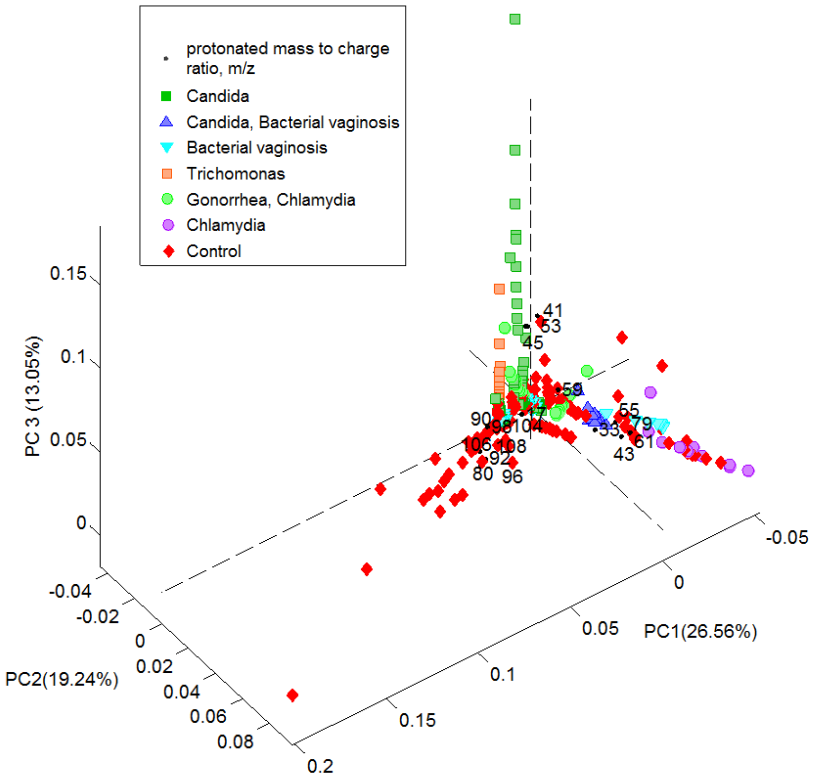



Figure 4.7: A principal component analysis biplot for the STI study of vaginal swab from infected subjects and healthy controls. The first principal component has been plotted against the second principal component and third principal component. The principal component analysis was performed using 17 *m/z* values and the data were pre-processed by auto-scaling.

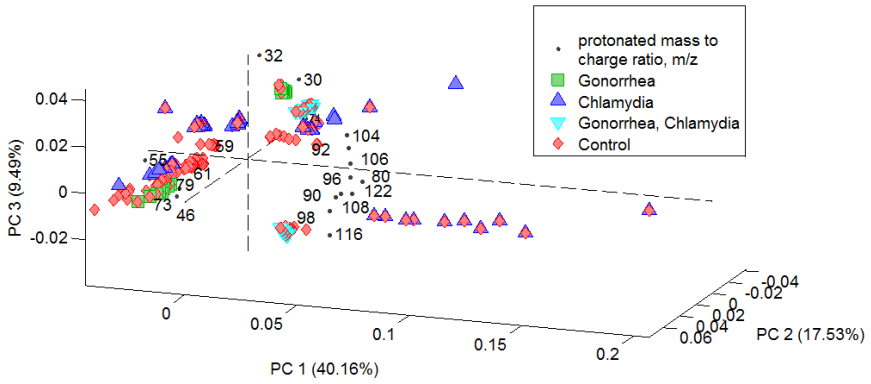



Figure 4.8: A principal component analysis biplot for the STI study of cervical swab from infected subjects and healthy controls. The first principal component has been plotted against the second principal component. The principal component analysis was performed using 16 *m/z* values and the data were pre-processed by auto-scaling.

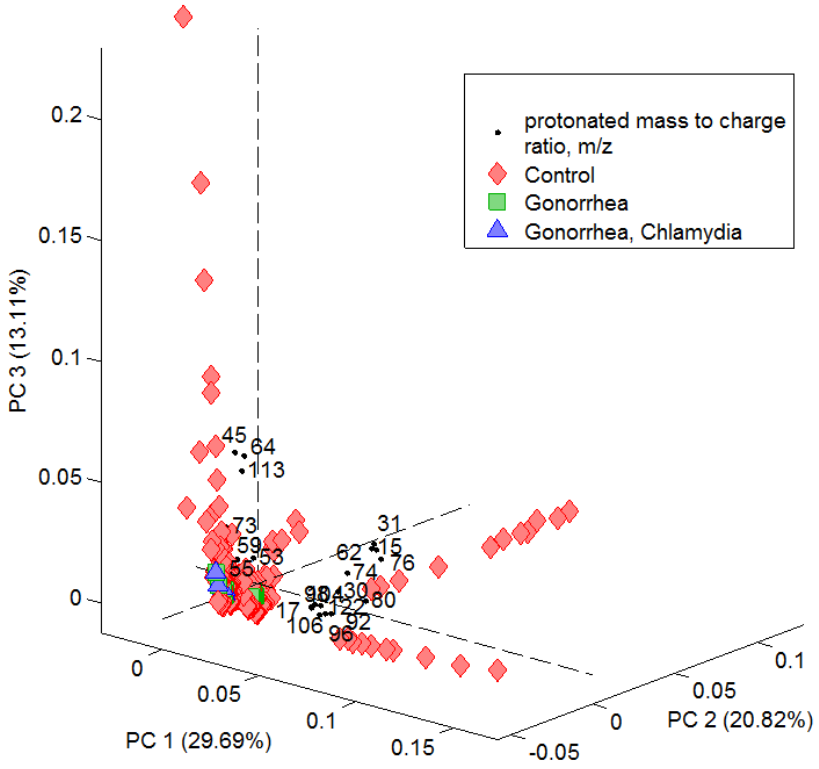



Figure 4.9: A principal component analysis biplot for the STI study of throat swab from infected subjects and healthy controls. The first principal component has been plotted against the second principal component. The principal component analysis was performed using 19 *m/z* values and the data were pre-processed by auto-scaling.

The measurement reproducibility is shown in Table 4.3 At higher mean signal intensity the reproducibility improves as a fraction of that mean. At greater signal intensity the statistical error in measurement becomes less effective, thus the reproducibility should improve for higher intensity ion signals. Healthy controls shows the best measurement reproducibility except for vaginal swabs as only twenty replicates were analysed. For the infected groups with one or two candidates showed lower reproducibility than the others owing to the small number of replicates. For the healthy controls the number of replicates

analysed for cervical and throat were more than 200 replicates indicating large number of samples were needed to obtain meaningful statistical outcome.
Some groups with lower number of replicates such as the candida infected swabs in the vaginal swab study had 110 replicates but gave high reproducibility percentage. This shows that certain infected swabs were able to give out significant amount of compounds to differentiate the group from the others.

Table 4.3: Table showing sample reproducibility for selected m/z values for vaginal, cervical and throat swab analysis.

| Vaginal swabs | m/z 53 | | m/z 80 | | m/z 96 | |
|---|---|---|---|---|---|---|
| | Mean, ncps | Reproducibility % | Mean, ncps | Reproducibility % | Mean, ncps | Reproducibility % |
| Healthy controls | 3585 | 64 | 406 | 67 | 66135 | 55 |
| Gonorrhea & Chlamydia | 6213 | 73 | 112 | 92 | 246 | 49 |
| Chlamydia | 83495 | 59 | 751 | 67 | 4647 | 75 |
| Candida | 6497 | 86 | 258 | 85 | 1685 | 83 |
| Bacterial vaginosis | 5082 | 77 | 1500 | 101 | 428 | 74 |
| Candida & Bacterial vaginosis | 6900 | 69 | 46316 | 49 | 245 | 88 |
| Trichomonas | 93 | 58 | 6468 | 86 | 2463 | 67 |

| Cervical swabs | m/z 80 | | m/z 92 | | m/z 96 | |
|---|---|---|---|---|---|---|
| | Mean, ncps | Reproducibility % | Mean, ncps | Reproducibility % | Mean, ncps | Reproducibility % |
| Healthy controls | 45325 | 87 | 4530 | 103 | 412 | 99 |
| Gonorrhea & Chlamydia | 1553 | 65 | 16647 | 77 | 5521 | 85 |
| Chlamydia | 1522 | 90 | 6215 | 55 | 4621 | 94 |
| Gonorrhea | 411 | 76 | 4795 | 72 | 1222 | 88 |

| Throat swabs | m/z 53 | | m/z 80 | | m/z 104 | |
|---|---|---|---|---|---|---|
| | Mean, ncps | Reproducibility % | Mean, ncps | Reproducibility % | Mean, ncps | Reproducibility % |
| Healthy controls | 4631 | 114 | 4796 | 76 | 793 | 88 |
| Gonorrhea & Chlamydia | 6593 | 89 | 7932 | 49 | 461 | 65 |
| Gonorrhea | 747 | 74 | 121 | 55 | 4766 | 74 |

### 4.4 Discussion and summary

The VOCs emitted by the STI swabs may include alkanes, methylated alkanes, aldehydes, alkenes, ketones and nitrogen and sulphur containing compounds. The VOCs (product ions) are assumed to be protonated molecular ions or protonated molecular fragment ions as detected by PTR-ToF-MS which may also include water clusters of protonated molecular ions or protonated molecular fragment ions due to the nature of the samples which contain high moisture levels. Most of the VOCs were recurring in all three analysis. Chemical identification was attempted with tentative chemical assignments where and if possible.

Chemical identification of the significant mass peaks selected from the statistics performed could not be done for all of the peaks. Following the inspection of the data, the *m/z* values of interest shows either elevated levels in the STI swabs of one or more individuals, compared to the levels in the corresponding healthy control individuals measured from the swabs collected.

A total of 42 vaginal swabs were collected with 22 of the swabs were controls, 11 swabs positive for candida infection, 4 swabs positive for bacterial vaginosis infection and 1 swab positive for trichomoniasis. For the cervical swabs study, 32 swabs were taken in total with 23 controls, 5 chlamydia swabs and 2 gonorrhoea swabs. The throat swabs were collected from 31 female and 2 male subjects. There were 30 throat swabs treated as control (female participants), 2 gonorrhoea infected swabs and 1 tested

positive for chlamydia and gonorrhoea infections. Infected participants had either single or multiple infections.

The results from the study show that there may could be some differences in the metabolite VOC profiles of STI patients when compared to healthy controls in the vaginal and cervical swab analysis. But this is difficult to say from the small sample size. More specimens need to be included in the GU trials to reach a meaningful statistical outcome and to identify the VOCs that are produced from the metabolic changes caused by STI, specifically the positive infected individuals.

Small sample size and high variance in the VOC emission in controls and infected swabs made it difficult to interpret the data obtained. The headspace VOCs of vaginal, cervical and throat swabs were measured to investigate whether patients with STI can be distinguished from healthy controls, this was not really possible due to the small sample size. In terms of the determination of whether identification of species-specific infections was possible – most if not all infected swab samples emitted VOCs as discussed in earlier sections at about the similar intensity. High variance in the VOC emission in controls also made it much more difficult to compare with infected swabs. Itshould be highlighted that not much differences can be observed in the infected group of a single or multiple infection between each other.

The small number of samples analysed limits the ability to make reliable conclusions from the data. This pilot study shows that larger sample size is needed to come to a meaningful statistical result. If larger sample size could be achieved, more information can be gathered to determine whether patients with STI can be distinguished from healthy controls and whether identification of species-specific infections was possible. This is ‘proof of principle’ based study and if the challenges are overcome, there could be some potential to exploit STI vaginal and cervical swabs as a method for diagnosing individuals with STI.

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# Chapter 5

# VOLATILE ORGANIC COMPOUNDS IN EXHALED BREATH IN WOMEN WITH OVARIAN CANCER: A PILOT STUDY

## 5.1 Introduction

Exhaled breath contains trace quantities of VOCs that can potentially be used as markers for biochemical processes within the body [1]. Detected VOC patterns can be expected to vary to reflect different disease states. The analysis of exhaled breath offers, in principle, a very convenient method of disease diagnosis since sample collection is non-invasive, painless and does not require skilled medical staff. While there are undoubted challenges in using breath volatiles as disease markers [2], there is already evidence of their clear potential in clinical diagnosis [3].

One area where VOCs measurements in breath may contribute to clinical diagnosis is the detection of cancer [4]. Previous work has focussed on lung [5] and breast [6] cancer and in those cases diagnosis was shown to be dependent on multiple VOCs (a profile) rather than on unique compounds [7].

