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MARTYN LEE HUMPHREYS

VOLATILE DIAGNOSTIC TECHNIQUES FOR VENTILATOR ASSOCIATED PNEUMONIA

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SUPERVISORS: **DR CATHERINE KENDALL, PROF NARESH MAGAN, PROF HUGH BARR.**

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Abstract
Ventilator associated pneumonia (VAP) is a significant challenge for the Intensive Care doctors worldwide. It is both difficult to diagnose accurately and quickly and to treat effectively once the diagnosis has been established. Current diagnostic microbiological methods of diagnosis can take up to 48 hours to yield results. Early diagnosis and treatment remain the best way of improving outcome for patients with VAP.

In this study we look at novel diagnostic techniques for VAP. Electronic nose (Enose) technology was used to identify to identify the presence of microorganisms in bronchoalveolar lavage (BAL) fluid samples taken from the respiratory tracts of ventilated patients. The results were compared with standard microbiological culture and sensitivities. The Enose was able to discriminate 83% of samples into growth or no growth groups on samples grown in the lab. When the technique was employed to samples taken directly from patients the accuracy fell to 68.2%. This suggests that patient related factors may be reducing the accuracy of the Enose classification. The use of antimicrobial drugs prior to patient sampling is likely to have played a major role.

The second part of this study used Gas Chromatography-Mass Spectrometry (GC-MS) analysis of patient's breath in an attempt to identify patients with VAP. Breath samples were taken at the same time as the bronchoalveolar lavage samples described above. The use of this technique did show differences between the breath samples of patients who did not have any microbiological growth from their BAL samples and those that did.

Leave one out cross validation of a PC fed LDA model showed 84% correct classification between healthy volunteers, no growth and growth groups.

Finally, we evaluated the Breathotron, which is a breath analysis device designed and built at Cranfield Health. It allows for analysis of breath samples using a single sensor system as opposed to a sensor array employed in traditional Enose devices. This allows it to be more portable and cheaper to build. The Breathotron also allows collection of breath onto sorbent cartridges for GC-MS analysis. Its single sensor did not allow for accurate discrimination between samples.

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Nomenclature

ARDS	Acute respiratory distress syndrome.
BAL	Bronchoalveolar Lavage
CHF	Congestive heart failure,
COPD	Chronic Obstructive Pulmonary Disease
CPIS	Clinical Pulmonary Infection Score
CT	computed tomography
Enose	Electronic nose
GC-MS	Gas Chromatography-Mass Spectrometry
HAP	Hospital acquired pneumonia
ICU	Intensive Care Unit
LDA	Linear Discriminant Analysis
LDF	Linear Discriminant Function
MOS	Metal oxide semiconductor
MOSFET	Metal oxide silicon field- effect sensors
MRSA	Methicillin resistant *Staphylococcus aureus*
NNIS	National Nosocomial Infection Surveillance
PCA	Principal Components Analysis
PCT	Procalcitonin
PSB	Protected specimen brush
sTREM-1	Soluble triggering receptor expressed on myeloid cells-1
VAP	Ventilator Acquired Pneumonia
VOCs	volatile organic compounds

Chapter 1

Introduction

1.1 Introduction

"Hospital acquired pneumonia (HAP) is an infection of the lungs, usually due to bacterial, viral or fungal pathogens, that is defined as occurring more than 48 hours after admission to hospital"[1]. In the USA, HAP is the second most common hospital-acquired infection but leads to the greatest number of nosocomial-related deaths[1]. Over and above increased morbidity and mortality, HAP also results in extended hospital stays and is frequently managed with prolonged antibiotic treatment, resulting in additional financial burdens and antibiotic resistance in hospitals. Favourable outcomes centre on the prevention of HAP when possible and the prompt use of appropriate antibiotics in the presence of infection[2]. This can be challenging when the aetiology of HAP is unknown.

Since the 1970's the potential of breath analysis as a way of detecting disease in the human body has been recognised. These early studies were primarily performed using GC-MS. In 1971 Pauling et al.[3] demonstrated that several hundred compounds were present in human breath and that some of these could be associated with abnormal physiological states or disease.

When considering breath analysis, diseases of the lung and respiratory tract are the obvious choice for pathologies to be investigated as the vast majority of expired breath gas has been produced in the lung. A small proportion will be dead-space gas, some of which may have come from the upper gastrointestinal tract.

All the methods for breath analysis rely on the detection of volatile organic compounds or VOCs. Organic compounds are those which contain carbon and are found in all living things. VOCs are organic compounds that easily become gases or vapours; as such they can be detected in breath and bodily fluids. Many studies, including those using GC-MS (Gas Chromatography-Mass Spectrometry) have shown that for certain pathological conditions (infection, malignancy, liver and cardiopulmonary disease) specific patterns of VOCs can be detected. These patterns are known as “Volatile Fingerprints”. It is hoped that these fingerprints will provide the basis for accurate breath screening for disease in the future. Although we have so far discussed using various technologies to analyse breath, these same technologies can also be employed to smell the headspace above bodily fluids such as blood and urine. This adds another potential medical application to the uses of the technologies we have looked at so far.

1.2 Research Objectives

As can be seen from the literature review there has been extensive research into the application of various technologies for volatile detection in the field of medicine. VAP is an obvious area for the application of this technology, which to this point in time has only been explored on a superficial basis. The fact that early diagnosis and treatment of VAP improves outcome and that Enose technology in particular offers the ability to perform rapid, near patient diagnosis make the application of this technology to this important clinical problem an exciting prospect.

There are two distinct sections to this piece of work, which examine the use of Enose technology and GC-MS in the diagnosis of VAP:

The diagnosis of VAP using Enose analysis of BAL fluid:

In this section we compare the detection of infection in BAL fluid by Enose analysis with accepted microbiological culture and sensitivities.

The diagnosis of VAP by using GC-MS analysis of exhaled breath from ventilated patient:

In this section we look at the breath profiles from patients who have a positive BAL culture and compare them with breath profiles from patients who do not. We hope to be able to detect the presence of compounds in the breath which may act as a "volatile fingerprint" for the presence of microbiological growth.

Chapter 2

Ventilator Associated Pneumonia

2.1 Introduction

Ventilator associated pneumonia (VAP), one form of HAP, specifically refers to pneumonia developing in a mechanically ventilated patient more than 48 hours following endotracheal intubation[2]. Although not included in this definition, some patients may require intubation after developing severe HAP and should be managed in the same way as those with VAP.

"There are approximately 300,000 cases of HAP and VAP annually in the USA, representing approximately 5-10 cases per 1,000 hospital admissions"[1]. Based on data from over 14,000 intensive care patients in the USA, HAP and VAP represent the second most common nosocomial infection, affecting 27% of all critically ill patients[4]. HAP accounts for up to 25% of all intensive care unit infections and more than 50% of all antibiotic use[4]. VAP occurs in 9-27% of all intubated patients[2, 5]. Among intensive care patients, nearly 90% of episodes of HAP occur during mechanical ventilation.

In mechanically ventilated patients the incidence of VAP increases with the duration of ventilation. The risk of VAP is highest early in the course of hospital stay and is estimated to be 3% per day during the first 5 days of ventilation, 2% per day during days 5-10 of ventilation and 1% per day after this[6]. Since most mechanical ventilation is short term, approximately half of all episodes of VAP occur within the first four days of mechanical ventilation.

In patients with respiratory failure, VAP is the primary cause of hospital acquired mortality. HAP is associated with approximately 60% of all deaths in patients with nosocomial infections[7], and the mortality rate is increased in critically ill patients and those patients developing VAP.

2.2 Pathophysiology of VAP

Nosocomial pneumonia (of which VAP is a type) can occur by four routes:

1. Haematogenous spread from a distant focus of infection
2. Contiguous spread
3. Inhalation of infectious aerosols
4. Aspiration

By far the most important cause is aspiration of the pathogenic Gram-positive and Gram-negative bacteria that colonise the oropharynx and gastrointestinal tract. The other routes of infection are much less significant[8].

After reaching the lung, microorganisms multiply and cause invasive disease. The host defence, including humidification and filtration of air in the upper airways, epiglottic and cough reflexes, ciliary transport phagocytes and opsonins in the distal lung and systemic immunity prevent bacterial invasion[9]. In the ICU host defences are compromised as a result of underlying disease and the treatments given. Patients are unable to cough

effectively due to sedation, pain or underlying disease. Also, when intubated the endotracheal tube holds the vocal cords open which facilitates aspiration.

2.3 Risk Factors

Endotracheal intubation is the single most important risk factor for VAP; associated with 3-21 fold increase in risk[10-13]. The risk is increased by the following methods:

1. Sinusitis and trauma to the nasopharynx (nasotracheal tube).
2. Impaired swallowing of secretions.
3. Acting as a reservoir for bacterial proliferation.
4. Increasing bacterial colonisation of the airways.
5. Presence of a foreign body traumatising the oropharyngeal epithelium.
6. Ischaemia secondary to cuff pressure.
7. Impaired ciliary clearance and cough.
8. Leakage of secretions around the cuff.
9. The use of suction to remove secretions.

Adapted from[14].

Microorganisms can adhere to the surface of the endotracheal tube and form a biofilm. This provides a reservoir of microorganisms, and they are highly resistant to both host defence and antimicrobial drugs[15].

Duration of mechanical ventilation is an important risk factor for the development of VAP. Cook et al.[6] reported a cumulative increased risk of VAP with time, with 3% per day in the first week, 2% per day in the second week and 1% per day in the third week. Other studies have confirmed that the risk of VAP increased as the duration of mechanical ventilation increased and that the risk is highest in the first 8-10 days[11, 16, 17]. Reintubation, urgent intubation and massive aspiration are also associated with a high incidence of VAP[18]. The table below summarises the independent risk factors for VAP.

Host factors	Intervention factors	Other factors
Age > 60 yr	Duration of MV	Season: Autumn, Winter
Severity of illness	Reintubation	
Organ Failure	PEEP	
Poor Nutritional state	Frequent ventilator circuit changes	
Upper abdominal/thoracic surgery	Nasogastric tube	
ARDS	Intracranial pressure monitoring	
Chronic lung disease	Paralysis, sedation	
Neuromuscular disease	H2 blockers and/or antacids	
Trauma, burns	>4 unit blood transfusion	
Coma, decreased conscious level	Supine head position	
Large volume aspiration	Transport out of ICU	
Upper respiratory tract colonisation		
Gastric colonisation and pH		
Sinusitis		

Table 2.1 Risk factors for VAP. Adapted from [18].

Nasogastric tubes are commonly used in the ICU for both feeding and drainage of stomach contents; especially in surgical patients. These tubes impair the function of the gastro-oesophageal sphincter and increase the risk of sinusitis, oropharyngeal colonization and reflux, all of which may lead to migration of bacteria and pneumonia[9, 19]. Enteral feeding via the nasogastric route may predispose to VAP by elevating gastric pH, leading to gastric colonisation and increasing the risk of reflux and aspiration by causing gastric distension[6, 20, 21].

2.4 Aetiology of VAP

The causative organisms vary depending on patient demographics, methods of diagnosis, duration of hospital and ICU stay and the local antibiotic policy. Gram-negative bacteria are the most common causative agent reported in several studies[18]. In National Nosocomial Infection Surveillance (NNIS) data, although *Staphylococcus aureus* was the most commonly reported isolate (17%), 59% of all reported isolates were Gram-negative[22]. It is worth discussing some of the important individual pathogens that cause VAP.

Streptococcus pneumonia is a Gram-positive diplococcus that colonises the upper respiratory tract and invades the lung after microaspiration of oropharyngeal secretions. It is the most common cause of community acquired pneumonia[23]. Although antibiotic resistance is a growing problem,[24, 25] most S. pneumonia isolates remain susceptible to traditional β-lactam antibiotics[26, 27]. It causes VAP predominantly in the early days

after intubation and is rapidly cleared after beginning antibiotic therapy[28]. The main risk factors for VAP caused by this pathogen are smoking, chronic obstructive pulmonary disease (COPD) and the absence of prior antibiotic therapy.

Haemophilus influenza is a small Gram-negative coccobacillus. In common with *S. pneumoniae* it is fastidious and easily eradicated by antibiotic therapy[28] and causes VAP most often early after the initiation of mechanical ventilation. Risk factors as a cause of VAP include COPD and the absence of prior antibiotic therapy[29].