There has been very little work on the measurement of endogenous VOCs in exhaled breath for the detection of gynaecological cancers such as ovarian cancer [8]. Around 7,100 women are diagnosed with ovarian cancer in the UK each year. This makes ovarian cancer the 5th most common cancer in women, after breast, lung, bowel and womb cancer [9]. Many cancers are detected in late stages with consequential high mortality rates. For example, the majority of human ovarian carcinomas are diagnosed

in stage III or IV, and 70% of these patients will die within five years. Thus, it is essential to develop inexpensive and simple methods for early diagnosis [9,10].

An ongoing trial (UK Collaborative Trial of Ovarian Cancer Screening [UKCTOCS]) is assessing multimodality screening with ultrasound and CA-125 versus either ultrasound alone or no screening, with preliminary results suggesting that multimodality screening is more effective at detecting early-stage ovarian cancer [11]. However, two separate studies assessing screening with transvaginal ultrasonography and CA-125 did not find that screening increased detection of early-stage cancer and that CA-125 did not increase the detection of cancer compared with ultrasound alone [12,13]. There is an urgent need to develop new methods in screening ovarian cancer in early stages for early diagnosis and metabolic profiling of exhaled breath may contribute to this.

Reports of cancer diagnosis from canine scent detection [28] suggest that cancers may produce distinctive odours. Dogs, which are thought to have odour thresholds in the pptv range [29], have been applied to the detection of lung and breast cancers through the scent of breath samples [29]. In place of a canine nose, some studies have applied electronic noses or sensor-array technology to show that the lung cancer breath has distinct chemical characteristics [30-33]. Mass spectrometry techniques have been applied to identify some of the VOCs present in the breath of cancer patients, of which many are aliphatic, aromatic hydrocarbons, or oxygenated compounds. Phillips et al. identified a group of 22 breath VOCs to distinguish between patients with lung cancer and healthy subjects. The VOCs included alkanes, methylated alkanes, benzene derivatives, alkenes, and the aldehydes; hexanal and heptanal [5]. Similarly, Gaspar et al. reported that a group of C14 – C24 linear and branched hydrocarbons had the can be used to distinguish between healthy subjects and lung cancer patients [35].

Phillips et al. also used the measurement of alkanes and methylated alkanes in breath to identify breast cancer patients [36]. 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 [7]. The aromatic compound o-toluidine has

been identified as being elevated in the breath of lung cancer patients [38] and those with other forms of cancer [39], although its reliability as a marker has been questioned [40]. 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 [41]. The prediction of breast cancer has also been based on a group of 5 oxygen-containing breath VOCs which included heptanal and 2-propanol [42]. 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 [42]. Formaldehyde has also been detected in the breath of breast cancer patients [37] and has been identified in the urine headspace of bladder and prostate cancer patients [21]. 1-Butanol and 3-hydroxy-2- butanone were found to be elevated in the breath of patients with lung cancer [14].

A common trend now 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 VOC fingerprints [23]. The aim of this pilot study was to investigate whether significant masses that may be biomarkers of ovarian cancer could be identified from the exhaled breath VOCs, and to assess the difference between cancerous ovarian cells and benign cysts found in the patients by biopsy using the exhaled VOC profiles. A small cohort study has been undertaken and the findings are presented here.

## 5.2 Methods

### 5.2.1 Study participant details

The study was carried out between June and November 2011 at Leicester Royal Infirmary which included 7 patients with histologically confirmed ovarian cancer, 5 patients with benign cysts and 12 healthy controls (Table 5.1). The median age of the cancer patients was 59 years (range 42-67), and BMI 23 (range 21–30). The median age of the control group was 61 years (range 53-95), and BMI 27 (range 19–40). The

median age of the patients with benign cysts was 47 years (range 40-51), and BMI 30 (range 21–44). All the patients in the control group were postmenopausal women. The cancer group consisted of 6 postmenopausal women and 1 pre-menopausal woman while the benign cysts group had 3 postmenopausal women and 2 pre-menopausal women.

All participants were above 18 years old. Patients and healthy subjects who smoked, have diabetes, respiratory, thyroid, liver or renal disease or those who consumed two or more units of alcohol per day were excluded from the study. Women were also excluded if they were fasting for more than 6 hours at the time of sample collection, or if they were on weight loss diets. Also excluded were patients and healthy controls on statins, insulin, oral hypoglycaemics, HRT, hormonal contraception, thyroxin, or pharmacological doses of anti-oxidant supplements (e.g. vitamin C and E). Potential healthy subjects were ovarian cancer free and were chosen among family members of the patients accompanying them as to represent the general population. Hospital staff were not allowed to participate as healthy subjects, as the VOCs in their exhaled breath would be from clinical environment and not representing the general population. Since hospital staffs works in a controlled clinical/hospital environment, their breath will have greater concentrations of some compounds for example, acetone, ethanol, isoprene, and ammonia as reported by Boshier *et.al*. [34]. The study was approved by the Derbyshire Research Ethics Committee, and participants gave written informed consent. The paper work regarding this study is in Appendix 2. The demographics of the clinical study participants is shown in Table 5.1.

Table 5.1: Demographics of the cancer group (P1 – P7), healthy control group (C1 – C12) and the patients with benign cysts group (B1 – B5).

| Patient ID | Group | Description | Age | BMI |
|---|---|---|---|---|
| P1 | Cancer – Stage IIIc | Postmenopausal | 55 | 22.9 |
| P2 | Cancer – Stage IV | Postmenopausal | 83 | 22.2 |
| P3 | Cancer – Stage IIIc | Postmenopausal | 59 | 28.6 |
| P4 | Cancer – Stage IIIc | Postmenopausal | 67 | 20.7 |
| P5 | Cancer – Stage Ic | Postmenopausal | 65 | 25.2 |
| P6 | Cancer – Stage IIc | Postmenopausal | 53 | 21.4 |
| P7 | Cancer – Stage IIIc | Normal proliferative phase | 42 | 22.9 |
| C1 | Healthy control | Postmenopausal | 53 | 27.7 |
| C2 | Healthy control | Postmenopausal | 55 | 27.1 |
| C3 | Healthy control | Postmenopausal | 62 | 30.4 |
| C4 | Healthy control | Postmenopausal | 57 | 27.5 |
| C5 | Healthy control | Postmenopausal | 58 | 26.6 |
| C6 | Healthy control | Postmenopausal | 95 | 28.6 |
| C7 | Healthy control | Postmenopausal | 56 | 37.2 |
| C8 | Healthy control | Postmenopausal | 62 | 19.6 |
| C9 | Healthy control | Postmenopausal | 63 | 40.0 |
| C10 | Healthy control | Postmenopausal | 67 | 24.2 |
| C11 | Healthy control | Postmenopausal | 60 | 22.5 |
| C12 | Healthy control | Postmenopausal | 92 | 27.3 |
| B1 | Benign cysts | Postmenopausal | 82 | 40.5 |
| B2 | Benign cysts | Normal secretory phase | 47 | 21.4 |
| B3 | Benign cysts | Normal proliferative phase | 51 | 37.8 |
| B4 | Benign cysts | Postmenopausal | 47 | 44.3 |
| B5 | Benign cysts | Postmenopausal | 40 | 22.9 |

### 5.2.2 Off-line breath sampling protocol

All breath samples were collected in 10 L Tedlar bags (SKC Ltd., UK) with standard polypropylene fittings [14]. A total of 18 bags were available for the study, all were used bags and so had to undergo thorough flushing process before initial use in this study. All bags were labelled for identification and cleaning purposes. Before collection of breath samples, all bags were thoroughly cleaned to remove any residual contaminants by flushing three times with high-purity nitrogen. The bag was then filled again with nitrogen before heating the filled bag under a heating device, a lamp heater installed with two 500 W tungsten halogen lamps, for 20 minutes. The bag was then again was flushed three times with high-purity nitrogen. The full details of the cleaning procedure and its effectiveness can be found in the thesis of K.A. Willis [15]. The cleaning procedure as detailed above were found to be effective in reducing breath-relevant compounds up to 70% by the end of the cleaning process.

The breath sampling apparatus consisted of a disposable mouthpiece and bacterial filter which was connected to a non-return valve. The Tedlar bag was attached to this valve via a piece of flexible marprene tube. The non-return valve allowed exhaled breath flow into the bag whilst preventing any loss.

Breath samples were collected at the Gynaecology Outpatient Clinic at Leicester Royal Infirmary. Patients were rested for at least 10 minutes before breath collection. Patients were then requested to provide an end-exhaled breath sample by breathing into the mouthpiece with full and repeated exhalations, each time excluding the first portion of the breath (dead space air) until the bag was filled. The bags were sealed before removing the breath collection apparatus. All subjects were able to provide breath samples without difficulty, despite the resistance caused by the small inner diameter of the bag fitting. An ambient air sample was obtained concomitant to breath sampling by filling a separate bag using an electric pump. The samples were then transported the short distance to the Chemistry Department at the University of Leicester for VOC analysis (always within 6 hours of breath collection). The study design flow chart is shown in Figure 5.1.

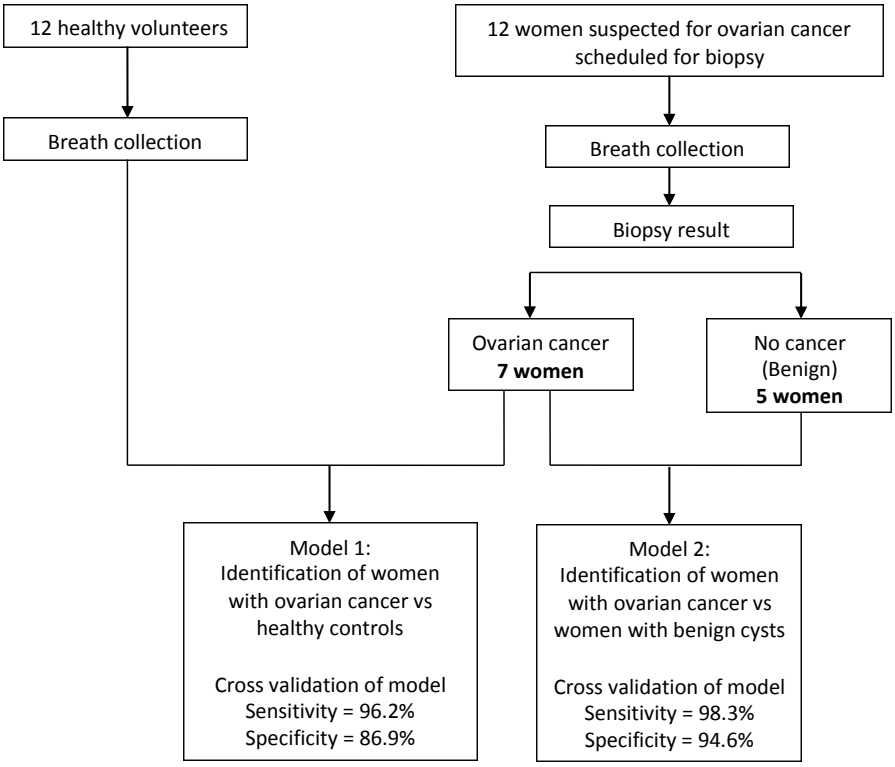


Figure 5.1 Study design

### 5.2.3 Instrumental operating settings

The Leicester-PTR-ToF-MS was used in this analysis [16-20]. The tedlar bag contents were analysed for 30 minutes (1 minute integration times, 15 – 200 amu) at an *E/N* of 80/170 Td with an headspace sampling flow rate of 220 ml $min^{-1}$ into the PTR-ToF-MS instrument. Other PTR-ToF-MS operating conditions were as follows: drift tube voltage, 1660 V; drift tube pressure, 6 mbar and drift tube temperature, 37°C.

### 5.2.4 Statistical Analysis

Each bag containing exhaled breath were analysed for 30 minutes at 1 minute integration times. Initial statistical analysis involved the use of two-sided Mann-Whitney test, in order to assess whether the mass peaks (*m/z*) identified from exhaled breaths were significantly different ($p<0.05$) from those of ambient air or which might arise from the Tedlar bags and the PTR-TOF-MS instrument. Breath samples from all of the participants were compared to ambient air measurement first and mass peaks that were found to be significantly different by Mann-Whitney were used for further comparison to exclude any possible exogenous VOCs present in the breath samples. The breath samples from cancer patients were compared to healthy controls and only VOCs significantly different were reported. Compared also were the breath samples from cancer patients to patients with benign cysts.

The identified mass peaks were assessed for variations in the overall pattern of exhaled compounds in the breath samples using a multivariate statistical analysis technique, principal component analysis (PCA) by the leave-one-out method. A leave-one-out cross-validation of the patients' classification was performed by partial least squares discriminant analysis (PLSDA), which is a multivariate regression method employed in this case to classify classes of samples based on the selected VOC masses. PLSDA was used to predict whether a patient belonged to the control group or had ovarian cancer (model 1), and ovarian cancer (malignant) or benign cysts (model 2) based on the breath VOC statistical model derived from all the other patients in the study.

## 5.3 Results and Discussion

### 5.3.1 Cohort Study

Using the Mann-Whitney tests, for ovarian cancer patients, 31 different mass-to-charge ratios (in the range of $15 \leq m/z \geq 150$) were identified as significantly different from healthy controls and from ambient air in the study (model 1). PCA biplots were then constructed using the 31 selected peaks, as illustrated in Figure 5.2. The same statistical technique were performed for comparison of ovarian cancer patients to patients diagnosed with benign cysts and based on the Mann-Whitney test, 16 peak masses were identified as significantly different which was used for further PCA and PLSDA analysis. The result of the PCA analysis of model 2 is shown in Figure 5.3. The VOCs may include alkanes, methylated alkanes, aldehydes, alkenes, ketones and nitrogen and sulphur containing compounds to identify patients with cancer from those without cancer. The VOCs (product ions) are assumed to be protonated molecular ions or protonated molecular fragment ions as detected by PTR-ToF-MS which may also include water clusters of protonated molecular ions or protonated molecular fragment ions due to the nature of the samples which contain high moisture levels.

Table 5.2 shows the selected peaks for model 1 and model 2 generated from Mann-Whitney test of significance. 13 m/z from model 2 were also identified in model 1 shows that most of the VOCs were recurring in both model and could be potential biomarkers for ovarian cancer. Chemical identification was attempted with tentative chemical assignments where possible based on previous reported VOCs of interest for cancer and PTR-MS detection of fragmented ions and protonated ions with water clusters.

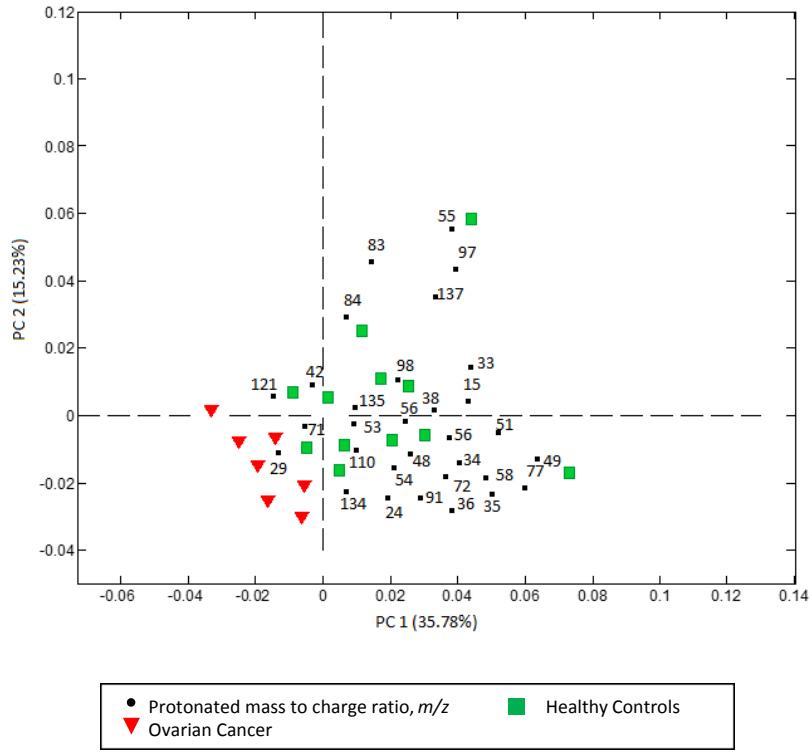


Figure 5.2 A principal component analysis biplot for the cohort study of exhaled breath from ovarian cancer patients and healthy controls. The first principal component has been plotted against the second principal component. The principal component analysis was performed using 31 *m/z* values and the data were pre-processed by auto-scaling.

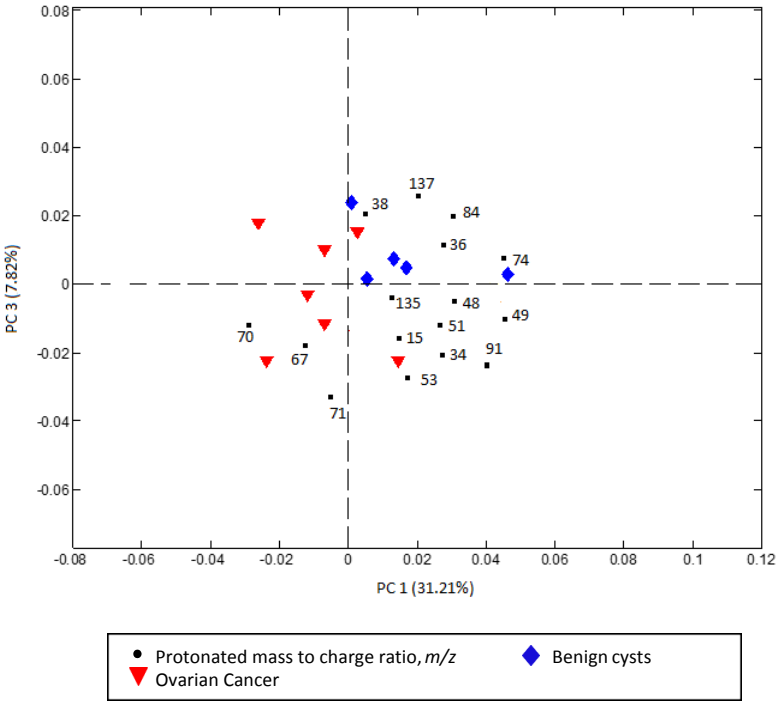


Figure 5.3 A principal component analysis biplot for the cohort study of exhaled breath from ovarian cancer patients and patients with benign cysts. The first principal component has been plotted against the second principal component. The principal component analysis was performed using 16 *m/z* values and the data were pre-processed by auto-scaling.

Table 5.2: Selected VOCs, *m/z* (M+1) used in PCA and PLSDA analysis

| **Model** | **VOC (*m/z*)** |
|---|---|
| Model 1: Ovarian vs controls | 15, 24, 29, 33, 34, 35, 36, 38, 42, 48, 49, 51, 52, 53, 54, 55, 56, 58, 71, 72, 77, 83, 84, 91, 97, 98, 110, 121, 134, 135, 137 |
| Model 2: Ovarian vs benign | 15, 34, 36, 38, 48, 49, 51, 53, 67, 70, 71, 74, 84, 91, 135, 137 |

The main finding of this study was that the breath test conducted able to identify women with ovarian cancer, when a combination of VOCs was employed in a statistical analysis, against healthy controls and women with benign cysts. The PCA biplots show that the ovarian cancer group were separated from the controls, and women with ovarian cancer were distinguishable from women with benign cysts. The cross-validation PLSDA of the breath VOCs built from the 31 selected mass peaks for model 1 predicted ovarian cancer with 96% sensitivity and 87% specificity; and for model 2, based on 16 selected mass peaks, PLSDA predicted a sensitivity of 98% and specificity of 94% in ovarian cancer.

Analysis of exhaled breath VOCs enables the understanding of biochemical processes that occurs in the body in a non-invasive way. In 1971, Pauling et al. [22] reported more than 200 volatile organic compounds in normal human breath using gas chromatography. The breath is a complex mixture of nitrogen, oxygen, carbon dioxide, water, inert gases and more importantly the trace components that may be generated in the body from metabolic processes which could provide some insights into the patient's clinical condition using breath biomarkers.

Phillips et al. used a combination of 22 breath markers that included alkanes, methylated alkanes, benzene derivatives, alkenes and aldehydes to identify patients with lung cancer from those without lung cancer [5]. In another study related to patients with breast cancer, Phillips et. al used VOC fingerprinting of breath biomarkers to

derive a model which detected correctly patients with breast cancer with a sensitivity of 75.3% and specificity of 84.8% in the training set [24].

Oxidative stress which involves increased oxygen free-radical activity in cancerous cells by lipid peroxidation [5] may explain part of the findings in this study. Lipid peroxidation breaks down cell membranes and converts the polyunsaturated fatty acids to volatile alkanes that are excreted in the breath [25]. Alkanes are also cleared from the body by oxidation to alkyl alcohols [5]. Most of the breath VOCs used in this study were either alkanes or alcohols; and high levels of alkanes in the breath have been reported previously in breast cancer [24], gastrointestinal and liver diseases [25], and lung cancer [5] which is consistent to the mechanism of oxygen free radical activity in cancerous cells.

Peak mass identification by PTR-ToF-MS is based on the detection and attribution of protonated product ions (neutral analyte molecule plus 1 amu). The resolution in our PTR-ToF-MS instrument is insufficient to distinguish ions with the same nominal (integer) mass but different accurate masses. Consequently, it is not easy to identify the specific VOC(s) responsible for a specific peak in the mass spectrum and it is impossible when two or more compounds have the same nominal mass. Identification of compounds can further be complicated by fragmentation and clustering of product ions. An attempt to identify all the number of compounds underlying the statistical analysis was not made, and therefore to do this further work would be required, such as a GC-MS analysis. Instead here a tentative identification is provided for some of the more significant peaks seen in the mass spectra.

Acetone, in this study it was tentatively identified as water clusters of acetone (*m/z* 77), are normally present at significant concentrations in breath. The acetone levels were found higher in the breath of cancer patients than of the age matched controls. Acetone is derived from lipid peroxidation and has been shown to be elevated in fasting subjects [3]. However, given that the breath samples were collected from non-fasting patients, elevated levels of acetone in the breath cancer patients may reflect increased oxidative stress. Acetone was not identified in model 2 as the concentration of acetone may be

similar in both ovarian and benign group. This finding suggests that oxidative stress is increased in women with benign cysts as well even though no tumour was detected.

Chemical identification of the significant mass peaks selected from the statistics performed could not be done for all of the peaks. Following the inspection of the data, the *m/z* values of interest shows either elevated levels in the breath of three or more individuals or at least in one cancer patient, compared to the levels in the corresponding ambient air samples measured from the breath collection site. Figure 5.4, 5.5, 5.6 and 5.7 summarises the data for each of the selected *m/z* values from the statistics performed, displaying the levels measured in the breath of each individual with the corresponding ambient levels.

*m/z* 15 levels were overall lower in the breath of the cancer group in comparison to that of the control group. Methanol (*m/z* 33) levels were significantly lower in the breath of the cancer group compared to healthy control group. This trend was also mirrored by the levels of the methanol-water cluster ions (*m/z* 51). The significance of lower level methanol detected in cancer group is unknown and the metabolic pathway that may contribute to this is still uncertain. The methanol levels in ambient air were much lower than in the breath samples.

From Figure 5.4, 5.5 and 5.6, the plots mostly shows that the levels of *m/z* of interest were higher in healthy controls compared to the cancer group and to benign cysts group. For example, *m/z* 49 which is normally assigned to methanethiol. From Figure 5.7, the last three plots displaying the measured signals from breath samples of cancer group and benign cysts patients shows that *m/z* 67 and *m/z* 70 levels were elevated in the breath measured from cancer patients in comparison to the levels found in the breath of benign cysts patients. *m/z* 67 can be tentatively assigned as disulfide. However, the opposite was observed with *m/z* 74 whereby this protonated mass was found to be higher in benign cysts patients compared to cancer patients. The levels in cancer breath patients were lower than the level found in the corresponding ambient air.