Staphylococcus aureus is a Gram-positive coccus that is one of the most important causes of nosocomial infections generally and of VAP[30]. Staphylococci cause VAP throughout the course of critical illness. The prevalence of methicillin-resistant *S. aureus* (MRSA) strains is increasing, even in community isolates[31]. Proven risk factors for VAP caused by methicillin-sensitive *S. aureus* include younger age, traumatic coma and neurosurgical problems[32]. Risk factors for VAP caused by MRSA include COPD, longer duration of mechanical ventilation, prior antibiotic therapy, prior steroid treatment and prior bronchoscopy. The likelihood that VAP is due to MRSA becomes nearly certain if the patient has received antibiotic treatment and the onset of VAP is later in the hospital course[33]

Enterobacteriaceae or enteric Gram-negative bacilli normally reside in the lower gastrointestinal tract. Antibiotic therapy and critical illness can suppress the normal bacterial flora and lead to overgrowth of these species in the gut and colonisation of the

skin and the respiratory and upper GI tracts. Some of these organisms have acquired resistance to penicillin and cephalosporin antibiotics[32]. This has implications for antibiotic therapy.

Pseudomonas aeruginosa is an aerobic nonfermenting Gram-negative bacillus and is intrinsically resistant to many classes of antibiotics. It is the most common antibiotic resistant pathogen causing VAP[2]. Unlike many other causes of VAP, Pseudomonas is consistently associated with a measurable attributable mortality[32]. Pseudomonas VAP is unusual early in the hospital course in previously healthy patients. It typically occurs only if risk factors are present, including COPD, prolonged duration of mechanical ventilation and prior antibiotic therapy. It is difficult to eradicate from the airways and persistent or recurrent episodes of pneumonia are common[28].

Acinetobacter species are aerobic nonfermenting Gram-negative bacilli that are widely distributed in soil and fresh water sources. *Acinetobacter* species have traditionally been considered to be low virulence, and clinical isolates have often been considered to represent colonisation rather than infection[34]. Recently, there has been increasing recognition of these species as important causes of nosocomial infection, particularly in critically ill patients[32]. They are readily spread between patients and have the ability to survive on health care workers hands and inanimate environmental surfaces[32]. They also have intrinsic resistance to many common antibiotics[32].

The figure below shows the relative frequencies of routine microbiological pathogens in VAP.

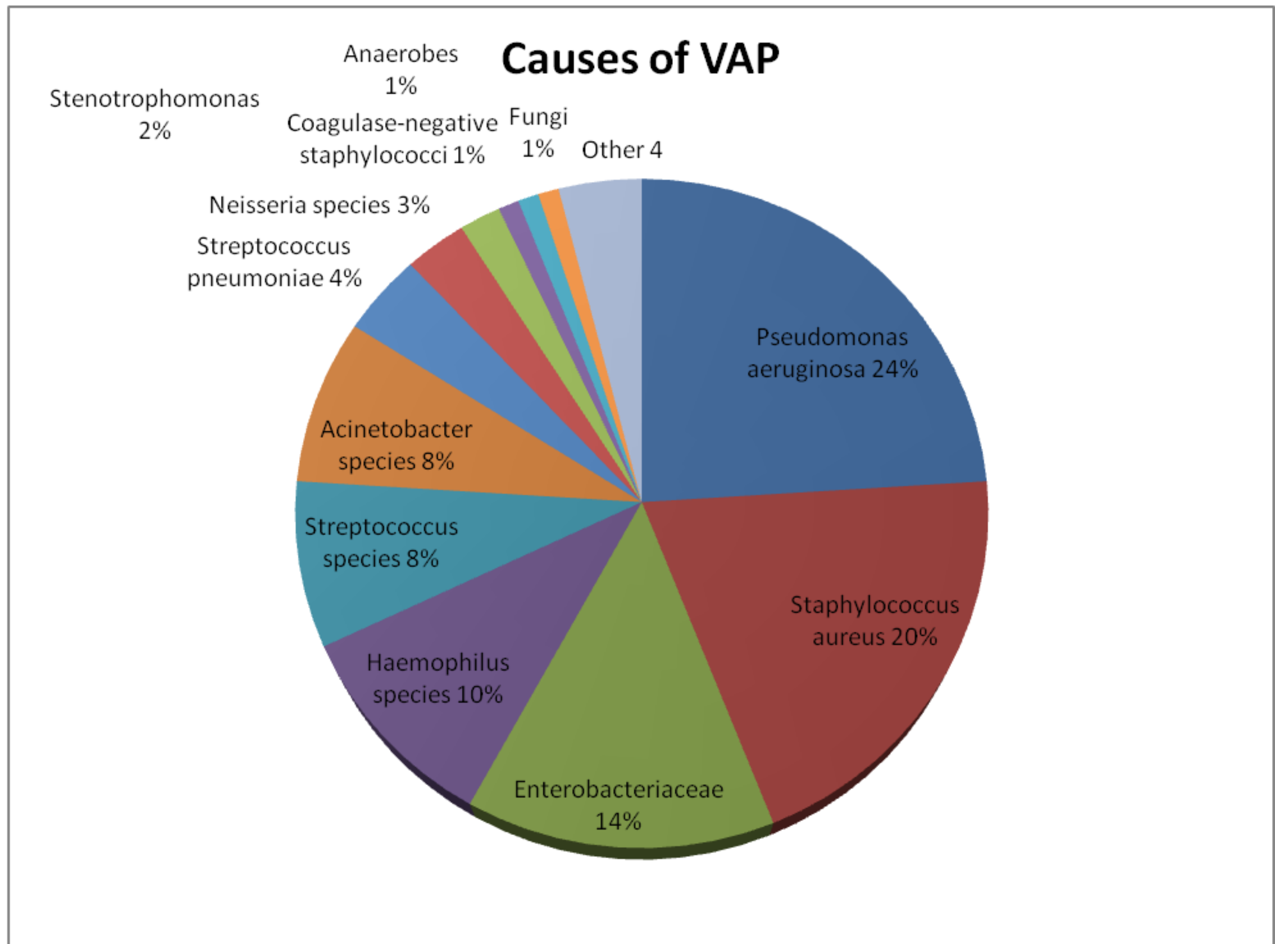

Fig. 2.1 The relative proportions of common causes of VAP are shown from 1,689 bronchoscopically confirmed cases involving 2,490 individual isolates reported in 24 published studies. Data from Chastre 2002[2].

2.5 Polymicrobial Infections

Polymicrobial infection occurs in many cases of VAP. Combes and colleagues have reported a series of VAP cases using the first episode of VAP as the unit of analysis. Forty eight percent of their 124 cases were polymicrobial, with up to 4 separate important isolates from individual patients. In these cases, the clinical features and outcomes and the prevalence of specific individual pathogens in the polymicrobial cases did not appear to differ from cases with only one infecting organism[35].

2.6 Multidrug-Resistant VAP Pathogens

Many of the organisms that cause VAP, especially Pseudomonas, Acinetobacter, and Stenotrophomonas species, and MRSA typically display high levels of antibiotic resistance. These organisms, and enteric Gram-negative bacilli expressing extended-spectrum β-lactamases, have been termed "potentially drug-resistant" pathogens[36] or "multidrug resistant" pathogens[37]. In order to account for this the empiric antibiotic regime must include multiple agents with an extremely broad spectrum of activity[36]. However, unnecessarily broad antibiotic coverage can have adverse consequences. These include encouraging the development of more resistant bacterial strains, higher rates of antibiotic-related complications and increased costs[38]. A major goal of VAP management is to minimise the unnecessary use of antibiotics, but it is essential to be able to predict when antibiotic coverage for multiple drug-resistant pathogens is necessary, in order to avoid under treatment of these infections[32].

The duration of mechanical ventilation preceding the onset of pneumonia is the single most important determinant of the causative agent(s) of VAP and the likelihood of multidrug resistant organisms being responsible. VAP has traditionally been classified as either "early-onset" (within 4-7 days of intubation), or "late-onset" (if it occurs after 4-7 days). Early-onset VAP is commonly caused by *Haemophilus* species, streptococci including *S. pneumoniae*, MRSA, and sensitive strains of *Enterobacteriaceae*. These organisms can also be responsible for late-onset VAP, but multidrug resistant organisms are more commonly encountered in this period[37]. In some studies the early/late onset distinction is quite apparent. In one study all 11 potentially multidrug resistant pathogens (out of a total of 40) occurred after 5 days of mechanical ventilation[39]. The presence of multidrug resistant organisms in the early period should prompt a search for other risk factors for this type of infection[40].

Extended periods in hospital prior to the requirement for mechanical ventilation could also be a risk factor for the development of multi drug resistant VAP in the early period [37, 41, 42]. Medical co-morbidities including HIV infection, malignancy and COPD as well as pre-hospital intubation predicted infection with multidrug resistant organisms in a study of trauma patients[43]. The use of antibiotics prior to intubation appears to be a "double edged sword". It may decrease the risk of early VAP due to antibiotic sensitive Gram-positive cocci and *H. Influenza*, but it increases the risk of VAP due to *Pseudomonas*, MRSA, and other multi drug resistant pathogens in the later stages of the hospital stay[36, 44, 45]. Trouillet et al. identified three variables independently

associated with infection by a multi drug resistant pathogen: duration of mechanical ventilation more than 7 days (odds ratio 6.0), prior antibiotic use (odds ratio 13.5) and prior broad spectrum antibiotic use (odds ratio 4.1)[36].

2.7 Antibiotics and VAP

The use of antibiotics for the treatment of VAP is an area of much debate within the Intensive Care community. The main areas of debate are:

1. When to initiate antibiotic therapy
2. The use of empirical antibiotic therapy
3. Descalation and duration of antibiotic therapy
4. Antibiotic resistance

2.7.1 When to initiate antibiotic therapy

This decision is crucial in the management of VAP as it has been shown that delays in the initiation of appropriate therapy are associated with increased morbidity and mortality [46-48]. The difficulty in making the diagnosis in the first place and the unreliability of clinical diagnosis make this an area of ongoing debate. Many units have developed their own local protocols based on a variety of diagnostic techniques including CPIS, radiological investigations and microbiology techniques.

The diagram overleaf demonstrates a diagnostic and therapeutic management strategy based on the use of invasive sampling techniques such as BAL. The use of such a strategy means that therapeutic decisions are tightly protocol driven based on the results of direct examination of distal pulmonary samples and results of quantitative cultures. It is argued that the use of such a strategy improves diagnostic accuracy and facilitates decisions on whether or not to treat. This in turn improves outcome and reduces unnecessary use of antibiotics.