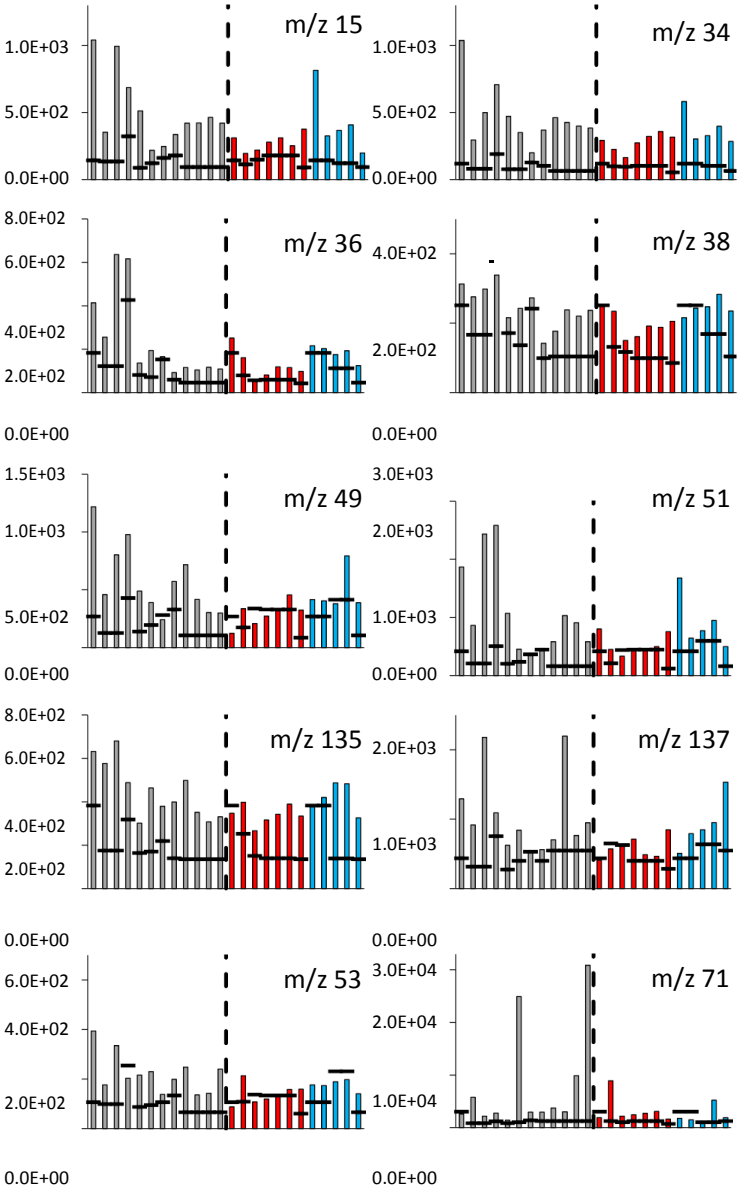


Figure 5.4 Plots displaying the measured signals (y-axis, ncps) in all of the breath samples for the 11 selected *m/z* values that were found in cancer patients, benign cysts patients and controls. The first 12 bars of each plot display the measurements for the healthy controls, next 7 bars represent the cancer patients and the 5 bars on the far right display those of patients with benign cysts. The black markers over each bar represent the corresponding ambient air levels.

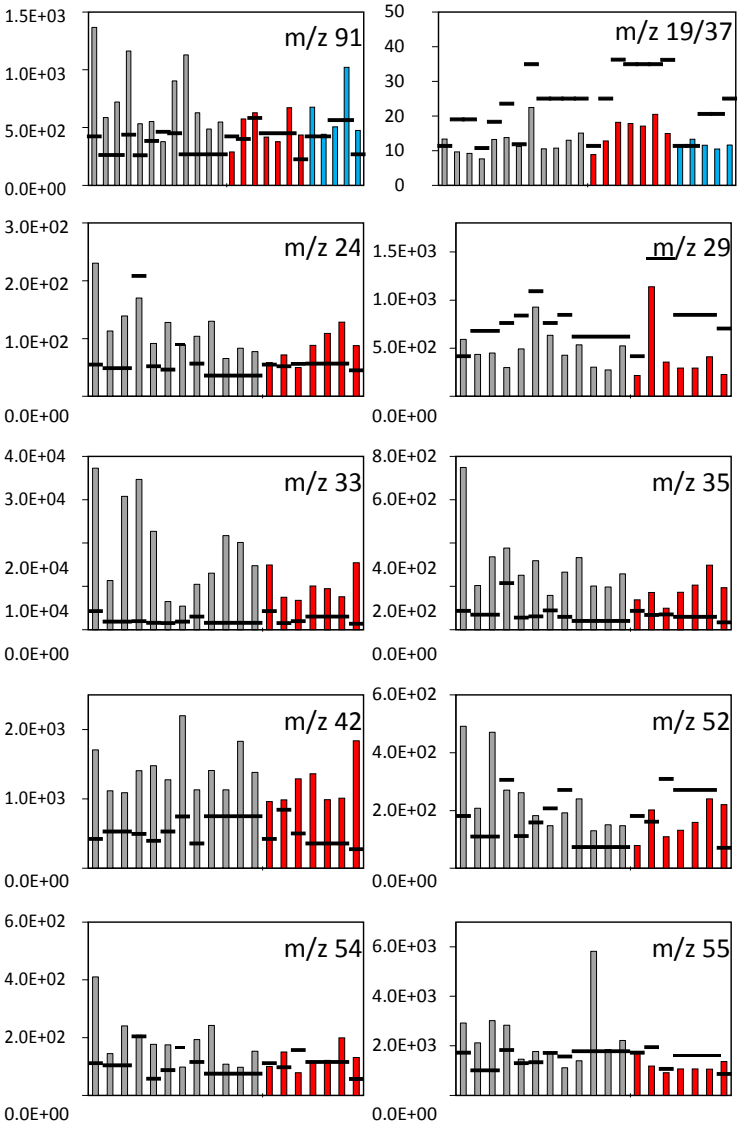


Figure 5.5 Plots displaying the measured signals (y-axis, ncps) in all of the breath samples for the 11 selected *m/z* values that were found in cancer patients, benign cysts patients and controls. The first 12 bars of each plot display the measurements for the healthy controls, next 7 bars represent the cancer patients and the 5 bars on the far right display those of patients with benign cysts. The black markers over each bar represent the corresponding ambient air levels. Also shown plot of m/z (19/37) ratio that provides an indication of sample humidity.

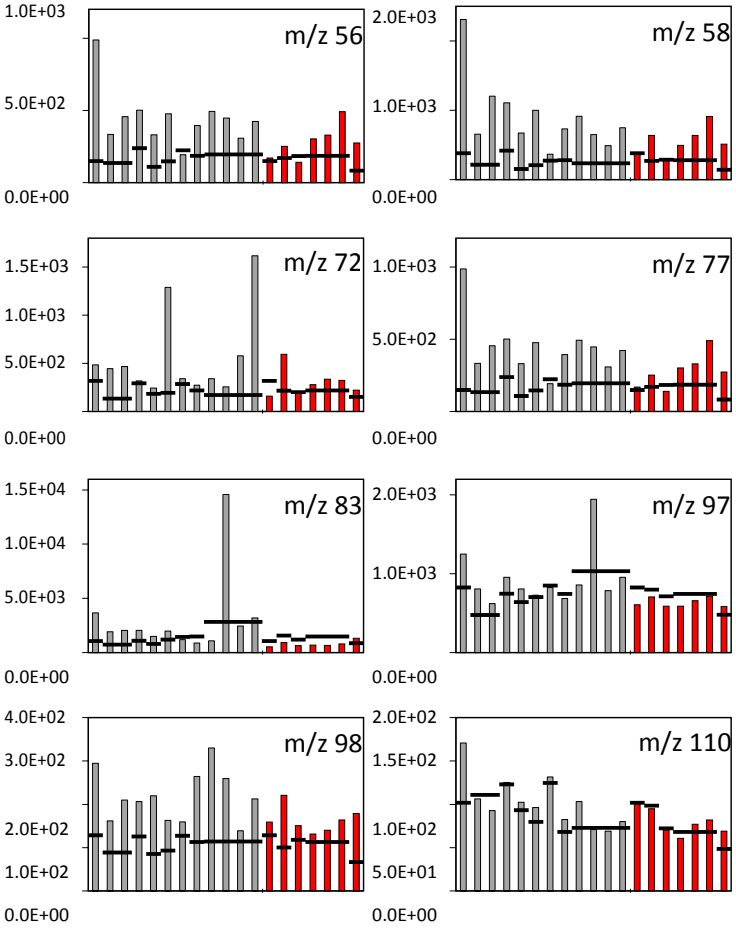


Figure 5.6 Plots displaying the measured signals (y-axis, ncps) in all of the breath samples for the 12 selected *m/z* values that were found in cancer patients and healthy controls. The first twelve bars of each plot display the measurements for the healthy controls and the seven bars on the far right represent the breath measurement from cancer patients. The black markers over each bar represent the corresponding ambient air levels.

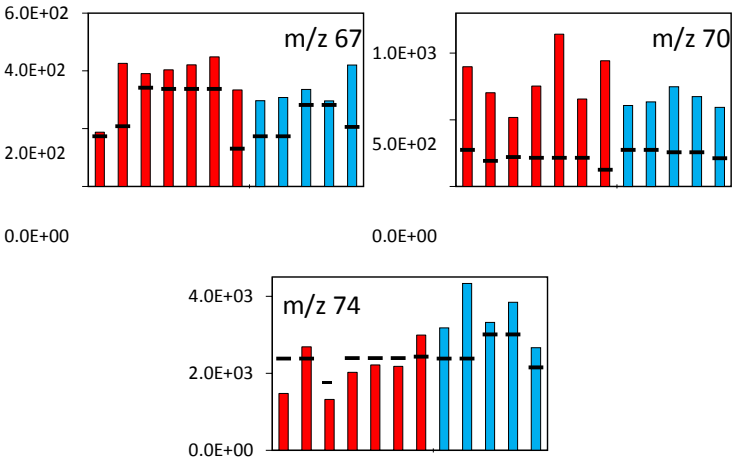


Figure 5.7 Plots displaying the measured signals (y-axis, ncps) in all of the breath samples for the 4 selected *m/z* values that were found in cancer patients and healthy controls. The first twelve bars of each plot display the measurements for the healthy controls and the seven bars on the far right represent the breath measurement from cancer patients. The black markers over each bar represent the corresponding ambient air levels. Also included 3 *m/z* values of interest displaying the measured signals in the breath samples of cancer patients and patients with benign cysts (*m/z* 67, 70 and 74).

### 5.3.2 Reproducibility Analysis

Since the study was unable to recruit large number of volunteers, the result have been analysed toward a discussion of the consistency of sampling and measurement technique. Five m/z values have been selected for analysis. Ions at m/z 33, 49, 51, 67 and 77 were selected from the selected mass peaks for both models. These ions were chosen to reflect the reproducibility of the technique over a range of intensity.

For a comparison of reproducibility it is useful to have a standard method to calculate a value by which the reproducibility may be judged. The method suggested by Bland and Altman [43] is used for this purpose. For a series of repeat measurements of the same subject, it is generally assumed that the measurements are not same caused by changes in the subject, the sample as a reflection of the subject, or the accuracy of the measurement procedure itself. For a set of repeat measurements an average value can be calculated. If the distribution of measurements is assumed to be normal, the mean should reflect a true value for the measurement. The standard deviation of these measurements will give an understanding of the reproducibility of the technique; 68 % of measurements should lie within one standard deviation of the mean. This study can examine two sources of error affecting the reproducibility; the measurement accuracy and the sample consistency. The reproducibility of measurements taken from the same sample should be affected only by the measurement accuracy. Whereas, repeat samples will be affected by the accuracy of the measurements, the sample's consistency and the patient's natural variability.

The analysis of repeat measurements from the same sample has been performed by finding the mean and variance for each set of repeats. The average variance across all samples was used to find the average standard deviation. The average of the mean was also taken for each m/z value. These values are shown in Table 5.3. In accordance with work by Bland and Altman [43], the samples reproducibility and reproducibility coefficient was also calculated and is shown in Table 5.3 for the five investigated m/z values.

Each bag containing exhaled breath were analysed for 30 minutes at 1 minute integration times. Each breath analysis was divided into ten portions, creating ten data points for each breath analysed. The identified mass peaks from 5.3.1 were used for principal component analysis (PCA) by the leave-one-out method for model 1: control group vs. ovarian cancer, and model 2: ovarian cancer (malignant) vs. benign cysts.

PCA biplots for the repeat analyses were constructed using the 31 selected peaks, as illustrated in Figure 5.8 for model 1 and for model 2 as depicted in Figure 5.9 using 16 selected *m/z*.

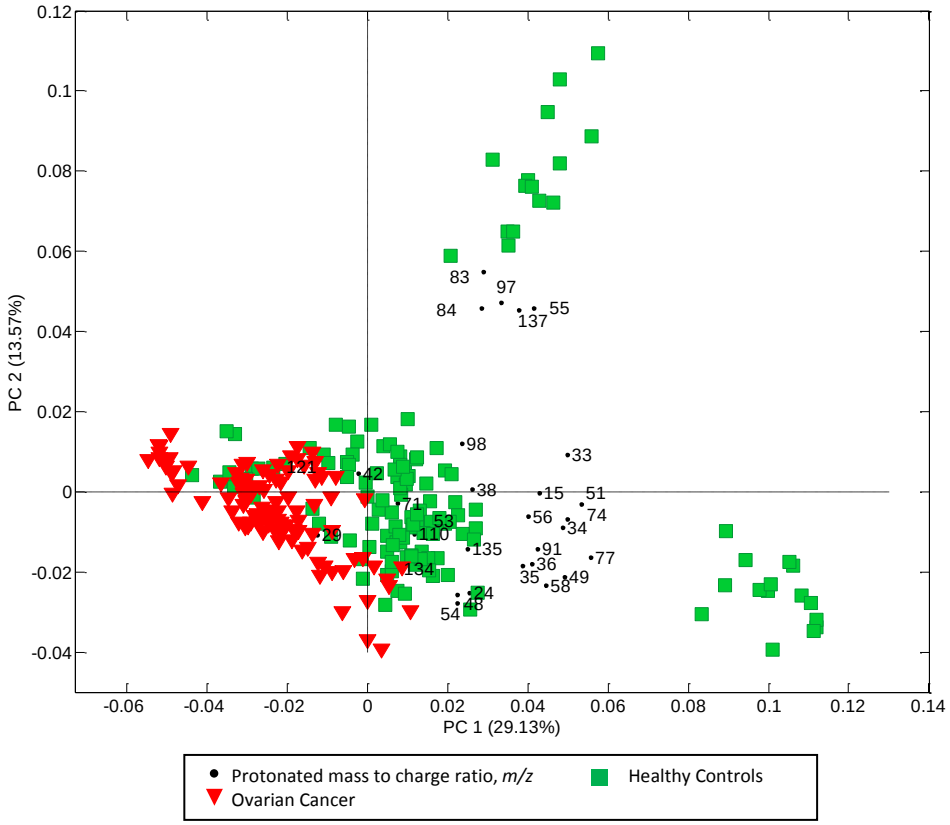


Figure 5.8: A principal component analysis biplot for the cohort study of exhaled breath from ovarian cancer patients and healthy controls. The first principal component has been plotted against the second principal component. The principal component analysis was performed using 30 *m/z* values and the data were pre-processed by auto-scaling.

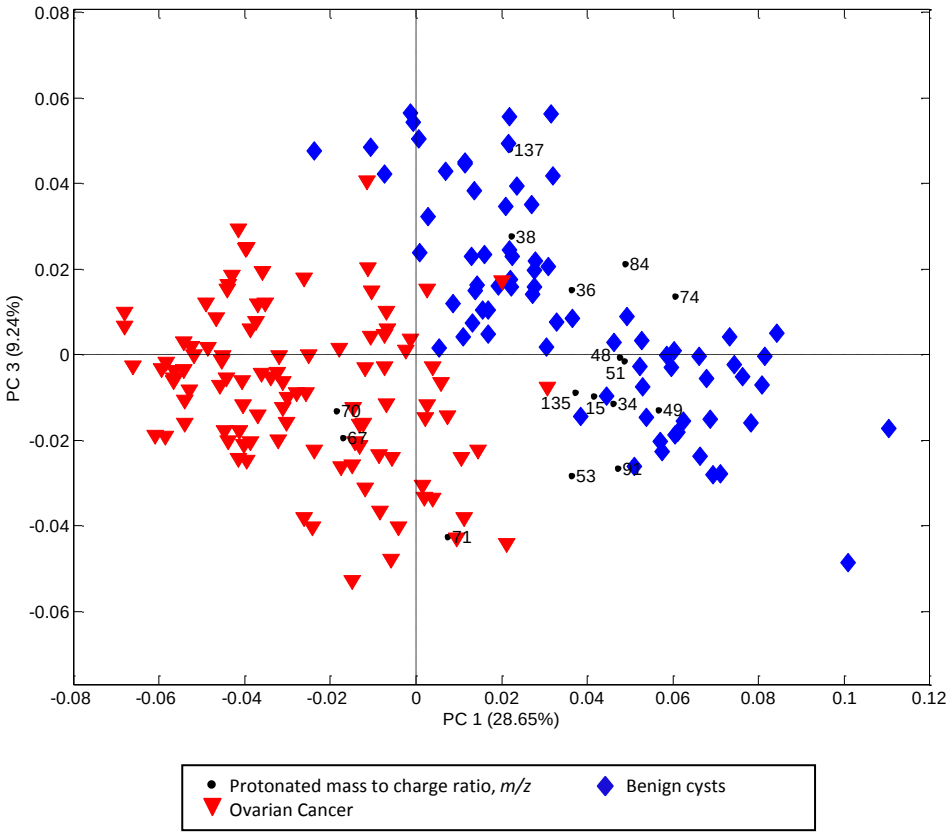


Figure 5.9: A principal component analysis biplot for the cohort study of exhaled breath from ovarian cancer patients and patients with benign cysts. The first principal component has been plotted against the second principal component. The principal component analysis was performed using 16 *m/z* values and the data were pre-processed by auto-scaling.

The measurement reproducibility is shown in Table 5.3 At higher mean signal intensity the reproducibility improves as a fraction of that mean. At greater signal intensity the statistical error in measurement becomes less effective, thus the reproducibility should improve for higher intensity ion signals. m/z 33 shows the best measurement reproducibility as a fraction of the mean and the highest signal intensity of all five compounds examined. For the benign group, all m/z measured showed good reproducibility. For the cancer group the variance were larger producing lower percentage of reproducibility. The reproducibility of the sample is less clearly dependent on the mean intensity, with the nature of the compound also affecting its reproducibility. This implies that the certain m/z in repeat samples is more variable than the others. Overall the reproducibility for all five m/z selected is quite good showing that the experiment can be repeated to obtain similar reslts in the future. With ten replicates in each sample analysed, a total of 120 measurement in control group, 70 measurement in cancer group and 50 measurement in benign group were analysed. High percentage of reproducibility observed in benign group may indicate that minimum of 50 independent samples may be needed in a study to obtain a meaningful statistic.

Table 5.3: Table showing sample reproducibility for five selected m/z values for breath analysis of control group, cancer group and benign group.

| **Group** | **m/z** | **33** | **49** | **51** | **67** | **77** |
|---|---|---|---|---|---|---|
| **Control** | Mean, ncps | 18257 | 789 | 2685 | 504 | 4089 |
| | Reproducibility % | 116 | 101 | 79 | 67 | 80 |
| **Ovarian cancer** | Mean | 14551 | 171 | 875 | 198 | 1044 |
| | Reproducibility % | 82 | 59 | 44 | 71 | 46 |
| **Benign** | Mean | 16614 | 484 | 933 | 315 | 983 |
| | Reproducibility % | 124 | 115 | 108 | 79 | 116 |

## 5.4 Summary

The results from the study show that there were differences in the breath VOC profiles of cancer patients when compared to healthy controls and women with benign cysts. Malignant tumours were clearly discriminated form benign cysts suggests partial discrimination of the ovarian cancer group from the healthy controls has been achieved, but again more patients need to be included in the breath trials to reach a meaningful statistical outcome and to identify the VOCs that are produced from the metabolic changes caused by cancer. Despite the limitation of small number of samples, encouraging results were obtained. This pilot study shows that there is potential to exploit exhaled breath as a non-invasive means for differentiating people with ovarian cancer from healthy individuals. The outcome of this study should certainly be considered as tentative but it justifies further validation studies to investigate the cancer trial.

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# Chapter 6

# SUMMARY AND FUTURE DIRECTIONS

## 6.1 Introduction

This thesis described the application of PTR-ToF-MS to the analysis of small molecular weight VOCs in bacteria and clinical studies of genitourinary infections and ovarian cancer. Most of the work performed and detailed in this thesis has been on a 'proof of principle' basis, which has provided interesting results that could be used as a starting point to initiate further investigations. During the time taken to complete the work covered in this thesis, the development of PTR-ToF-MS system for metabolic profiling has come a long way, from the design of sampling protocol and the first instrument measurements of biological specimens, to the participation in clinical trials and the search for potential markers of infections and disease.

## 6.2 PTR-ToF-MS performance

PTR-ToF-MS analysis of VOCs requires high sensitivity on a short timescale. Both PTR-ToF-MS instruments, the Leicester-PTR-ToF-MS and Medi-PTR-ToF-MS, used in the thesis was optimised through a series of calibration experiments, using a range of chemical species and sample humidity for the determination of the settings that provided the greatest sensitivity for the compounds of interest. The Leicester-PTR-ToF-MS instrument showed high sensitivity and low detection limit ranging from 0.4 – 34 ppbv for the compounds analysed for bacterial headspace analysis and for breath analysis from 0.1 – 32 ppbv for the compounds analyzed. The Leicester-PTR-ToF-MS sensitivities at 70% relative humidity using acetic acid and formic acid showed that the higher the sample humidity, the lower the amount of the compound detected. It

appears that these two compounds were highly dependent on the humidity of the sample.

The Medi-PTR-ToF-MS showed that the switch from dc to rf mode improved the sensitivity by between 1 and 2 orders of magnitude for the majority of compounds analysed. Improvements were also observed for LOD for all compounds with the shift to rf mode. In almost all cases the LOD was reduced from several hundred parts-per-trillion by volume in the dc mode to ≤200 pptv in the rf mode, and in some cases considerably better. Except for methanol and trans-2-butene, where these compounds did not fare well in terms of LOD. Methanol high LOD caused by contamination from $O_2^+$and trans-2-butene, with LOD near 350 pptv may partly caused by low polarizabilty and the lack of a permanent electric dipole moment for this molecule which will reduces the proton transfer rate. Furthermore, trans-2-butene has the lowest proton affinity of all of the molecules analysed and therefore was more susceptible to back-reaction with water vapour.

The PTR-ToF-MS has the capability to generate alternative chemical ionisation reagent ions. The use of alternative reagent ions such as $NH_4$+, $NO^+$ and $O_2^+$ may provide a means to analyse 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. As for breath analysis, the benefit of these alternative reagents for the analysis of breath has not yet been established since the high humidity causes significant interference from $H_3O^+$ and its ionisation products. Alternative reagent ions could be used if a method for suppressing the $H_3O^+$ interference could first be established.

The down side to efficiency of the instrument is mass resolution. Even though the Leicester PTR-ToF-MS have an excess of 1000 mass resolution, but it was insufficient to distinguish ions with same nominal mass. Consequently, it is was not easy to identify the specific VOC(s) responsible for a specific peak in the mass spectrums obtained and it is impossible when two or more compounds have the same nominal mass. Identification of compounds can further be complicated by fragmentation and clustering of product ions. In future, an attempt should be made to use GC-MS to

complement the PTR-ToF-MS to identify peak masses observed. As such, more information would be available for identification and interpretation purposes.

### 6.3 Bacterial headspace analysis

VOCs have been extensively studied in recent years because of their ability to be used in bacterial identification and differentiation. The qualitative and quantitative characteristics of microbial VOC patterns allow researchers to identify bacterial VOCs as sensitive and specific biomarkers for rapid bacterial detection although more research to be done to improve biomarkers discovery. The application of bacterial VOCs for developing non-invasive and rapid in-situ bacterial detection methods offers enormous promises in clinical diagnosis of infections and real time monitoring of disease development as well as the effect of treatment.

The work described in Chapter 3 was to put forward a suggestion that the detection of emitted VOCs by PTR-ToF-MS may have potential as a rapid means of identifying *C. difficile* infection. Also, the VOCs may be markers for different active metabolic pathways in specific ribotypes.

The presence or absence of VOCs in both pathogenic and non-pathogenic ribotype could provide information on the different active metabolic pathways existing in various *C. difficile* ribotypes. The different intensities of the metabolic VOCs released by the *C. difficile* ribotypes may explain the biological functions that occur in each ribotype and how this would affect infectious behaviour. This may bring some light to the question as to why R027 is far more aggressive than the other ribotypes. PCR ribotype R027 has found to be the cause of many hospital-related *C. difficile* infections and outbreaks. So, the study of VOC emissions may answer this question and data of this type may ultimately provide useful clues.

The ten *C. difficile* ribotypes were successfully distinguished from one another and the scanned VOC patterns were consistent to identify each of the ribotypes tested. The profiles of detected metabolites from the different ribotypes strongly suggested that VOC pattern profiling may provide a useful indication as to the identification of the

ribotypes investigated. The results presented here based on metabolic VOCs distinguished these ribotypes and would provide a foundation for a *C. difficile* biomarker library that could one day serve as an information base and diagnostic tool in identifying *C. difficile* infections.

The approach detailed here may lead to a clinical diagnostic test based on the VOCs released from faecal samples of patients infected with *C. difficile*. Such an approach would have many obvious advantages when coupled with PTR-ToF-MS as a rapid detection method. The investigation could also be extended to the analysis of mixed culture plates to investigate whether VOC production changes in response to the presence of other microorganisms.

Another suggestion for future studies could involve the analysis of the effect of bacteriophage on *C. difficile*. This method, once established can be used to observe bacteriophage effect on *C. difficile* that can be used to complement currently used method of absorption spectrometry to monitor bacteriophage analysis used by microbiologists. PTR-ToF-MS enables a thorough monitoring of bacteriophage attack against bacteria for a duration compared to data taken at various time-point to measure the bacteriophage and bacteria amount. Data collected from continuous monitoring may provide useful information for the interpretation of bacteriophage action. This method could be used for other bacterial and bacteriophage analysis. Some analysis was carried out on the effect of bacteriophage on *C. difficile*, where data were collected overnight for a total of six experiments but none of the data proved useful. This was caused by uncontrollable factors such as computer breakdown during data collection and contamination of the instrument from other studies.