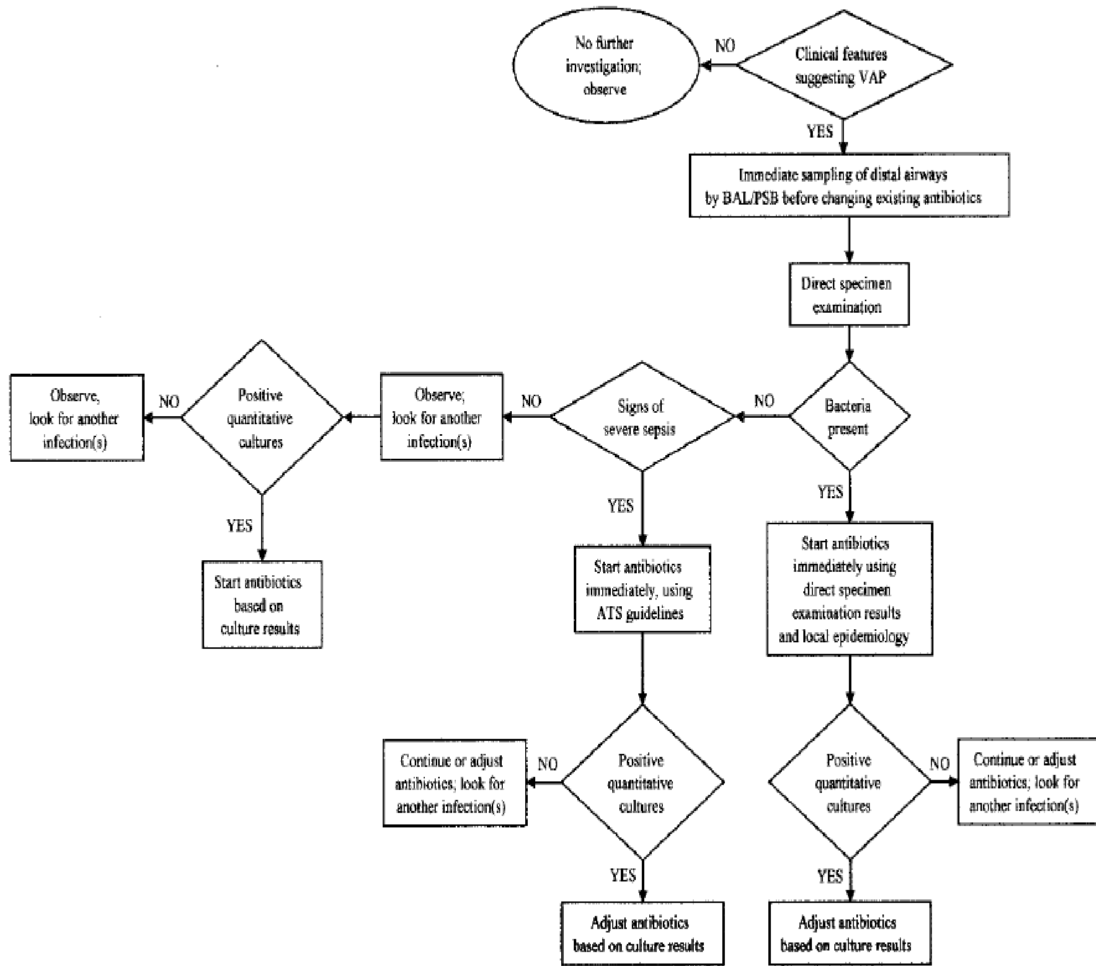

Fig 2.2. Diagnostic and therapeutic strategy applied to patients with a clinical suspicion of VAP according to the "invasive" strategy. From Chastre 2005[49].

However Muscedere et al.[50] reviewed studies that examined the impact of invasive versus noninvasive techniques (such as endotracheal aspirates) on patient important outcomes. They did not find that the use of invasive sampling techniques with quantitative cultures in the populations studied improved clinical outcome. In addition,

as these techniques are more intrusive, expensive, and less universally available, they did not recommend their routine use.

The following points must be considered when evaluating this recommendation. Firstly, it is not possible to distinguish between the various invasive techniques in use to obtain distal pulmonary tree samples for quantitative cultures as these have not been studied individually with regard to their ability to affect clinical outcome. Second, this recommendation does not apply to immunocompromised patients. Third, this recommendation is based on the assumption that a decision has already been made to treat with appropriate antibiotics until culture results are available. This is general but not universal practice throughout the world. Finally, one study demonstrated potential benefit to reducing antibiotic use by including the findings from immediate Gram stain of bronchoscopic samples into the decision on whether or not to initiate antibiotics[51]. However the validity and clinical reproducibility of these data are challenged by subsequent publications demonstrating a high error rate if decisions to withhold antibiotics are made on the basis of Gram stain findings[50].

For VAP treatment, the recommendation that antibiotics be initiated at the time that VAP is suspected is supported by one randomised clinical trial[47]. Although this trial showed only a trend towards improved outcomes with empiric therapy at the time of VAP suspicion, there is a large amount of supporting evidence in the form of observational data that associates delays in instituting adequate therapy with worse outcomes[46, 52-55]. Similarly, a randomised controlled trial in which a large proportion of the patients had VAP, showed the institution of empiric antibiotic therapy at the time of suspicion of infection resulted in improved outcomes as compared to culture-directed therapy[56].

2.7.2 Empirical antibiotic therapy

Empirical antibiotic therapy is defined for our purposes as the initiation of antibiotic therapy at the time of VAP suspicion and delayed culture-directed therapy is defined as the initiation of antibiotic therapy for VAP when culture reports are available. We have already discussed that there is little evidence to support the use of delayed antibiotic therapy, indeed there is considerable evidence to show it results in patient harm.

One of the difficulties with the use of empiric antibiotic therapy is the selection of antibiotic drug or drugs. The initiation of empiric therapy is essentially the use of "best guess" antibiotic based on local policies and resistance patterns. This usually involves the use of broad spectrum drugs which have activity against a wide range of pathogens. The downside of this is that it can encourage the emergence of drug resistant organisms.

Broad spectrum antibiotic use also increases the rate of *Clostridium Difficile* infection in patients receiving these antibiotics.

Muscedere et al[50] reviewed 5 trials that compared empiric broad spectrum monotherapy to combination therapy in patients with VAP. They concluded that there is no advantage to combination therapy. These trials did not demonstrate any differences in mortality or clinical response rates. However, the benefit of a reduction in antibiotic use and cost favours monotherapy. Empiric therapy should be based on local resistance patterns and patient risk factors. In situations where high rates of resistance are present, 2 or more antibiotic agents may be justified to ensure that each potential pathogen is covered by at least one agent.

Once treatment has been started the prompt cessation of antibiotic therapy if pneumonia is not confirmed has several advantages. Reduced costs, side effects and drug interactions are all important advantages as well as the reduced likelihood of the emergence of resistant organisms. Other sites of infection requiring definitive therapy may be more likely to be discovered because their signs are not being masked by antibiotics.

Several studies have shown that it may be safe to withdraw the empiric therapy when quantitative cultures are sterile or show a bacterial concentration that is lower than the threshold used to define infection[51, 57, 58]. These thresholds must not be used in patients who had recent changes or new antibiotics prior to sampling. It should be

recognised that these studies were not designed to specifically address the issue of withdrawing therapy, and the decision to stop antibiotics was made by the clinician on both clinical status of the patient and culture results.

The level of clinical suspicion defines the context in which microbiology data should be interpreted. Only a test with a high likelihood ratio (for example a positive blood culture) should make the clinician continue antibiotics in the setting of low clinical suspicion. On the other hand, a test with a lower likelihood ratio such as a positive tracheal aspirate culture might be dismissed as a contaminant.

2.7.3 Descalation and the duration of antibiotic therapy

Descalation is the switch from broad spectrum empirical antibiotic therapy to narrow spectrum antibiotics based on positive culture results.

The traditional approach to infection has been to narrow the antibiotic spectrum to the most specific, safest and least costly drug based on definitive culture result. This approach is more difficult in the context of VAP because of concerns about sensitivity and specificity of virtually all culture techniques.

The most clear cut situation in which bacteria are cultured from blood, open lung biopsy or pleural fluid is rare. In these scenarios treatment can be based on culture results with confidence. With somewhat less confidence, treatment can be adjusted or focused on the basis of invasive (PSB or BAL) cultures. In cases where these were performed prior to

any antibiotic changes, narrowing the antibiotic spectrum based on the quantitative cultures (descalation) is logical. In patients who had antibiotic changes before bronchoscopy, patients who are clinically deteriorating despite "subthreshold" or negative cultures, or patients who are found to be on effective empiric therapy but are worsening nonetheless, the results of invasive cultures should be interpreted with caution.

The duration of antibiotic therapy for VAP is another controversial area. Deciding on the length of treatment requires the balancing of the risk of either failure or relapse with short treatment courses against the concerns surrounding antibiotic overuse with more prolonged courses of antibiotics[59]. The belief that longer antibiotic courses pose no risk to the patient as long as the drugs used are effective against the infecting organisms is false. Despite initial resolution of clinical parameters of infection within 6 days of instituting appropriate antibiotic therapy, Denneson et al.[28] noted that Gram negative pathogens re-grew to colonise the trachea during the second week of therapy. This led to recurrence of VAP but now with strains that were resistant to the original antibiotics used[28].

Shorter, 7 day courses of antibiotics have been proposed for VAP[28]. Chastre et al. [59] compared discontinuing antibiotics at 8 days with 15 days. There were no significant differences in mortality, length of stay, or duration of mechanical ventilation. The shorter course was associated with a reduction in antibiotic use and a reduction in the emergence of resistance. A higher percentage of patients treated with 8 days of antibiotics developed recurrence of lung infection secondary to nonfermenting Gram negative

bacteria, but this was not associated with worsened clinical outcomes. Among those who developed a recurrent VAP, multiresistant organisms emerged significantly less often in the group who received 8 days of therapy.

Therefore in patients who receive adequate initial antibiotic therapy, a total of 8 days of antibiotic therapy can be recommended[50].

2.7.3 Antibiotic Resistance

Many of the issues surrounding this point have been discussed in the preceding text; including the use of empirical antibiotic therapy and descalation. Trouillet et al.[36] prospectively and specifically evaluated risk factors for infection with potentially drug resistant pathogens in 135 cases of bronchoscopically proven VAP. Multivarate analysis identified 3 variables independently associated with infection by a potentially drug resistant pathogen:

1. Duration of mechanical ventilation> 7 days. (odds ratio 6.0)
2. Prior antibiotic use. (odds ratio 13.5)
3. Prior broad spectrum antibiotic use. (odds ratio 4.1)

These findings have serious implications for empiric antibiotic choice. The antimicrobial susceptibility profiles of the isolates recovered in this study became increasingly resistant with increased duration of mechanical ventilation and with prior exposure to antibiotics.

2.8 Current methods of Diagnosing VAP

VAP is usually suspected when a patient develops a new or progressive pulmonary infiltrate with fever, raised white blood cell count and purulent tracheobronchial secretions[60]. However these signs are not specific to VAP and the diagnosis can be difficult to make.

2.8.1 Clinical Pulmonary Infection Score (CPIS)

CPIS was originally proposed as a way of combining clinical parameters with microbiological testing as a way of diagnosing VAP [61]. A modified version of the original CPIS, used in this work is shown below. The accuracy of CPIS has been questioned[62].

VAP clinical diagnosis criteria

Temp °C

>or equal to 36.5 and <or equal to 38.4 = 0 points

>or equal to 38.5 and <or equal to 38.9 = 1 point

>or equal to 39 and <or equal to 36.0 = 2 points

White cell count/mm^3

>or equal to 4000 and <or equal to 11000 = 0 points

<4000 or > 11000 = 1 point

Tracheal secretions

No secretions = 0 points

Non purulent secretions = 1 point

Purulent secretions = 2 points

Oxygenation: PaO_2/FiO_2, kPa

>31.6 or ARDS = 0 points

< or equal to 31.6 and no ARDS = 2 points

CXR findings

No infiltrates	= 0 points
Diffuse/patchy infiltrates	= 1 point
Localised infiltrates	= 2 points

Progression of infiltrates

No radiographic progression	= 0 points
Radiographic progression	= 2 points

(after exclusion of CHF and ARDS)

Culture of tracheal aspirates

Pathogenic bacteria cultured in rare or light growth/no growth	= 0 points
Pathogenic bacteria cultured in moderate/heavy growth	= 1 point
Pathogenic bacteria on Gram stain	= ADD 1 point

TOTAL SCORE: /12 (Score 6 or more significant)

Adapted from Pugin et al. 1991

CHF is congestive heart failure; ARDS is acute respiratory distress syndrome.

2.8.2 Radiological Investigations

Chest x-ray is a routine and accepted investigation frequently used in the diagnosis of VAP. However chest x-ray changes can take days to become apparent after infection. Computed Tomography (CT) scanning of the chest has a higher sensitivity in diagnosing VAP than chest x-ray. In one study CT scan detected 26% of opacities that were not detected on portable chest x-ray[63]. However it has the distinct disadvantage that patients need to be transported from the relative safety of the ICU to the CT scanner. This is not always possible when the patient is unstable. Both of these investigations have a false positive rate and can be normal in the presence of VAP. The overall radiographic sensitivity of a pulmonary opacity consistent with pneumonia on chest x-ray is only 27%-35%[63].