## 6.4 Genitourinary infections - Clinical study

The genitourinary clinical study involved a group of patients with sexually transmitted infections (STI) consisting of gonorrhoea, bacterial vaginosis, trichomonas, chlamydia and candida. The headspace VOCs of vaginal, cervical and throat swabs were measured to investigate whether patients with STI can be distinguished from healthy controls, and to determine whether identification of species-specific infections was possible.

A total of 42 vaginal swabs were collected with 22 of the swabs were controls, 11 swabs positive for candida infection, 4 swabs positive for bacterial vaginosis infection and 1 swab positive for trichomoniasis. For the cervical swabs study, 32 swabs were taken in total with 23 controls, 5 chlamydia swabs and 2 gonorrhoea swabs. The throat swabs were collected from 31 female and 2 male subjects. There were 30 throat swabs treated as control (female partcipants), 2 gonorrhoea infected swabs and 1 tested positive for chlamydia and gonorrhoea infections. Infected participants had either single or multiple infections.

Certain challenges were encountered with this experiment, including:

1. The small sample size for each group did not provide enough information to distinguish infected samples from controls and the different infections analysed. Furthermore, each group had more controls than infected swabs so less information was obtained to give any distinct interpretation. Some groups had only 1 swab for one type of infection. Any weightings cannot be given to a single sample to represent a large population or substantially use it for comparison purposes.

2. Unlike breath, urine, blood or skin samples, where VOC emission from bacteria or infection can still be detected and differentiated, it seems that vaginal and cervical swabs have more complex VOC fingerprints compared to other biological/clinical specimen. This may be due to the presence of flora, other bacteria and processes occurring in the vaginal and cervical region emitting

large abundance of VOCs that overlaps the VOCs emitted by the infections in study. This complicates the interpretation of data as there is no sufficient information on control swabs and no past literature for references.

3. The control vaginal, cervical and throat swabs showed high variances of VOCs within each group. This may be caused by factors such as presence of flora which is very different for each individual in the vaginal or cervical region. As for throat swabs, factors of food intake, drink, and hygiene may contributed to the high variances within the control group. But it is interesting though that breath samples are less complicated than throat swabs as the exhalation comes through the same pathway.

Because of the challenges of small sample size, high variance in the VOC emission in controls, this made it much harder to interpret the data obtained. The headspace VOCs of vaginal, cervical and throat swabs were measured to investigate whether patients with STI can be distinguished from healthy controls. The small sample size hindered the ability to achieve this objective; and to determine whether identification of species-specific infections was possible, high variance in the VOC emission in controls made it much more difficult to compare with infected swabs. It should be highlighted that not much variances can be observed in the infected group of a single or multiple infection such as the two gonorrhoea and one gonorrhoea + chlamydia samples in the throat group analysis.

### 6.5 Ovarian cancer - Clinical study

The clinical trial of VOC analysis in exhaled breath of 7 ovarian cancer patients, 5 patients with benign cysts and 12 healthy controls was performed. The results from the study using 31 *m/z* values show that there were some differences in the breath VOC profiles of cancer patients when compared to healthy controls and women with benign cysts. Using 16 *m/z* values, malignant tumours were could be discriminated from benign cysts suggesting partial discrimination of the ovarian cancer group from the healthy controls.

This study demonstrated on a basis of 'proof of principle' that the metabolic profiling of exhaled breath may have some potential for more studies. In this pilot study, the size of this preliminary investigation was too small to reach a meaningful statistical outcome and to identify the VOCs that are produced from the metabolic changes caused by ovarian cancer. Despite the limitation of small number of samples, encouraging results were obtained. This pilot study shows that there is potential to exploit exhaled breath as a non-invasive means for differentiating people with ovarian cancer from healthy individuals and to follow the many researches done for the other breath VOC based cancer studies. The outcome of this study should certainly be considered as tentative but it justifies further validation studies to investigate the use of breath biomarkers in the general population for ovarian cancer.

### 6.6 Breath sampling

The breath sampling device used in the cancer clinical study was built out of standard, wide-bore components that produced a low-resistance, comfortable piece of apparatus. Tedlar bags with standard fittings were used for off-line breath collection and together with breath adapter allowed repeated end-exhaled breaths to be collected. Several problems with this sampling technique were found including the variability in breathing pattern, and the difficulty in monitoring the performance of the procedure during breath collection. 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 [1], and would improve 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.

Another approach would be nasal exhalation collection. A comparison study can be performed between nasal and oral breath sampling for the studies currently undertaken by the research group. This would include developing a nasal collection device, setting up breathing protocols for on-line breath analysis.

## 6.7 Online, real-time breath analysis - Diagnostics Development Unit (DDU)

The experimental work discussed in this thesis used the off-line breath analysis method as the PTR-ToF-MS instrument was not able to be located at the site of breath collection. To explore the online, real-time breath analysis especially for the cancer clinical studies, it is suggested that the facilities of the Diagnostic Development Unit (DDU) at the Leicester Royal Infirmary (LRI) be used in further investigation of the cancer clinical studies.

The DDU, is a non-invasive disease detection research facility, developed by the University of Leicester, a collaboration between Chemistry, Physics and Astronomy, Space Research Centre, Cardiovascular Sciences, Emergency Medicine, Infection, Immunity and Inflammation and IT services in August 2011. This unit is located in the Accident and Emergency Department at the Leicester Royal Infirmary (LRI). The DDU research aims to use a combination of non-invasive diagnostics methods to develop a holistic assessment of patient state, which then can be used to build better diagnostic capabilities leading to better patient care.

The DDU is based on the concept of 'smelling', 'looking' and 'feeling' diseases by using various combinations of instruments installed in a resuscitation bay at the hospital. The breath analysis instruments based on the 'smelling' concept include PTR-ToF-MS, a spirometer, gas sampling instrument for single and multiple breath measurement and a nitric oxide analyser (NOA). By utilizing the breath analysis instruments together with spectral imaging equipment and body state monitors, a multiplexed view of the patient parameters should become available which may allow the clinician to reach a diagnosis of a particular disease on a shorter time scale. The DDU project has received ethical approval for initial clinical studies of 500 patients. An initial project for study includes respiratory infections, cardiovascular dysfunction and sepsis among others. The study objectives are (1) to identify biomarkers in the exhaled breath profile of patients admitted in DDU for the conditions and diseases approved for study, and (2) to investigate the exhaled breath profile of normal healthy individuals.

The breath analysis incorporates the use of PTR-ToF-MS for the analysis of VOCs in exhaled breath to detect the metabolic products and underlying body states. Nitrogen oxides measures $NO_x$ and exhalation pressure/flow to monitor patient's breath exhalation. The capnograph (spirometer) installed in the breath sampling adapter measures $CO_2$ level in the breath exhaled by patients. The single and multilple analyser collects single deep breath exhalation and multiple breath exhalation respectively, together with $CO_2$ level, exhalation pressure and flow. The instruments were moved to the LRI late August 2011 for setting up before starting clinical studies in October 2011. Before clinical studies can be initiated, a breath sampling protocol and instrumentation set-up was developed.

The DDU research facility comprises of two separate rooms. The patient bay has the bed where the patient will be sitting or lying down, the single and multiple breath adapters connected to the gas sampling instruments (GSI), and a laptop to control the NOA instrument and record nitric oxide measurement. The equipment room, which is next door, houses the PTR-ToF-MS instrument, pumps and gas cylinders. Single and multiple breath adapters are connected to the GSI using 1/8 inch tubing line which is heated to 40 °C to minimize breath condensation in the sampling lines. A toggle valve installed between the single and multiple breath sampling line for ease of switching sampling lines between single and multiple breath measurement.

The planned online breath sampling protocol begins with the research nurse explaining to the patient of the breathing technique required and allows the patient to practice before the actual sampling. The patient is required to provide a repeated tidal exhalation over a defined amount of time for the multiple breath analysis and a single forced exhalation for the full exhalation breath analysis. The breath sampling adapters are installed with disposable bacterial filter mouth pieces to prevent contamination from one patient to another. Every participant will be required to wear the nose clip during the breath collection. A background spectrum is acquired using the PTR-ToF-MS. Once the nurse has recorded the patient ID on the instruments, the sampling is performed by starting NOA and GSI-multiple acquisition whereby the GSI-multiple triggers the PTR-TOF-MS program for analysis. After the multiple breath measurements are taken, the instrument is prepared for the single breath exhalation

sampling. The same instrumentation protocol is used but this time single breath exhalations are taken using the GSI-single breath adapter. This will be repeated two more times to obtain three replicates of single breath samples.

### 6.8 Final comments

The work covered here represents exploratory studies that tested the capabilities of PTR-ToF-MS for metabolic analysis research. While a number of interesting preliminary results have been reported, follow-up investigations are required to substantiate these early findings. The bacterial studies provided new data input and new ways to analyse and monitor bacterial behaviour that could provide useful information for researchers in better understanding the metabolic pathways in bacteria. For the clinical studies, long-term studies and larger groups of participants may enable better profiling of disease and infection biomarkers. The development of standardised breath sampling protocols is currently one of the key goals for the field of breath research. This is a necessity for breath tests to progress into clinical practice, but will allow the comparison of data between different research groups and may promote multicentre research trials. The pursuit of bringing metabolic biomarkers 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 potentially saving lives in the process through early detection and treatment. As such, metabolic profiling for medical diagnosis is both a highly interesting and highly rewarding field of research.

**References**

1. O'Hara ME, O'Hehir S, Green S, Mayhew CA. (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 1 – Right to access to conduct research through University Hospitals of Leicester NHS Trust**

University Hospitals of Leicester NHS
NHS Trust

Research & Development Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

**DIRECTORATE OF RESEARCH & DEVELOPMENT**

**Director:** **Professor D Rowbotham**
**Assistant Director:** **David Hetmanski**
**R&D Manager:** **Carolyn Maloney**

Direct Dial: (0116) 258 4199
Fax No: (0116) 258 4226

3rd February 2012

Mrs Sharmilah Kuppusami
PhD Student
Department of Chemistry
University of Leicester
LE1 7RH

Dear Sharmilah,

This letter confirms your right of access to conduct research through **University Hospitals of Leicester NHS Trust** for the purpose and on the terms and conditions set out below. This right of access commences on **3rd February 2012** and ends on **30th September 2014** unless terminated earlier in accordance with the clauses below.

You have a right of access to conduct such research as confirmed in writing in the letter of permission for research from this NHS organisation. Please note that you cannot start the research until the Principal Investigator for the research project has received a letter from us giving permission to conduct the project.

The information supplied about your role in research at **University Hospitals of Leicester NHS Trust** has been reviewed and you do not require an honorary research contract with this NHS organisation. We are satisfied that such pre-engagement checks as we consider necessary have been carried out.

You are considered to be a legal visitor to **University Hospitals of Leicester NHS Trust** premises. You are not entitled to any form of payment or access to other benefits provided by this NHS organisation to employees and this letter does not give rise to any other relationship between you and this NHS organisation, in particular that of an employee.

While undertaking research through University Hospitals of Leicester NHS Trust, you will remain accountable to your Employer **University of Leicester** but you are required to follow the reasonable instructions of **Professor Tim Coats** in this NHS organisation or those given on her/his behalf in relation to the terms of this right of access.

Where any third party claim is made, whether or not legal proceedings are issued, arising out of or in connection with your right of access, you are required to co-operate fully with any investigation by this NHS organisation in connection with any such claim and to give all such assistance as may reasonably be required regarding the conduct of any legal proceedings.

You must act in accordance with **University Hospitals of Leicester NHS Trust** policies and procedures, which are available to you upon request, and the Research Governance Framework.

You are required to co-operate with **University Hospitals of Leicester NHS Trust** in discharging its duties under the Health and Safety at Work etc Act 1974 and other health and safety legislation and to take reasonable care for the health and safety of yourself and others while on **University Hospitals of Leicester NHS Trust** premises. You must observe the same standards of care and propriety in dealing with patients, staff, visitors, equipment and premises as is expected of any other contract holder and you must act appropriately, responsibly and professionally at all times.

You are required to ensure that all information regarding patients or staff remains secure and *strictly confidential* at all times. You must ensure that you understand and comply with the requirements of the NHS Confidentiality Code of Practice (http://www.dh.gov.uk/assetRoot/04/06/92/54/04069254.pdf) and the Data Protection Act 1998. Furthermore you should be aware that under the Act, unauthorised disclosure of information is an offence and such disclosures may lead to prosecution.

You should ensure that, where you are issued with an identity or security card, a bleep number, email or library account, keys or protective clothing, these are returned upon termination of this arrangement. Please also ensure that while on the premises you wear your ID badge at all times, or are able to prove your identity if challenged. Please note that this NHS organisation accepts no responsibility for damage to or loss of personal property.