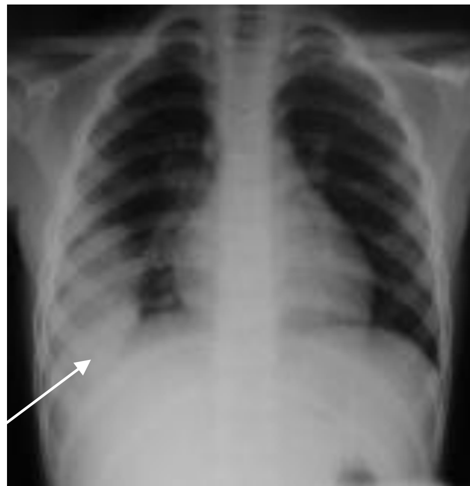

Fig. 2.3 Radiographic changes in VAP. Area of consolidation is indicated by the arrow.

2.8.3 Microbiological Investigations

Upper airway secretions can be cultured simply by collecting sputum samples from the ventilated patient. This method of testing has the advantage of being easily reproducible and requires little technical expertise. The fact that the upper airways can rapidly become colonised by potentially pathogenic micro-organisms after endotracheal intubation, even when pneumonia is not present detracts from the value of these studies. Thus if an organism is cultured or noted on Gram stain, it is impossible to know if it is the cause of a pneumonia or simply colonisation.

In a study of 48 patients with respiratory failure, agreement between tracheal non quantitative cultures and cultures of lung tissue from open lung biopsy was only 40%. In the same study, of those patients with pneumonia on lung histology, endotracheal aspirate had a sensitivity of 82% but a specificity of only 27% [63].

In order to improve the specificity of the diagnosis of VAP numerous studies have evaluated the role of quantitative cultures of respiratory secretions. These have included both "blind" and bronchoscopic methods of sampling. Bronchoalveolar Lavage (BAL) and protected specimen brush (PSB) are methods of sampling fluid from the distal airways, which can be performed "blind" or bronchoscopically guided. Studies have shown no significant difference in the diagnostic accuracy of either of these methods[49]. Protected specimen brush testing is an alternative to BAL sampling. The preferred method of sampling is uncertain [64, 65].

VAP spreads to the blood or pleural space in <10% of cases. Despite this, if an organism known to cause pneumonia is cultured on a clinical background of suspected pneumonia, treatment is warranted. It is important to note that the sensitivity of blood cultures for the diagnosis of VAP is 26%[66], but also, that when positive, the organisms may originate from an extrapulmonary site of infection in as many as 64% of cases; even in the presence of VAP[63].

2.9 New Diagnostic Markers of VAP

2.9.1 Procalcitonin

(http://www.procalcitonin.com/default.aspx?tree=_2_3&key=aboutpct22)

Procalcitonin (PCT) is a 116 amino acid peptide which has been shown to play a role as an inflammatory mediator in systemic infection. Its sequence is identical to the prohormone of calcitonin. Under normal metabolic conditions, calcitonin is produced and secreted by the C-cells of the thyroid gland. Normally the circulating levels of calcitonin are very low (< 0.05ng/ml).

Following bacterial infections, PCT is released from all parenchymal tissues and differentiated cell types throughout the body. Concentrations of PCT up to 1000ng/ml can be detected in cases of severe bacterial infection or sepsis. Parenchymal cells (including liver, lung, kidney, adipocytes and muscle) provide the largest tissue mass and principal source of circulating PCT in sepsis. The inflammatory release of PCT can be induced either directly via microbial toxins (eg. endotoxin) or indirectly via a humoral or

cell-mediated host response (eg. IL-1ß, TNF-alpha, IL-6). The induction can be attenuated by cytokines also released during a viral infection (eg. interferon-gamma).

PCT increases after 2-3 hours following induction e.g. by endotoxin and may increase to levels up to several hundred nanogram per ml in severe sepsis and septic shock. After successful treatment intervention the procalcitonin value decreases, indicating a positive prognosis. Persistingly high or even further increasing levels are indicators for poor prognosis. After induction, PCT increase is observed within 2-3 hours. Levels then rise rapidly, reaching a plateau after 6-12 hours. PCT concentrations remain high for up to 48 hours, falling to their baseline values within the following 2 days. The half-life is about 20 to 24 hours.

PCT has also been shown to be elevated in conditions other than sepsis such as surgery[67] and transplant rejection[68] amongst others. Low levels of PCT can also be seen in the early stages of bacterial disease or when infection is localised[69-71]. The use of PCT as a marker of infection therefore relies on follow up and re-evaluation of levels, rather than a one off measurement.

In lung infection and injury circulating levels of PCT rise rapidly. This is most likely in response to cytokine release from pulmonary neuro-endocrine cells and/or mononuclear cells[72, 73]. Duflo et al [74] stated that serum PCT had a role as a diagnostic marker of VAP and also that serum levels were greater in patients that died compared with those that survived. With a cutoff of 3.9ng/ml sensitivity was 61% and specificity 100%.

These results have been questioned by other studies which have shown a poor sensitivity of PCT for diagnosing CAP or VAP[75, 76]. This controversy suggests that PCT should be used as a complementary tool for the diagnosis of VAP alongside established diagnostic methods.

2.9.2 The soluble triggering receptor expressed on myeloid cells-1 (sTREM-1)

This has been identified as a molecule involved in the inflammatory response which belongs to the immunoglobulin superfamily. It is expressed on the surface of neutrophils, mature monocytes and macrophages[77]. TREM-1 is not upregulated in samples from patients with noninfective or inflammatory conditions such as psoriasis, ulcerative colitis or vasculitis caused by immune complexes[77]. Besides TREM-1 (which is the membranous form) a soluble form (sTREM-1) exists that is specifically released in several inflammatory processes[76]. The detection of this soluble form in body fluids shows promise as a diagnostic test, especially during severe sepsis and pneumonia, and in patients who have received previous antimicrobial drugs, since its determination is quick, cheap and can be performed in batches or for individual samples. No sTREM-1 assay is commercially available at present.

The diagnostic importance of sTREM-1 in pneumonia has been investigated in mechanically ventilated patients with a clinical suspicion of pneumonia[78]. The detection of sTREM-1 in BAL fluid was more accurate than clinical findings or laboratory testing (including CPIS and Procalcitonin) in identifying the presence of bacterial pneumonia. However, sTREM-1 was detected in the BAL fluid in 6/64 patients who did not have pneumonia.

Determann et al.[79] measured sTREM-1 levels in the BAL fluid on alternate days in 28 critically ill, mechanically ventilated patients from the day ventilation was initiated until weaning. Nine patients developed VAP whilst nineteen did not. In those who developed VAP, an increase in sTREM-1 levels were seen in BAL fluid until the day of diagnosis. In contrast sTREM-1 was barely detectable in the BAL fluid of those who did not develop VAP. Using a cutoff value of 200pg/ml on the day of VAP diagnosis has a diagnostic sensitivity of 75% and a specificity of 84%. On this basis sTREM-1 appears to be a reliable marker for VAP but its sensitivity is not 100%.

2.9.3 Determination of bacterial endotoxin in BAL fluid

More than 80% of VAP is caused by Gram-negative bacteria, which are associated with a high mortality. It has been postulated that the measurement of endotoxin in BAL fluid may permit the rapid diagnosis of Gram-negative bacterial pneumonia. Pugin et al.[80] reported that patients with VAP due to Gram-negative bacteria could be identified accurately by having an endotoxin concentration of more than 6 endotoxin units/ml in their BAL fluid. Kollef et al.[81] performed a study in which 63 patients underwent 71 episodes of BAL sampling and quantitative cultures. A cutoff value of more than 5 endotoxin units/ml yielded a 100% sensitivity and 75% specificity for the diagnosis of Gram-negative pneumonia. The findings of these and other studies indicate that this method may be a useful adjunct for the rapid diagnosis of Gram-negative pneumonia.

Several biomarkers are showing promise as early and accurate tests for the presence of lung infection in the mechanically ventilated patient. These assays may be used as screening tools to identify those who may benefit from microbiological testing and

empirical antibiotic therapy. Raised PCT and sTREM-1 also indicate a host inflammatory response to infection, and could be markers of those at risk of organ dysfunction and failure. Neither of these markers however can be used as a surrogate for bacterial infection. As with all diagnostic tests, the results must be interpreted with care in the context of the clinical state of the patient.

Chapter 3

Breath Analysis for the Detection of Disease

3.1 Introduction

In this chapter we will focus on the science that underlies the principles of operation of electronic noses (Enoses), Gas Chromatography-Mass Spectrometry (GC-MS) and Selected Ion Flow Transfer-Mass Spectrometry (SIFT-MS). These are the technologies that are currently at the forefront of breath analysis. Breath analysis is a technique that is gaining popularity as the search for novel diagnostic methods gathers pace. Breath analysis is attractive because it offers the potential for rapid, non invasive near patient diagnosis. The list of disease applications below is by no means exhaustive. It is merely meant to demonstrate the wide range of applications for this technology in both laboratory and clinical medicine.

3.1.1 Lung cancer

In 1999 Phillips et al.[82] found a combination of 22 VOCs in the breath of lung cancer patients which could be used to identify those with the disease. The compounds were identified using GC-MS and were predominantly alkanes, alkane derivatives and benzene derivatives. This was a cross-sectional study which looked at breath samples from 108 patients with abnormal chest x-rays. Patients then underwent bronchoscopy and histological or cytological sampling. 60 patients were confirmed to have lung cancer. Patients underwent breath sampling within 24 hours prior to bronchoscopy. The breath samples were analysed using GC-MS techniques.

Forward stepwise discriminant analysis was used to identify VOCs that could discriminate between patients with and without lung cancer. 67 VOCs were common to the breath samples of 62 patients, of these VOCs 22 were selected by discriminate analysis. Using this approach for the detection of stage 1 lung cancer the technique had 100% sensitivity and 81.3% specificity. Cross validation of the technique correctly predicted the diagnosis in 71.7% with lung cancer and 66.7% of those without.

The small number of patients with stage 1 lung cancer may have skewed the results in this paper as there seemed to be no increase in the predictive values of the technique as the lung cancer stage became more advanced. This goes against what would be expected. Also as this is a cross sectional study, the predictive values of this technique when applied to screening an unselected population is unknown.

In 2003 Di Natale et al.[83] used an electronic nose to identify lung cancer patients. A total of 62 breath samples were taken from 60 individuals by the bag collection method. 35 patients had lung cancer, 18 were used as reference subjects and 7 patients were post surgery for lung cancer. 100% of the lung cancer patients were correctly classified, 94% of the controls were correctly classified and 44% of the post surgical patients were also correctly classified. The misclassified post surgical patients were classified as healthy controls.

This study is promising but contains small sample numbers. It should also be mentioned that the cancer patients all had similar stage cancer, but no information about what stage of the disease the patients had is mentioned.

In 2005 Machado et al.[84] also used an electronic nose to analyse breath samples from lung cancer patients. This study used a group of 14 cancer patients and 45 non cancer patients (although some had lung pathology) to establish a discovery and training group. All 14 lung cancer patients then went into the validation group. The validation group consisted of the same 14 cancer patients and 68 non cancer patients. The non cancer patients were a mixture of healthy volunteers and patients with non cancer lung pathology. A total of 330 of 388 breath samples collected from this group were correctly classified; an overall accuracy of 85%. The electronic nose had 71.4% sensitivity and 91.9% specificity.

The cancer patients used to create the training model consisted of 13 patients with non small cell lung cancer and 1 patient with small cell lung cancer. In the validation model patients with small cell lung cancer were misclassified; this suggests the volatile fingerprints of small cell and non small cell lung cancer may be different. Also the VOC's chosen were identified using GC-MS, there is no evidence that the Enose detected the VOCs described in the paper.

3.1.2 Breast Cancer

In 2003 Phillips et al.[85] used 22 C4-C20 alkanes found in breath to identify women suffering with breast cancer from those without breast cancer. Two hundred breath samples were obtained from women with abnormal mammograms who subsequently underwent breast biopsy. Fifty one cases of breast cancer were detected in 198 consecutive biopsies.

The breath test distinguished between women with breast cancer and healthy volunteers with a sensitivity of 94.1% (48/51) and a specificity of 73.8% (31/42) (cross-validated sensitivity 88.2% (45/51), specificity 73.8% (31/42)). Compared to women with abnormal mammograms and no cancer on biopsy, the breath test identified breast cancer with a sensitivity of 62.7% (32/51) and a specificity of 84.0% (42/50) (cross-validated sensitivity of 60.8% (31/51), specificity of 82.0% (41/50)). The negative predictive value (NPV) of a screening breath test for breast cancer was superior to a screening mammogram (99.93% versus 99.89%); the positive predictive value (PPV) of a screening mammogram was superior to a screening breath test (4.63% versus 1.29%).