We may terminate your right to attend at any time either by giving seven days' written notice to you or immediately without any notice if you are in breach of any of the terms or conditions described in this letter or if you commit any act that we reasonably consider to amount to serious misconduct or to be disruptive and/or prejudicial to the interests and/or business of this NHS organisation or if you are convicted of any criminal offence. Your substantive employer is responsible for your conduct during this research project and may in the circumstances described above instigate disciplinary action against you.

**University Hospitals of Leicester NHS Trust** will not indemnify you against any liability incurred as a result of any breach of confidentiality or breach of the Data Protection Act 1998. Any breach of the Data Protection Act 1998 may result in legal action against you and/or your substantive employer.

If your current role or involvement in research changes, or any of the information provided in your Research Passport changes, you must inform your employer through their normal procedures. You must also inform your nominated manager in this NHS organisation.

Yours sincerely

Carolyn Maloney

**Carolyn Maloney**
**R&D Manager**

**cc:** **Copy to UoL HR (Lesley Green)**
**Nicola Junkin HR UHL**
**Copy for File**
**Tim Coats UHL**

## Appendix 2 – Ethical approval for Genitourinary infections - Clinical study

NHS
Health Research Authority

**NRES Committee East Midlands - Leicester**
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839435
Fax: 0115 8839294

19 October 2012

Dr Vendela McNamara
Associate Specialist
University Hospitals Leicester
Dept Genitourinary Medicine
Leicester Royal Infirmary
Infirmary Square
LE1 5WW

Dear Dr McNamara

**Study title:** **Use of Real Time Air Finger-printing Technology (RAFT) in the Diagnosis of Female Genitourinary (GU) Infections**
**REC reference:** **12/EM/0063**
**Protocol number:** **Pending**
**Amendment number:** **7**
**Amendment date:** **02 October 2012**

The above amendment was reviewed on 17 October 2012 by the Sub-Committee in correspondence.

**Ethical opinion**

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

**Approved documents**

The documents reviewed and approved at the meeting were:

| Document | Version | Date |
|---|---|---|
| Participant Consent Form: Group 2 | 4 | 01 October 2012 |
| Participant Consent Form: Group 1 | 4 | 01 October 2012 |
| Participant Information Sheet: Group 2 | 3 | 01 October 2012 |
| Participant Information Sheet: Group 1 | 3 | 01 October 2012 |
| Protocol | 6 | 10 February 2011 |
| Notice of Substantial Amendment (non-CTIMPs) | 7 | 02 October 2012 |

**Membership of the Committee**

The members of the Committee who took part in the review are listed on the attached sheet.

**R&D approval**

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| 12/EM/0063: | Please quote this number on all correspondence |
|---|---|

Yours sincerely

**Dr Carl Edwards**
**Chair**

E-mail: georgia.copeland@nottspct.nhs.uk

*Enclosures:* *List of names and professions of members who took part in the review*

*Copy to:* *Ms Carolyn Maloney, University HOspitals Leicester*

## Appendix 2 – Consent form for Genitourinary infections - Clinical study

Version 4 1/10/12

Centre Number:

Study Number:

Patient Identification Number for this trial:

**CONSENT FORM**

| | |
|---|---|
| Title of project: | Use of Mass Spectrometry in diagnosis of Genitourinary Infections |
| Name of Researcher: | Dr V McNamara, Department of GU Medicine, University Hospitals of Leicester |

Please initial box

1. I confirm that I have read and understand the information sheet "Participant Information Sheet, patient group 2" dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving reason, without my medical care of legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and/or data may be looked at by responsible individuals from the study team, the Sponsor, NHS Trust or from regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to access my clinical records ☐

| | | |
|---|---|---|
| Name of patient | Date | Signature |
| Name of person taking consent | Date | Signature |

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

## Appendix 2 – Patient information sheet - Genitourinary infections - Clinical study

**Patient Information Sheet: Use of Mass Spectrometry in Diagnosis of Genitourinary Infections (Patient Group 2)**

We would like to invite you to take part in our research study. Before you decide, we would like you to understand why the research is being done and what it would involve for you.
One of our team will go through the information sheet with you and answer any questions you may have. This should take about 10 minutes.
If you agree to take part we will ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you would normally receive.

**Purpose of the Study**

The purpose of this study is to look at possible new ways of diagnosing genitourinary infections and STIs (sexually transmitted infections) using a technique called Mass Spectrometry (MS). The equipment used has been developed by a research team at the University of Leicester.
At the moment most sexually transmitted genital and throat infections are diagnosed by sending samples to the laboratory, and waiting 2-3 weeks for results.
MS looks at different gases present in a sample. Some infections in the vagina, such as bacterial vaginosis, are known to give off certain gases which can be identified by mass spectrometry. This study aims to see if other genital infections such as Chlamydia, gonorrhoea, Trichomonas and thrush can also be identified this way. It will also aim to see whether infections in the throat can be found on breath testing.
One advantage of MS is that results come up straight away, and so if this technique works, patients in the future would be able to get an instant diagnosis and be treated straight away.

**What Does Taking Part Involve?**

As you have attended the GU Clinic today for treatment of a known infection, you have been invited to take part in this study. Normally you would not be asked to have another examination today, but if you decide to take part in this study, you would be examined by a doctor to obtain vaginal and throat swab sample for the study (if you are female) or just a throat swab (if you are male). You will also be asked to provide a breath sample into a bag. These samples should only take an extra ten minutes of your time.
Involvement in this study will have no effect on the care you would normally receive at this clinic.

**Confidentiality**

All research records will be kept confidential. All samples taken from you will be labelled with a confidential patient number and not your name.
Your GP will not be informed of your involvement in this study.

**Further Information**

The costs of this study are covered by the Department of GU Medicine, University Hospitals Leicester.
All research in the NHS is looked at by an independent group of people called a Research Ethics Committee to protect your interests. This study has been reviewed and given a favourable opinion by the East Midlands-Leicester Research Ethics Committee.
Should you require any further information about your involvement in this study please contact Dr V. McNamara, c/- Department of GU Medicine, University Hospitals Leicester, Tel 0116 258 6653.
If you have any general questions or concerns about patient involvement in research, you can also contact the Patient Information and Liaison Service (PILS) of the Leicester Royal Infirmary on 0116 2588928.

**Version 3: 1/10/12**

## Appendix 2 – Study protocol sheet - Genitourinary infections - Clinical study

STUDY PROTOCOL:
Use of Mass Spectrometry in Diagnosis of Genitourinary Infections

Introduction:
Mass spectrometry (MS) is an analytical technique which can be used to assess volatile organic compounds (VOCs) in complex gaseous mixtures.
One common genitourinary (GU) infection, Bacterial Vaginosis (BV) is already known to be associated with release of amines detectable on vaginal discharge specimens by mass spectrometry, but VOCs from vaginal secretions associated with other GU infections (Candida, Chlamydia, gonorrhoea and Trichomoniasis) are not known.
Mass spectrometry has been used experimentally in the analysis of breath specimens from patients with respiratory disease and metabolic disorders. Bacterial Vaginosis has been found to be associated with changes in oral microflora, possibly secondary to receptive oral intercourse. Chlamydia and Gonorrhoea may be acquired in the throat following receptive oral intercourse. Breath analysis changes occurring with sexually acquired oral infections are completely unknown.
If detectable changes are found, MS offers the possibility of point of care testing for immediate diagnosis of STIs. Time delay in current laboratory diagnostic techniques for STIs carries the risk of delayed treatment and onward spread of infection in the community.

Aim:
Feasibility study to determine whether MS can assess differing patterns of volatile organic compound emission in common GU infections compared to healthy controls.

Method:
The potential diagnostic capabilities of MS will be analysed in a small feasibility study using cases and controls to establish sensitivity and specificity data which may then be used to calculate sample size in a subsequent larger scale study. It is hoped to recruit 50 women with Candida, 50 women with BV and as many women as possible with Chlamydia, Trichomonas and gonorrhoea over a 12 /12 period along with 100 negative controls. In addition male patients with positive throat swabs for gonorrhoea and Chlamydia will be recruited.
The GU infections listed are currently diagnosed by means of swab-taking during speculum examination at the GU Medicine Clinic. BV is diagnosed by microscopy in clinic, Candidiasis and Trichomoniasis by microscopy in clinic along with laboratory culture while Chlamydia and gonorrhoea are diagnosed by means of Nucleic Amplification Tests (NAATs) in the laboratory, with an average turnaround time of 2 weeks.
Prior to recruitment of participants, a culture sample obtained from the hospital microbiology laboratory for each of the infections under investigation will be analysed by MS in the atmospheric chemistry laboratory based at Leicester University. Positive cultures for Candida, trichomonas and gonorrhoea will be used to obtain baseline data on the potential pattern of VOCs which will inform assessment of subsequent clinical specimens. Culture is not undertaken in the diagnosis of BV and Chlamydia, and baseline data for these infections will be based on clinical specimens once the study has commenced. The atmospheric chemistry laboratory already processes other bacterial cultures in their equipment and have appropriate health and safety protocols for the handling of these specimens.

Recruits will fall into two groups. In the first, women attending the Department of GU Medicine for infection screening will be invited to participate in the study at the time of their initial appointment. An extra swab will be taken at the time of examination for MS analysis. All women will also be asked to provide a breath sample into a sealed plastic bag. Those women found on the day of attendance to have Candida or BV on microscopy will have samples analysed by MS for possible changes.
Asymptomatic women attending for screening will be recruited as controls.
In the second group, women diagnosed with genital or oral Chlamydia, gonorrhoea and Trichomonas in the laboratory, and men diagnosed with oral Chlamydia or gonorrhoea will be asked to provide samples for MS when re-attending clinic for treatment. This will require a second speculum examination for women to obtain MS swabs and throat swabs if not taken at their first appointment . Male patients with oral infection will be asked to provide a second throat swab for MS analysis. A breath sample will also be requested from patients in this group.
Swabs and breath samples for MS analysis will be processed in the MS equipment located onsite at the LRI Casualty Department, where they will be analysed by an atmospheric chemist with expertise in MS. Specimens will be transported by a member of the research team to the MS unit, and study participants will not be required to leave the Department of GU Medicine. In some instances the specimens may have to be transported by a member of the research team to the laboratory at Leicester University.
Recruits found positive on standard tests for any of the abovementioned infections will have MS analyses compared to those results for negative controls, and any differing patterns of VOCs identified. If changes are found on MS analysis, sensitivities and specificities will be calculated, and used as the basis for sample size calculation in a subsequent larger scale study.

Version7, 1/10/12

Appendix 2 – Survey form for Genitourinary infections - Clinical study

## Use of Mass Spectrometry (MS) in Diagnosis of Genitourinary Infections: Case Report Form

## UHL Study Ref: UHL 11158

**Date:**

**Participant ID:**

**Clinical History:**

**Slide Results:**

Gram stain:

Wet Prep:

**Laboratory Results:**

Throat Swab:

Endocervical Swab:

High Vaginal Swab:

BBI Screen:

**MS Samples:**

Throat:

Cervix:

Vagina:

Breath:

## Appendix 3 – Consent form for Ovarian cancer - Clinical study

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 254 1414
Fax: 0116 258 5631
Minicom: 0116 258 6878

# CONSENT FORM

*Three copies to be made: one for Participant, one for researcher, one for hospital notes*

**Measurement of Volatile Organic Compounds in Exhaled Breath in women: a pilot study**

Chief Investigator: Mr M Habiba,
Principal researchers: Dr A Akkad, Dr S Julian,
Co-investigators: Ms Kerry Willis, Professor P Monks and Dr Andrew Ellis

**Participant to initial each box – then sign and date this form**

- I have read and understand the study information sheet (version 1.0 dated 3rd January 2007). I have been given the chance to ask questions. ☐
- I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
- I understand that my research records may be looked at by people from organisations that monitor the way medical research studies are performed. I agree that these individuals can look at my records. ☐
- I understand that I can withdraw from the study at any time by advising the investigators named on this form. ☐
- I agree to take part in the above study. ☐

**Person taking consent and principal researcher to sign and date this form**

| | | |
|---|---|---|
| Name of Participant | Date | Signature |
| Name of Person taking consent (*if different from researcher*) | Date | Signature |
| Researcher | Date | Signature |


Trust Headquarters, Gwendolen House, Gwendolen Road, Leicester, LE5 4QF
Website: www.uhl-tr.nhs.uk

## Appendix 3 – Survey form for Ovarian cancer - Clinical study


**Measurement of Volatile Organic Compounds in Exhaled Breath in Women – A Pilot Study**

| | |
|---|---|
| Patient ID: | Date: |
| Patient sample bag code number: | Time of collection: |
| Background air bag code number: | Time of collection: |
| Age: | |
| Weight: | |
| Height: | |
| Parity: | |