Again, this is a promising pilot study with small numbers.

3.1.3 Tuberculosis

In 2004 Pavlou et al.[86] used a Bloodhound Systems BH114 electronic nose to distinguish sputum samples infected with Mycobacterium tuberculosis from sputum infected with other related bacteria. Using the Enose combined with a neural network they were able to classify 100% of the TB samples correctly.

3.1.4 Pneumonia

In 2004 Hockstein et al.[87] used Enose analysis of the breath of ventilated patients in an intensive care unit to diagnose ventilator associated pneumonia. He compared breath analysis to computed tomography (CT) of the chest. They report an accuracy of at least 80% when comparing e nose analysis to chest CT.

Adrie et al.[88] have reported increased levels of nitric oxide in the breath of patients with pneumonia compared to those without pneumonia.

3.1.5 Urinary Tract Infection

In 2001 Pavlou et al.[89] used a Bloodhound Systems BH114 Enose to identify samples of urine that were infected with bacteria. They were further able to identify the species of bacteria causing the infection.

The above are examples of the many potential applications for the expanding field of breath analysis and "smelling". They offer the potential for rapid and non invasive diagnosis, not currently available to the clinician. The next section looks at some of the technologies currently in use for breath analysis.

3.2 Electronic Noses (Enose)

Since the time of the ancient Greeks, physicians have been aware that it is possible to smell disease on the breath of those afflicted. An obvious example of this is the pear drop like smell of acetone in the breath of a diabetic. Liver failure can also result in the characteristic "fetor hepaticas" on the breath. Bodily fluids such as urine can also have distinctive smells, which are specific to disease, such as the honey like smell of urine in phenylketonuria

The search began for an alternative way to identify these volatile fingerprints using cheaper and less sophisticated equipment. Research into the interactions between various volatile compounds and commercially available sensors commenced. During the 1970's attempts were made to study the possibility of using redox reactions of volatile compounds and amplifying volatile conductivity and the contact between volatile molecules and sensor-based materials. This research resulted in the creation of the first "electronic nose" by Persaud & Dodd in 1981[90].

They attempted to simulate the various stages of the human olfactory process using biochemical sensors on which volatile compounds can react, amplification and treatment of the sensor signal responses, and using neural networks to evaluate the key useful components of the data, resulting in volatile odour recognition. Unlike GC-MS, which is quantitative; this approach is largely qualitative or semi-quantitative, it is however, suited to rapid screening because results can be obtained in minutes as opposed to days. The advances in this methodology over the past ten years have allowed the rapid development of sensor technology together with artificial intelligence approaches[91].

Electronic noses have appeared in several different design types. All these designs however, consist of the same three basic components. These are: a sensor array over which the volatile compounds are passed, the conductance of the sensor changes depending on the degree of binding and results in a set of sensor signals, which are linked to data processing software which produces an output.

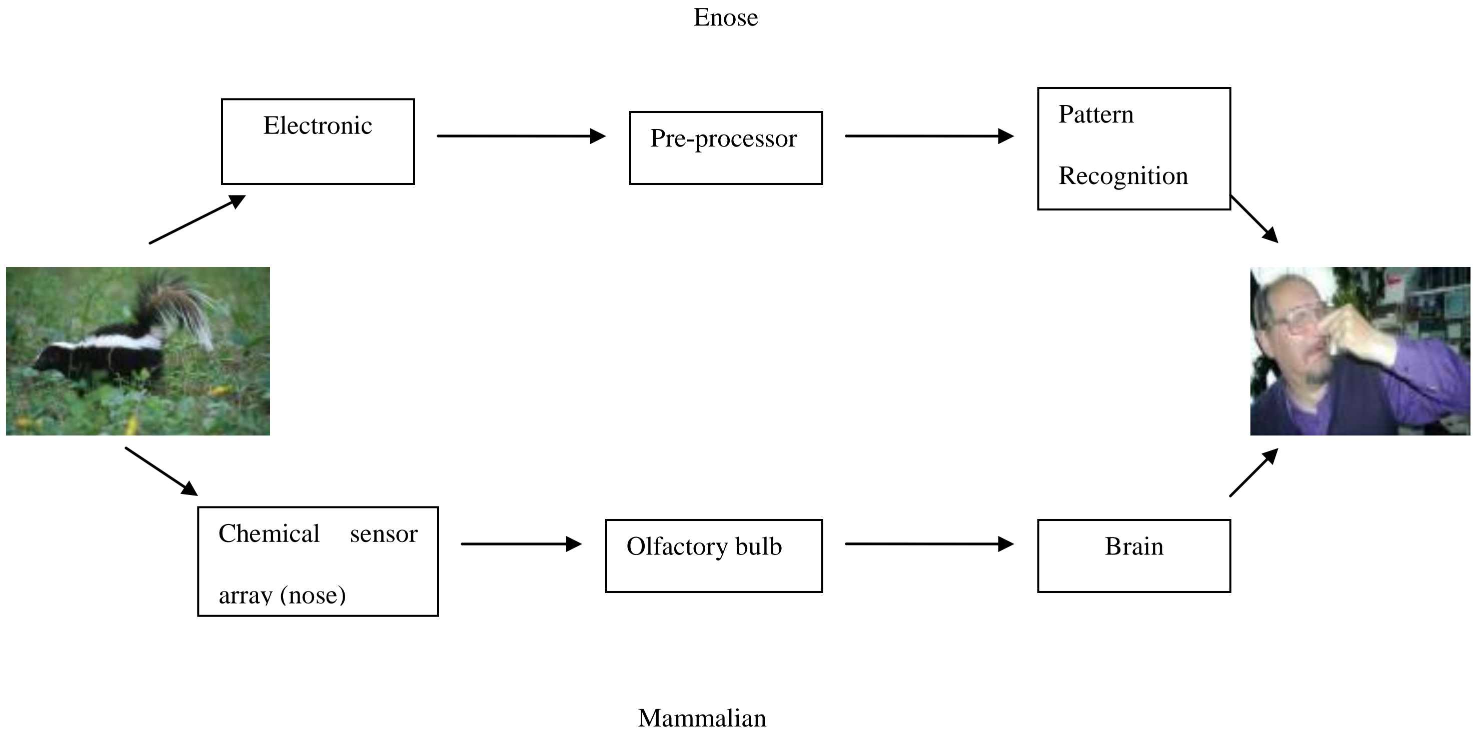

Fig 3.1 Schematic representation of the processing pathways in an Enose and the mammalian olfactory system.

3.2.1 Sensor Types

"A chemosensor is a device that is capable of converting a chemical quantity into an electrical signal and responds to the concentration of specific particles such as atoms, molecules, or ions in gases or liquids by providing an electrical signal"[92]. The type of chemosensors used in an electronic nose need to respond to odorous molecules in the gas phase, which are typically VOCs with different relative molar masses. The following descriptions of sensor types are adapted from Turner & Magan[93]

Metal Oxide Sensors

The oxides in these sensors contain chemically bound oxygen which can interact with the VOCs causing oxidation which alters the conductivity of the sensor. Altering the amounts of noble metals in the sensor or changing the operating temperature will alter the selectivity of the sensor. This type of sensor is resistant to humidity and ageing effects but they are susceptible to drift over time.

Conducting Polymer Sensors

This sensor type also exhibits a change in conductance when exposed to reducible or oxidizable gases. Organic conducting polymers show changes in conductivity when chemical substances adsorb and desorb from the polymer. The mechanism by which the conductivity is changed is not clear at present.

Metal oxide silicon field- effect sensors (MOSFET)

These are related to metal oxide sensors but the output signal is obtained from a change in potential when the volatile molecules react at a catalytic surface. They operate at 100-200 degrees centigrade.

Gravimetric Odour sensors

These are based on piezoelectric crystals coated with a suitable sorbent membrane. Detection of vapour molecules can then be achieved by their effect on the propagation of the acoustic wave causing changes in the resonant frequency and the wave velocity. The selectivity of these sensors is dependent on the thickness of the coating.

Surface acoustic-wave devices

These are an alternative to the above sensors and are based on waves that are emitted along the surface of a crystal by the electric field of surface-deposited aluminium electrodes.

Optical sensors

These function using an optical phenomenon in which incident light excites the volatile analyte. The resulting signal can be measured in the resulting absorbance, reflectance, fluorescence or chemiluminescence.

Electrochemical sensors

This class of sensor was amongst the first to be used in an electronic nose format. They contain electrodes and an electrolyte, the responses generated being dependent on the electrochemical characteristics of the volatile analyte. At the working electrode, the analyte is either oxidized or reduced, with the opposite reaction occurring at the counter

electrode. The voltage generated by the reactions between the electrodes is measured and has been used to detect CO_2, SO_2 and H_2S.

The most important point to remember is that total specificity is not required. Using multiple approaches it is sufficient for elements of the sensor array to react differently to various compounds allowing discrimination between samples.

As time goes on more is being demanded of sensor technology. The questions being asked are more complex and the challenge is to develop new sensor technologies to keep pace with the ever increasing demands. Without high quality sensors and good performance, we have little chance of collecting good data to answer these questions. New sensor types are being developed which will give improved consistency and sensitivity along with greater shelf life and reduced susceptibility to humidity and temperature variation.

3.3 Gas Chromatography

The first attempts to use breath as a way of gaining information on the physiological state of the human body were using GC-MS. As the name implies this method is actually two distinct technologies combined to form a powerful analytical tool. Gas chromatography separates the components of a mixture of chemicals and mass spectrometry characterises each of the components individually. By combining the two techniques as described it is possible to both qualitatively and quantitatively evaluate solutions or gases containing multiple chemical constituents.

Generally speaking, chromatography is used to separate mixtures of chemicals into their individual components; once these are isolated they can be individually evaluated. Separation occurs when the sample mixture is introduced (usually by injection) into a mobile phase. In gas chromatography, the mobile phase is an inert gas such as helium.

The mobile phase then carries the sample mixture through into a stationary phase. This is usually a chemical that can selectively attract components in a sample mixture. This is usually contained in a tube of some description which is referred to as a column. Columns can be made of glass or stainless steel and come in various sizes.

The mixture of compounds in the mobile phase then interacts with the stationary phase. Each compound in the mixture interacts at a different rate; those that interact the fastest will exit (elute from) the column first. By altering the characteristics of the mobile and stationary phases, different mixtures of chemicals can be separated. This separation process can be affected further by changing the temperature of the stationary phase or the pressure of the mobile phase.

The column is held in an oven that can be programmed to increase the temperature gradually (ramped) which helps with separation. As the temperature increases, compounds with lower boiling points elute from the column sooner than those with

higher boiling points. Therefore, there are two distinct separating forces, temperature and stationary phase interactions mentioned earlier.

As the eluted compounds leave the column they enter a detector, this is capable of generating an electronic signal whenever the presence of a compound is detected. The greater the concentration, the bigger the signal. This signal is then interpreted by a computer. The time from when the injection is made (time zero) to when elution occurs is referred to as the *retention time*.