**Meal/Drink:**

| | |
|---|---|
| Time of last meal/drink: | |
| Weekly alcohol intake: | *exclude if >2-3 units/day |

**Health information:**

| | |
|---|---|
| Smoker: | *exclude |
| Airway symptoms: | *exclude |
| Diabetes: | *exclude |
| Liver disease: | *exclude |
| Renal disease: | *exclude |
| Thyroid disease: | *exclude |
| LMP/age at menopause: | |

**Medication:**

| | |
|---|---|
| Current medication: | *exclude statins/insulin/ hypoglycemics |
| Antioxidant supplements: (e.g. vitamin C and E): | *exclude |

University of Leicester — atmospheric chemistry

**Primary Diagnosis:**

1- Normal menstrual cycle
   Proliferative phase (sample 1)
   Secretory phase (sample 2)

2- Postmenopause
   HRT *exclude

3- Ovarian cancer
   Stage of disease

4- Endometrial cancer
   Stage of disease

5- Pregnancy
   1st trimester
   2nd trimester
   3rd trimester

6- Preeclampsia
   Gestational week
   Blood pressure (ACOG criteria)
   Proteinuria (ACOG criteria)

7- IUGR
   Gestational week
   Growth centile (customised growth centile)

**Measurement of Volatile Organic Compounds in Exhaled Breath in women - a pilot study**

| | |
|---|---|
| Study Serial Number | |
| Age | |
| Weight | |
| Height | |
| Parity | |
| Smoker | * exclude |
| Weekly alcohol intake | * exclude if >2-3 units/day |
| Diabetes | * exclude |
| Time last meal/drink | * exclude if >6 hours ago |
| Current medication | |
| Statins/insulin/oral hypoglycemics | * exclude |
| Airway symptoms | * exclude |
| Antioxidant supplements (e.g. vitamin C and E). | * exclude |
| Liver disease | * exclude |
| Renal disease | * exclude |
| Thyroid disease | * exclude |
| LMP/age at menopause | |
| **Primary Diagnosis** | |
| 1- Normal menstrual cycle | |
| Proliferative phase (sample 1) | |
| Secretory phase (sample 2) | |
| 2- Postmenopause | |
| HRT | * exclude |
| 3- Ovarian cancer | |
| Stage of disease | |
| 4- Endometrial cancer | |
| Stage of disease | |
| 5- Pregnancy | |
| 1st trimester | |
| 2nd trimester | |
| 3rd trimester | |
| 6- Preeclampsia | |
| Gestational week | |
| Blood pressure (ACOG criteria) | |
| Proteinuria (ACOG criteria) | |
| 7- IUGR | |
| Gestational week | |
| Growth centile (customised growth centile) | |

## Appendix 3 – Patient information sheet for Ovarian cancer - Clinical study

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 254 1414
Fax: 0116 258 5631
Minicom: 0116 258 6878

**Measurement of Volatile Organic Compounds in Exhaled Breath in women - a pilot study**

You are invited to take part in this study. The study aims to examine whether some medical conditions can be diagnosed by analysing small molecules in the breath.

**What is the purpose of the study?**

Our study will look to see if analysis of 'breath' can give an indication of disease. We will analyse the pattern of volatile compounds expired in the breath from normal women and from some ill patients. We will then compare the patterns to see in what way they are different. We may then be able to develop this into a test that could be used in medical diagnosis.

**Why have I been chosen?**

You have been chosen because you are either healthy or because you have one of the conditions we wish to investigate. In order to make valid comparisons we need to exclude some groups of women like those with diabetes, smokers and those who drink too much alcohol.

**Do I have to take part?**

It is up to you whether or not you want to take part. If you decide not to take part we won't ask why.

If you decide to take part, you can still leave our study any time you want, without saying why. If you leave we will keep any information we have already collected. This information won't have your name on it, so nobody will know it is about you.

**How much time will it take up if I join your study?**

The whole study will take a few minutes. You need to have rested for a few minutes after your journey to hospital. You also need to read the information leaflet and sign the consent form.

**Can you give me more details about what will happen?**

The study involves you providing a 'breath test' by simply blowing into a disposable mouthpiece or a collection bag. We will use a special machine to analyses the breath. We will then compare the breath pattern and examine how it relates to your clinical condition (whether you are healthy or have a clinical condition or illness). We will need some information about your health. Mostly, this will be information you can provide yourself, but we may need to look into your medical notes to verify some information or to obtain exact blood or test results.

For women with medical problems we will ask for one breath sample. Women who do not have health problems (normal controls) will be asked for two samples one before and one after menstruation.

**Are there disadvantages or risks if I join your study?**

There are no disadvantages to you from joining the study, apart perhaps from the imposition on your time. We will arrange the breath test at the same time as you're coming to hospital for other reason(s).

**Are there benefits if I join your study?**

There are no benefits for you personally, but the information we get from this study may lead to improved treatment in the future.

### What if something goes wrong?

We think it is very unlikely that anything will go wrong in our study, so we have made no special arrangements for compensation in this study. If you are harmed because someone is negligent, you may be able to take legal action – but you might have to pay for it.

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, or your treatment, the normal National Health Service complaints mechanisms is available to you. You should contact the Patient Advice and Liaison Service on 0116 258 8295.

### Will my details be kept confidential?

Nobody will find out from us that you have taken part in this study. Information about you will be kept strictly confidential, and your name and address will be taken off before the sample is analysed so no-one will know it is about you. The result of your test will not be disclosed to anyone. When we analyse the sample it will have a study reference number only.

### What will happen to the results of your study?

The results will be presented at medical conferences and published in medical journals. All this published information will be anonymous – you will not be identified in any way.

It may be quite a while before we present any information in this way. If you want to know the results of our study, tell us and we will send them to you as soon as they are ready

### Who is organising the study?

Dr Marwan Habiba is organising the study. He is a Senior Lecturer and gynaecologist working at the University of Leicester.

### Has anyone approved the study?

We have been given a favourable opinion by an NHS Research Ethics Committee. Studies like ours can't go ahead without being approved by an NHS Research Ethics Committee if they involve NHS patients or staff, information from NHS medical records, NHS premises or NHS facilities.

This approval does not guarantee your safety. But it does mean the Ethics Committee believes your rights will be respected and that risks have been reduced to a minimum and balanced against possible benefits. The Ethics Committee also checks you have been given the information you need to make an informed choice about whether or not you want to join our study.

### Where can I go for more information

If you have any questions, you can talk us on the numbers below:
Mr Marwan Habiba Principal Investigator Consultant Obstetrics & Gynaecology 0116 2523170
Dr Andrea Akkad Consultant Obstetrics & Gynaecology Tel 01162586962
Dr Sophia Julian Specialist Registrar Obstetrics & Gynaecology Tel 01162541414

***Thank you again for your interest in our work***

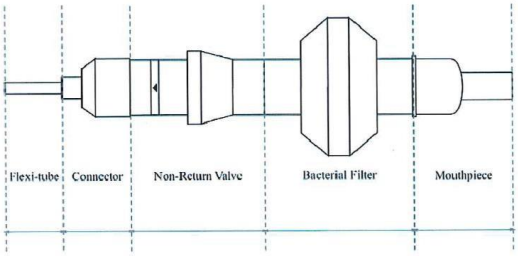
## Appendix 3 – Breath collection procedure sheet for Ovarian cancer - Clinical study

University of Leicester — atmospheric chemistry

**Breath Collection Procedure**

***Equipment:***

a) Breath collection apparatus (as shown below)
b) 1 clean 10 L Tedlar® bag per patient
c) 1 clean 10 L Tedlar® bag per sampling group
d) Pump for background air sampling




The breath collection apparatus for use with Tedlar® sample bags

***Breath Sampling:***

1. Patients should be rested for at least 10 minutes before breath collection. While waiting, fill out the details in the form.
2. Note down the Tedlar® bag code number for the patient breath collection.
3. Attach a new mouthpiece and bacterial filter to the breath collection apparatus and connect the flexi-tube to the Tedlar® bag nozzle.
4. Open the tap on the Tedlar® bag (unscrew the polypropylene tap 1½ full turns).
5. Note the time of breath collection on the bag.
6. The patient can then provide an end-exhaled breath by breathing into the mouthpiece:

b. Take full and repeated exhalations, each time excluding the first portion of the breath until the bag is 80% full (do not overfill).
c. A small amount of resistance may be experienced; however subjects should be able to provide samples without difficulty.

7. Close the tap on the Tedlar bag before removing the breath collection apparatus.
8. Dispose of the mouthpiece and bacterial filter.
9. Remove the non-return valve from the apparatus and flush before re-using (use air from the pump outlet, or purified air or nitrogen if available).
10. Store the sample bag in a black liner until analysis.

***Background air sampling:***

1. Take a sample of background air for every group of patients that provide breath samples.
2. Note down the Tedlar® bag code number.
3. Connect the Tedlar® bag to the pump using the tubing provided.
4. Note the time of collection on the bag.
5. Open the tap on the Tedlar® bag and fill the bag to approximately 80% (do not overfill).
6. Close the tap on the Tedlar® bag before removing the bag from the pump.
7. Store the Tedlar® bag in a black liner until analysis.

* Breath samples must be transported to the laboratory and analysed within 6 hours to prevent sample degradation.

Appendix 4

| Seminars Attended | Date |
|---|---|
| *The HOOO Radical: An Experimental & Theoretical Challenge*<br>Craig Murray | 20/10/10 |
| *Integrating Genomic Scale Data: Exploring the Galaxy*<br>Richard Badge | 25/10/10 |
| *Surface Redox Chemistry of Insulating Materials*<br>Katherine Holt | 08/11/10 |
| *An Introduction to 'R'*<br>Eran Tauber | 29/11/10 |
| *The Application of Forensic Engineering to Forensic Medicine*<br>Sarah Hainsworth | 06/12/10 |
| *Marine Trace Gases*<br>Lucy Carpenter | 09/02/11 |
| *Aqueous Dispersion Polymerisation*<br>Steve Armes | 23/02/11 |
| *Catalytic Challenges for Surface Chemistry*<br>Stephen Jenkins | 02/03/11 |
| *Modelling of Atmospheric Halogen Species*<br>Martyn Chipperfield | 24/06/11 |
| *The Weird Behaviour of Water: Ultrafast to Ultraslow Dynamics and Worm-hole Structures*<br>Klaas Wynne | 05/10/11 |
| *A Little Light Relief*<br>David Phillips | 07/10/11 |
| *Systems Biology Seminar*<br>University of Leicester: Point of Care & Integrated Diagnostics Development Unit | 19/10/11 |

| Seminars Attended (continued) | Date |
|---|---|
| *Practical Chemometrics for Analytical Scientists*<br>South East Region Analytical Division & The Chemometrics Group of the Royal Society of Chemistry | 25/10/11 |
| *Aerosol modelling in Manchester – from Single Particle to Regional Scales*<br>Gordon McFiggans | 26/10/11 |
| *Probing weak Intermolecular Forces through Rotational Polarization*<br>Matt Costen | 16/11/11 |
| *A Prototype Bacteria Detecting responsive wound Dressing*<br>Toby Jenskins | 24/11/11 |
| *The End of the Shuttle Program and the Future of Human Space Flight*<br>NASA Astronaut Jeff Hoffman | 13/12/11 |
| *Cold Molecules and Controlled Ultracold Chemistry*<br>Jeremy Hutson | 18/01/12 |

| Presentations | Date |
|---|---|
| Poster Presentation: *Can you smell the disease?*<br>FPGR 2011, University of Leicester | 16/06/11 |
| Poster Presentation: *Can you smell the disease?*<br>Department of Chemistry postgraduate Symposium 2011, University of Leicester | 06/07/11 |
| Poster Presentation: *Breath Analysis for Medical Diagnosis*<br>Department of Chemistry postgraduate Symposium 2012, University of Leicester | 26/06/12 |
| Oral Presentation: *Exhaled Breath Analysis using Proton Transfer Reaction Time-of Flight Mass Spectrometry: Volatile Organic Compounds in Exhaled Breath in Women with Ovarian Cancer - A Pilot Study*<br>Annual Department of Chemistry Postgraduate Research Symposium 2013 | 24/07/13 |

Publications

P. S. Monks, S. Kuppusami (2012) Simply Breath, *Chem Ind*, 76(3):40-43

S. Kuppusami, M.R.J. Clokie, T. Panayi, A.M. Ellis, P.S. Monks (2014) Metabolite Profiling of *Clostridium difficile* Ribotypes using Small Molecular Weight Volatile Organic Compounds, *Metabolomics*, DOI: 10.1007/S11306-014-0692-4

Awards

*Highly Commended for THES (Times Higher Education Supplement) Award Outstanding Contribution to Innovation and Technology 2012*
Diagnostics Development Unit