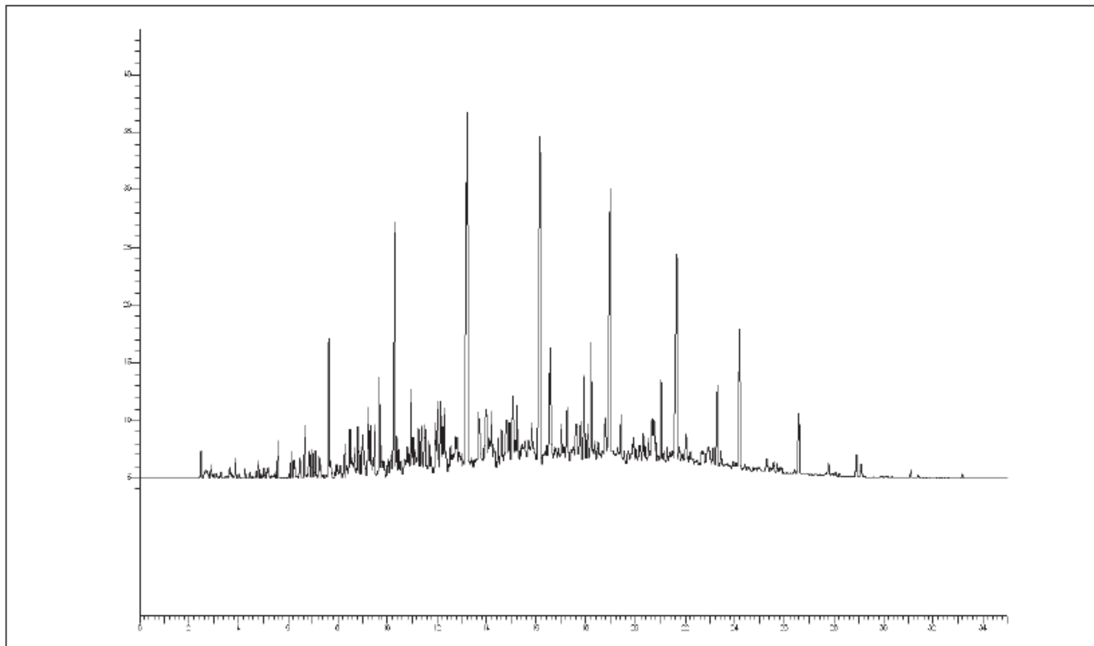

Fig 3.2 Chromatogram of Kerosene. From www.las.perkinelmer.com

The above figure shows a chromatogram generated by a GC for kerosene. Each of the peaks represents the signal generated when a compound elutes from the column into the detector. The x-axis shows the retention time (RT), and the y-axis shows the intensity (abundance) of the signal. Each peak represents an individual component separated from the kerosene.

If the GC conditions (temperature ramp, column type etc.) are the same, a given compound will always elute from the column at nearly the same RT. By knowing the RT for a given compound, we can make assumptions about the identity of that compound. However, compounds with similar properties will often have the same retention times. Therefore more information is needed before the identification of a compound in a sample containing a mixture of unknown compounds can be made.

3.4 Mass Spectrometry

As the individual compounds elute from the GC column, they enter the mass spectrometer (electron ionisation) detector. They are bombarded with a stream of electrons causing them to break apart into fragments. These are pieces of the original molecules of varying sizes.

The fragments are charged ions with a certain mass. The mass of the fragment divided by the charge is called the mass to charge ratio (M/Z). Since most fragments have a

charge of +1, the M/Z usually represents the molecular weight of the fragment. A group of four magnets (called a *quadropole)* focuses each of the fragments through a slit and into the detector. The quadropoles are controlled by the computer to direct only certain M/Z fragments through the slit. The rest are deflected away. The computer cycles the quadropoles through different M/Z's individually until a range of M/Z's are covered. This occurs many times a second. Each cycle of ranges is referred to as a *scan.*

The computer records a graph for each scan. The x-axis represents M/Z ratios. The y-axis represents the signal intensity (abundance) for each of the fragments detected during the scan. This graph is referred to as a *mass spectrum*.

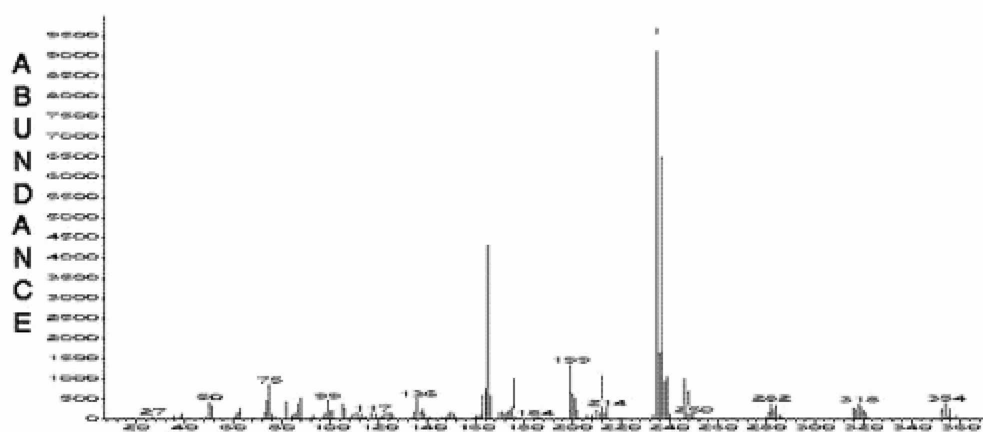

Fig.3.3 Mass spectrum of DDT.

From http://www.unsolvedmysteries.oregonstate.edu/MS_06

The mass spectrum generated by a given chemical compound is essentially the same every time. Therefore, the mass spectrum is a fingerprint for the molecule. This can then be used to identify the compound in question. The computer usually compares the mass spectrum from an unknown compound with a library of known mass spectra in order to identify the compound.

3.5 Gas Chromatography-Mass Spectrometry (GC-MS)

It can be seen from the above discussion that combination of these two analytical technologies produces a powerful tool for the identification of compounds. Not only can a researcher identify the individual components he or she can also determine the quantities (concentrations) of each of the components.

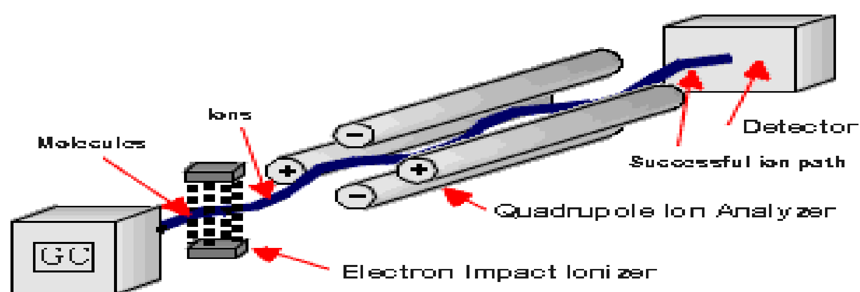

Fig. 3.4 Schematic illustration of a GC-MS machine.

From http://www.chem.arizona.edu/massspec/intro_html/intro.html

3.6 Origins of endogenous volatile biomarkers

Of the hundreds of VOCs that can be identified in human breath, most are of exogenous origin. Endogenous markers, which are commonly used for diagnostic purposes, are hydrocarbons such as ethane, pentane and isoprene; oxygen containing compounds such as acetone, acetaldehyde, methanol, ethanol and 2-propanol; sulphur containing compounds such as dimethylsulfide, methyl and ethyl mercaptans; and carbon disulfide and nitrogen containing substances such as ammonia and dimethyl/trimethylamine. In order to assess the physiological importance and diagnostic potential of these compounds, the biochemical pathways of production need to be understood[94].

3.6.1 Saturated hydrocarbons

Lipid peroxidation is a chain reaction initiated by the removal of an allylic hydrogen atom through reactive oxygen species (ROS). The radical generated in this reaction is conjugated, peroxidised by oxygen and then undergoes further reactions. Saturated hydrocarbons such as ethane and pentane are generated in this way from omega3 and omega6 fatty acids respectively. Aldehydes such as malondialdehyde are generated along the same pathway. Omega3 and omega6 fatty acids are basic components of cell membranes. In vitro and in vivo studies have demonstrated an association between clinical conditions with high peroxidative activity and the exhalation of ethane and pentane[95]. As a result, these hydrocarbons are considered as in vitro and in vivo markers of lipid peroxidation. Although there are other potential sources of hydrocarbons in the body, such as protein oxidation and colonic bacterial metabolism, these are apparently of limited importance and do not interfere with interpretation of the

hydrocarbon breath test for ethane and pentane[96]. Propane and butane are mainly formed from protein oxidation and faecal flora, and their role as markers of lipid peroxidation is uncertain.

Due to the physiological ratio of omega3 to omega6 fatty acids, four times more pentane than ethane is generated through lipid peroxidation. Pentane, but not ethane, may be easily metabolised by hepatic cytochrome P450 enzymes[97]. As a result, exhaled pentane concentrations must be interpreted cautiously whenever liver function varies between patients or during the sampling period.

Hydrocarbons as stable end products of lipid peroxidation show only low solubility in blood and are therefore excreted into breath within minutes of their formation in tissues. Hence, exhaled concentrations of ethane and n-pentane can be used to monitor the degree of oxidative damage in the body[98, 99].

3.6.2 Unsaturated hydrocarbons

Isoprene (2-methylbutadiene-1,3) is ubiquitous in human breath and thought to be formed along the mevalonic pathway of cholesterol synthesis. The parallel decrease in isoprene secretion and sterol synthesis caused by acute or chronic lovastatin administration (a drug that reduces cholesterol synthesis) suggests that breath isoprene is derived from the cholesterol synthesis pathway in humans in vivo[100]. A small fraction of exhaled isoprene may be of bacterial origin[101].

There is experimental evidence that isoprene exhalation may be related to oxidative damage to the fluid lining of the lung[102] and the body[103]. Human breath isoprene concentrations show a circadian rhythm with a maximum at around 06:00 and a minimum at around 18:00. Concentrations of breath isoprene also seem to be age dependent, being significantly lower in children[104].

3.6.3 Oxygen-Containing Compounds

Acetone is one of the most abundant compounds in human breath. It is produced by hepatocytes via decarboxylation of excess Acetyl-CoA. Acetone is formed by decarboxylation of acetoacetate, which derives from lipolysis or lipid peroxidation. Ketone bodies such as acetone are oxidised via the Krebs cycle in peripheral tissue. Ketone bodies in blood are increased in ketonemic subjects in times of fasting, starving or during diet. Breath acetone concentrations are increased in patients with uncontrolled

diabetes mellitus[105]. As acetone is produced by spontaneous decarboxylation of acetoacetate, it is impossible to quantify the fraction that arises from lipid peroxidation.

Acetaldehyde is probably produced by oxidation of endogenous ethanol. As a result, acetaldehyde concentrations in breath are always lower than corresponding ethanol concentrations.

Ethanol concentrations in breath of human subjects are normally very much lower than the levels found in human breath after alcohol ingestion. The potential source of endogenous ethanol is the intestinal bacterial flora[106]. 2-propanol is thought to be a product of an enzyme mediated reduction of acetone. As for acetaldehyde, concentrations of 2-propanol in humans are always lower than acetone concentrations[107]. The origin of breath methanol may also be the intestinal flora.

3.6.4 Sulphur-containing compounds

Compounds such as ethyl mercaptan, dimethylsulfide ordimethyldisulfide are responsible for the characteristic odour in the breath of cirrhotic patients[108]. Sulphur-containing compounds are generated in humans by incomplete metabolism of methionine in the transamination pathway[109]. Mercaptans are easily oxidised to their respective sulphides. Under normal conditions, concentrations of sulphur-containing compounds in human blood and breath are very low. Their concentrations are increased by impaired liver function.

3.6.5 Nitrogen-containing compounds

The characteristic odour of uraemic breath is due to elevated levels of dimethylamine and trimethylamine. Significant levels of ammonia will appear in the blood if the removal of ammonia by its conversion into urea is limited due to impaired liver function. Ammonia could also be identified in the breath of normal and in higher concentrations in uraemic patients[110].

3.7 Technical and methodological issues related to breath analysis

Breath analysis has not been introduced into mainstream clinical practice despite some promising results in the literature. The main stumbling blocks seem to surround technical problems such as sampling, preconcentration and analysis, as well as basic methodological issues such as normalisation and expression of data. Generally accepted standards of sampling, preconcentration, and analysis do not exist. As such, reproducibility and reliability of sampling methods and analytical procedures are of paramount importance.

3.7.1 Sampling

The collection of gas samples during breath analysis represents a fundamental issue. Dilution and contamination of samples with dead space gas and loss of analytes during the sampling procedure have resulted in huge variations of substance concentrations between different studies. There are two basic approaches to breath collection:

1. Mixed expiratory sampling means that total breath, including dead space air, is collected.
2. Alveolar sampling means that pure alveolar gas is collected.

The first method has been the most widely employed as it is the simplest to perform in spontaneously breathing subjects and requires no additional equipment. Endogenous VOC concentrations are two to three times higher in alveolar air than in mixed expiratory air samples. This is because there is no dilution by dead space gas. Alveolar gas samples also have the lowest concentrations of contaminants[111, 112].

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The figure below shows a schematic of a capnography trace illustrating the different phases of breathing. Phase I is anatomical dead space gas which does not contain any endogenous VOCs or CO_2. Phase II contains a mixture of alveolar and dead space gas. Phase III reflects alveolar emptying and as a result, minimally changing CO_2 concentration.

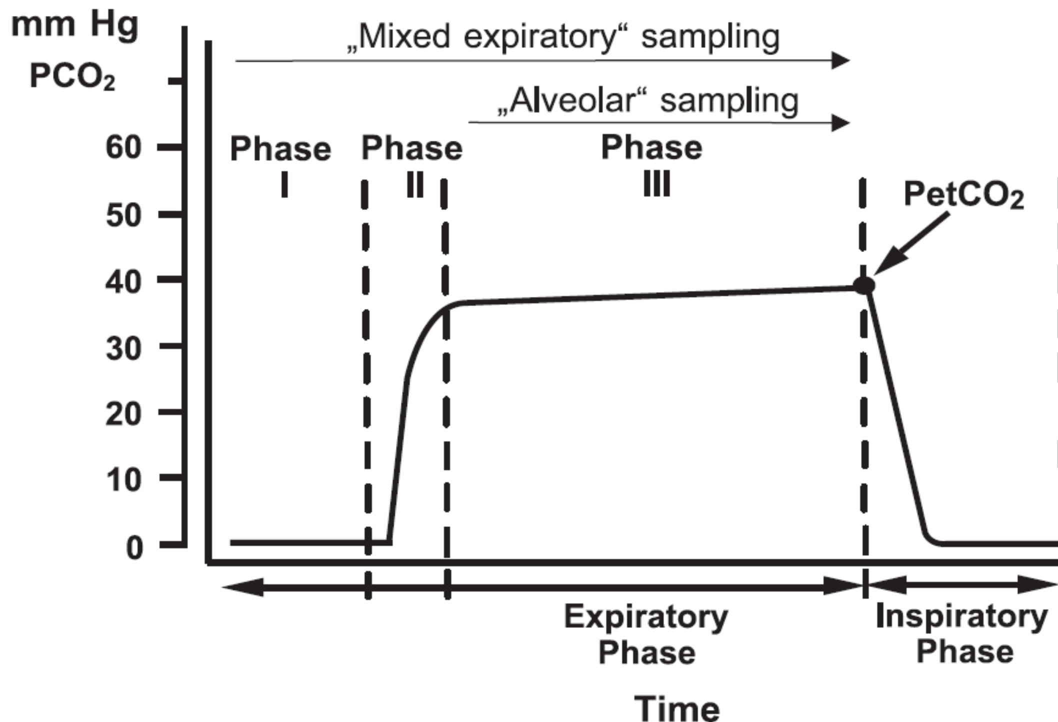

Fig 3.5 Schematic illustration of a capnography trace showing the different phases of sampling. From Miekisch et al 2004[94].

To allow distinction between exogenous and endogenous compounds some form of correction for background inspired air is necessary. Different approaches to this problem have been utilised. Substance concentration in ambient or inspired air have been measured, and an "alveolar gradient" calculated by subtracting inspiratory from expiratory concentrations[113]. Others have attempted to eliminate ambient contaminants by having patients breathe pure air for a time before sampling[98]. This approach is not only time consuming and cumbersome but cannot easily eliminate exogenous compounds merely by breathing pure air; as a result it is unlikely to be applicable in the clinical setting.

Subtraction methods such as the calculation of alveolar gradients are easier to perform, but they are too simplistic. They fail to take into account the complex nature of pulmonary adsorption and exhalation of volatile substances. This is a particular problem when the inspiratory concentrations are of the same order of magnitude as the expired concentrations of interest. Expired samples may be diluted or contaminated by inspiratory and/or dead space ventilation, which is dependent on the breathing pattern [114]. Also the ventilation/perfusion ratio in the lung and alveolar concentration gradient of the substances affect the excretion and intake of volatile substances[115].

As the metabolic pathways of many VOCs are not understood exogenous substances may appear in the breath having been ingested hours or days prior to the sampling, from locations remote to the sampling site. Therefore subtracting compounds from the inspired ambient air will not take into account these compounds which may then be falsely considered as endogenous. Some compounds of interest that have been identified in vivo may also be metabolised prior to excretion and therefore not detected. If the biochemical pathways involved in this metabolism are not understood then these metabolites may not be identified as compounds of interest.

Sampling may be single breath or time limited. If single-breath sampling is employed steps have to be taken to ensure that this single breath is representative for all subsequent breaths. This is particularly important in the spontaneously breathing subject as breath to breath concentrations may vary considerably.

3.7.2 Preconcentration and desorption

Because most substance concentrations in exhaled breath fall in the nmol/l-pmol range, preconcentration is necessary. This can be achieved by adsorption onto sorbent traps, coated fibres[116, 117] or by direct cryofocussation[118]. Variation in the boiling points of different VOCs means that adsorbents in sorbent traps have to be carefully selected to avoid breakthrough as well as memory effects. Organic polymers (e.g. Tenax TA)[119], activated charcoal[120], different types of graphitized carbon (e.g. Carbopack X), and carbon molecular sieves (e.g. Carboxen 1021) have been used[121]. Organic polymers are least affected by high water content in the samples but have low breakthrough volumes, especially for small hydrocarbons. In contrast, carbon molecular sieves and graphitized carbon have high breakthrough volumes for these compounds. The downside is possible memory effects when these compounds are used as the only adsorption material. Multibed sorbent traps can overcome these problems[121, 122]. For further analysis the VOCs must be liberated from the adsorption material. This can be achieved by heating the trap (thermal desorption) or by means of microwave energy.

3.7.3 Analysis and detection

Hydrocarbons in the nmol/l-pmol/l range are usually determined using GC coupled to flame ionisation or mass selective detection. The choice of separation column is crucial. Substances such as isopentane may coelute with pentane[123]. As a result mass spectrometric analysis has to be applied for the detection of unknown compounds. The high water content of breath samples can affect preconcentration, separation and detection of single compounds. This is a particular problem in mechanically ventilated patients who breathe humidified gases.

3.7.4 Normalisation/expression of data

Problems with this aspect of breath analysis has been one of the major causes of the significant variations in results seen in published studies. The normalization of data by the ratio of PCO_2 in the sample to end tidal PCO_2 is a minimum requirement when mixed expiratory and not alveolar samples are taken. This is necessary to account for the dilution of the sample by dead space air. Because the extent of this dilution cannot be quantified and may be variable, the only method to obtain reliable and comparable results is the normalisation of samples to alveolar concentration levels[98, 111].

Exhaled compound concentrations can be affected by physiological factors such as cardiac output, minute ventilation and ventilation/perfusion ratio in the lung. In healthy volunteers, these effects may be negligible. In patients with significant physiological dysfunction the effects can be marked.

3.8 Breath Markers in disease

The main disease targets for investigation have been lung diseases, inflammatory and malignant processes and specific disease states such as allograft rejection and renal failure. The requirements and problems with breath testing differ depending on the type of study undertaken:

In cross sectional studies a control group is compared to a patient or disease group. Breath markers are analysed in order to detect qualitative or quantitative differences between the two groups. Attempts are made to find biomarkers specific to the disease in question or predictive biomarkers.

In longitudinal studies, disease progression is investigated. Breath markers are observed during the timecourse of a disease or an intervention within a patient group. Variation of results is less of a problem than in cross sectional studies, because each patient can act as their own control.

Due to individual differences, concentrations of some volatiles may vary considerably even in the healthy state[82]. In spite of this some correlations between clinical conditions and breath marker concentrations have been described.

3.8.1 Lung Cancer

Approximately 180,000 people in the USA develop lung cancer every year. The 5 year survival over all is a depressing 14%. This can be increased to 50% if lung cancer is localised at the time of diagnosis and treatment is prompt. The detection of lung cancer at an early stage could be accomplished by breath analysis when useful markers have been determined[82]. Studies have been performed in attempts to characterise compounds in breath samples from patients with and without lung cancer. Samples were collected with a portable machine in which 10L of breath was passed through a sorbent trap containing activated carbon. VOCs were then thermally desorbed, concentrated by two stage cryofocussing and then quantified by GC-MS.

A combination of more than 20 breath VOCs, predominantly alkanes, alkane derivatives, o-toluene, analine, and benzene derivatives, along with altered lipid peroxidation activity [124-127], was shown to be able to discriminate patients with lung cancer with a probability of about 70%. More work is needed to confirm these findings in the general population.

3.8.2 Inflammatory Lung Diseases

Non-invasive monitoring of these conditions including asthma, cystic fibrosis, chronic obstructive pulmonary disease, bronchiectasis and interstitial lung diseases can be observed by various biomarkers in the breath. Nitric oxide, carbon monoxide, ammonia, and many non-volatile molecules (isoprostanes, leukotrienes, prostaglandins, cytokines, products of lipid Peroxidation, nitrite/nitrate, S-nitrosthiols, nitrotyrosine) have been measured in breath samples[128]. The analysis of exhaled breath may also be used to monitor a course of therapy. Increased concentrations of 8-isoprostane, hydrogen peroxide, nitrite, and 3-nitrotyrosine are found in exhaled breath in inflammatory lung diseases. In addition, increased levels of lipid mediators are found in these conditions, with a differential pattern depending on the nature of the disease process[129].

3.8.3 Nitric Oxide as a Marker of Pneumonia in Ventilated Patients

Nitric Oxide (NO) has been proposed as a marker of pneumonia in ventilated patients by Adrie et al[88]. Nitric Oxide is known to play a vital role in the control of vasomotor tone and blood flow in both the systemic and pulmonary circulation[130, 131]. NO is synthesised by different forms of nitric oxide synthase (NOS). A calcium independent isoform of NOS, known as inducible NOS (iNOS), can be activated by various cytokines and bacterial endotoxin. This can lead to excess production of NO in many inflammatory conditions. The consequence of this is inappropriate systemic vasodilatation, as seen in bacterial sepsis. Steudel et al.[132] proposed that iNOS is the principal contributor to exhaled NO synthesis. Adrie et al.[88] hypothesised that as inflammation results from

infection, which in turn releases NO, exhaled NO levels would be elevated in ventilated patients with pneumonia. NO may therefore act as a marker of pneumonia in these patients.

NO levels were measured using chemoluminescence. Plasma nitrite and nitrate levels were also measured, in order to investigate any correlation between exhaled NO levels and plasma levels of nitrite and nitrate. This was to test the hypothesis that nonspecific inflammation could induce iNOS expression specifically in the airway epithelium, which would be detected by a rise in NO levels in the nasal passages, or non-specifically as seen by an increase in plasma nitrite levels. NO contamination from inhaled gases was excluded by the use of specific filters.

Forty nine patients admitted consecutively within 72 hours to the intensive care unit were included. Twenty one of these patients were diagnosed as having pneumonia based on a clinical scoring system similar to CPIS. Significantly higher tracheal NO levels were observed in those diagnosed with pneumonia. The researchers proposed an end-expiratory tracheal threshold level of 5 ppb and a nasal NO threshold of 500 ppb as definitive for a diagnosis of pneumonia. Nasal NO levels were also elevated in this group, suggesting the presence of a generalised airway epithelium response rather than a specific distal airway response. The threshold levels established were then validated on a second group of 60 consecutive patients admitted to the intensive care unit.

This hypothesis was further supported by the finding of an inverse correlation between plasma NO_x levels and exhaled NO levels in patients with pneumonia. This was not seen in the total population. The authors argue that this could be specific for pneumonia as this was not seen in patients with other causes of sepsis. Delen et al.[133] showed that exhaled NO was elevated in chronic bronchitis but not in patients with Chronic Obstructive Pulmonary Disease (COPD). Exhaled levels of NO have been found in patients with asthma[134, 135] further supporting its role as a marker of airway inflammation.

3.8.4 Oxidative stress

Lipid peroxidation is a free radical mediated degradative process which involves polyunsaturated fatty acids in cells. This leads to the formation of lipid hydro peroxides, whose decomposition process gives rise to a wide variety of carbonyl secondary oxidation products[136, 137]. Malondialdehyde, one of the major products, has been found to be elevated under various conditions of oxidative stress, where reactive oxygen and nitrogen species play important roles.

Cancer has been shown to be accompanied by the increased production of oxygen free radicals that degrade membranes by lipid peroxidation, releasing VOCs that are seen in the breath. Heart transplant rejection is also accompanied by increased oxygen free radical activity, and breath VOC analysis may be able to detect markers of rejection. Increased levels of ethane and pentane have been related to oxidative stress in breast cancer[138], heart transplant rejection[139] and bronchial asthma[140] although they are of little value for diagnostic purposes owing to the lack of sensitivity and specificity. Methylated alkanes have also been proposed as markers of oxidative stress[141].

3.9 Selected Ion Flow tube- Mass Spectrometry (SIFT-MS)

Selected ion flow tube mass spectrometry (SIFT-MS) was conceived in 1976 by Adams & Smith[142]. It provides the opportunity for real time detection and quantification of the trace gases present in exhaled breath. GC-MS has been used to perform analysis on exhaled breath and detect compounds at parts per billion (ppb) and even parts per trillion (ppt) levels for several decades. However, it requires the collection of the gases of interest onto specialised adsorbtion or cryogenic traps. This means that GC-MS cannot be regarded as real time monitoring.

The other disadvantages of GC-MS are as follows:

(i) The fragmentation pattern of each component of the gas mixture must be known when the electron ionisation method is used. This is generally the case in GC-MS.

(ii) The instruments have to be calibrated for each trace gas to achieve accurate quantification.

(iii) Low molecular weight compounds such as formaldehyde and ammonia are not readily detected by GC-MS[143, 144].

SIFT-MS presents a real time breath analytical tool which does not suffer the drawbacks listed above.

3.9.1 How does SIFT-MS work?

A detailed description of the physics and chemistry underlying this technology is beyond the scope of this introduction but can be found in Smith & Spanel 2004[145]. A basic description follows:

The trace gases in the breath sample are ionised using a precursor ion species (either H_30^+, NO^+ or 0_2^+ are used depending on the trace gas compounds to be analysed) chosen from the mixture of ion species formed in a microwave discharge by a quadropole mass filter. The ions are injected into a fast flowing inert carrier gas (usually helium at a pressure of ~ 100Pa) through a Venturi-type orifice (diameter typically 1-2mm). The breath sample is introduced into the entry port of the SIFT-MS machine via a mouthpiece and then into the carrier gas at a known flow rate through a heated calibrated capillary. The precursor ions are thermalised to the carrier gas temperature (usually 300 K) by multiple collisions with the helium atoms before reacting with the trace gases in the breath sample. It is the ratio of the count rates of the product ions to the precursor ions that is essential parameter in the quantification of the particular trace gas compounds.

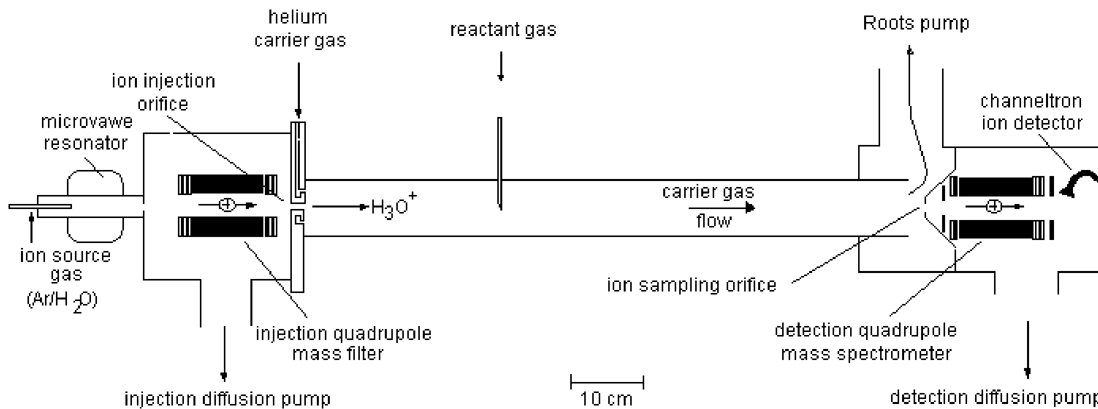

Fig 3.6 Schematic representation of the SIFT-MS instrumentation showing the main components. This diagram shows H_3O^+ as the precursor ions.

3.10 Pattern Recognition and Statistical Methods

Interpretation of the sensor data and the characteristics of individual odour patterns are critical to the functioning of an Enose. During odour classification, new sensor data is compared against a set of known volatile profiles. These form the "odour memory". A number of statistical methods have been employed in order to facilitate data reduction in order to study complex volatile mixtures. In these complex mixtures overlapping sensor sensitivities are partially correlated. In this piece of research we have focused on the use of Principal Components Analysis (PCA) and Linear Discriminant Analysis (LDA).

3.10.1 Principle Components Analysis (PCA)

(from http://home.ubalt.edu/ntsbarsh/stat-data/Topics.htm)

"Multivariate analysis is a branch of statistics involving the consideration of objects on each of which are observed the values of a number of variables. Multivariate techniques are used across the whole range of fields of statistical application: in medicine, physical and biological sciences, economics and social science, and of course in many industrial and commercial applications.

Principal components analysis is used for exploring data to reduce the dimension. Generally, PCA seeks to represent n correlated random variables by a reduced set of uncorrelated variables, which are obtained by transformation of the original set onto an appropriate subspace. The uncorrelated variables are chosen to be a good linear combination of the original variables, in terms of explaining maximal variance and orthogonal directions in the data. Two closely related techniques, principal component analysis and factor analysis, are used to reduce the dimensionality of multivariate data. In these techniques correlations and interactions among the variables are summarized in terms of a small number of underlying factors. The methods rapidly identify key variables or groups of variables that control the system under study. The resulting dimension reduction also permits graphical representation of the data so that significant relationships among observations or samples can be identified.

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The main applications of factor analytic techniques are:

1. To reduce the number of variables and
2. To detect structure in the relationships between variables, that is to classify variables.

Therefore, factor analysis is applied as a data reduction or structure detection method. Confirmatory factor analysis allows you to test specific hypotheses about the factor structure for a set of variables, in one or several samples.

Correspondence analysis is a descriptive/ exploratory technique designed to analyse two-way and multi-way tables containing some measure of correspondence between the rows and columns. These results provide information which is similar in nature to those produced by factor analysis techniques, and they allow one to explore the structure of categorical variables included in the table.

Combining Two Variables into a Single Factor. One can summarise the correlation between two variables in a scatterplot. A regression line can then be fitted that represents the “best” summary of the linear relationship between the two variables. If we could define a variable that would approximate the regression line in such plot, then that variable would capture most of the “essence” of the two items. Subjects’ single scores on that new factor, represented by the regression line, could then be used in future data

analyses to represent that essence of the two items. In a sense we have reduced the two variables into one factor. Note that the new factor is a linear combination of the two variables.

Principal Components Analysis. The example above, combining two correlated variables into one factor, illustrates the basic idea of principle component analysis. If we extend the two-variable example to multiple variables, then the computations become more involved, but the basic principle of expressing two or more variables by a single factor remains the same.

Extracting Principle Components. The extraction of principle components amounts to a variance maximising rotation of the original variable space. For example in a scatterplot we can think of the regression line as the original x-axis, rotated so that it approximates the regression line. This type of rotation is called variance maximising because the goal of the rotation is to maximise the variability of the "new" variable (factor), while minimising the variance around the new variable.

Multiple orthogonal factors: After we have found the line on which the variance is maximal, there remains some variability around this line. In PCA, after the first factor has been extracted, that is, after the first line has been drawn through the data, we continue and define another line that maximises the remaining variability, and so on. In this manner, consecutive factors are extracted. Because each consecutive factor is defined to maximise the variability that is not captured by the preceding factor,

consecutive factors are independent of each other. Put another way, consecutive factors are uncorrelated or orthogonal to each other.

How many factors to extract? As we extract consecutive factors, they account for less and less variability. The decision on when to stop extracting factors basically depends on when there is only very little "random" variability left. The nature of this decision is arbitrary; however, various guidelines have been developed."

3.10.2 Linear Discriminant Analysis (LDA)

(http://home.ubalt.edu/ntsbarsh/stat-data/Topics.htm)

"Linear discriminant analysis (LDA) and the related Fisher's linear discriminant are used in statistics to find the linear combination of features which best separate two or more classes of object or event. The resulting combinations may be used as a linear classifier, or more commonly in dimensionality reduction before later classification.

LDA is closely related to ANOVA (analysis of variance) and regression analysis, which also attempt to express one dependent variable as a linear combination of other features or measurements. In the other two methods however, the dependent variable is a numerical quantity, while for LDA it is a categorical variable (i.e. the class label).

LDA is also closely related to principal component analysis (PCA) and factor analysis in that both look for linear combinations of variables which best explain the data. LDA

explicitly attempts to model the difference between the classes of data. PCA on the other hand does not take into account any difference in class, and factor analysis builds the feature combinations based on differences rather than similarities. Discriminant analysis is also different from factor analysis in that it is not an interdependence technique: a distinction between independent variables and dependent variables (also called criterion variables) must be made.

Unlike PCA, LDA requires information as to how the variables are classified. LDA involves finding a Linear Discriminant Function that maximises the distance between two groups whilst minimising the distance between the members of each group. The LDF can therefore be thought of as a vector or direction.

The purpose of LDA is to classify objects into one of two or more groups based on a set of features that describe the objects. In general, we assign an object to one of a number of predetermined groups based on observations made on the object.

Note that the groups are known or predetermined and do not have order (i.e. nominal scale). The classification problem gives several objects with a set of features measured from those objects. We are looking for two things:

1. Which set of features can best determine group membership of the object?
2. What is the classification rule or model to best separate those groups?”

During the analysis of the data presented in this research we have used PCA, LDA and PCA fed LDA for the classification of Enose data.

Chapter 4

Methods

Chapter 4 Methods

This section will describe the general methods employed in the design and execution of this study. Detailed methods for each of the individual experiments are contained in the relevant sections.

Approval was gained from the Gloucestershire Local Research Ethics Committee for all the research experiments described in this thesis. The issue of gaining informed consent from patients receiving mechanical ventilation is a difficult area. The majority of these patients are unable to give informed consent as they are either unconscious or sedated to allow intubation and ventilation. UK law states that no adult can give consent for medical treatment on behalf of another adult. The ethics committee stated that where a close family member was available we were to gain assent (not consent) from that family member as an indication of the patient's wishes. Should the patient then recover and be able to give informed consent, this was gained retrospectively. If the patient subsequently refused to be included in the study, their samples and study data were to be confidentially destroyed. This scenario did not arise during the course of this study.

BAL samples and breath samples collected with the Breathotron were collected from the same patient in one sampling episode. Breath samples were collected prior to BAL samples in order to avoid the potential for the normal saline used during the BAL sampling to contaminate the breath samples.

BAL sampling was performed by instilling 20ml of sterile normal saline solution into the respiratory tract of the patient and aspirating back as much of the fluid as possible. The recovered fluid was then divided into two equal aliquots; one of which was sent for standard microbiological analysis and the other was sent to Cranfield for Enose analysis. Enose analysis is performed in an attempt to discriminate patients with evidence of microbiological growth in the BAL sample from those without. We compare this analysis with accepted microbiological methods. Further details can be found in Chapter 5.

The breath samples were collected by passing a fixed volume of breath (total of 5 litres) through a specially designed sorbent trap. This trap collects and pre-concentrates volatile compounds in the breath. The compounds are then analysed by means of a GC-MS machine in an attempt to identify the many individual compounds present in a single breath sample. This was undertaken in order to investigate whether we could identify compounds in breath that may act as biomarkers of VAP. More detail on this process can be found in Chapter 6.

At the same time that breath samples are being collected as described above, the Breathotron also uses a single MMOS sensor to analyse the breath being collected. This is an attempt at using a single sensor Enose type approach to breath analysis instead of employing a sensor array as found in most commercial Enose devices. If a single sensor has the ability to discriminate breath samples with acceptable accuracy then this could mean a smaller and cheaper device could be developed for near patient testing. More details can be found in Chapter 7.

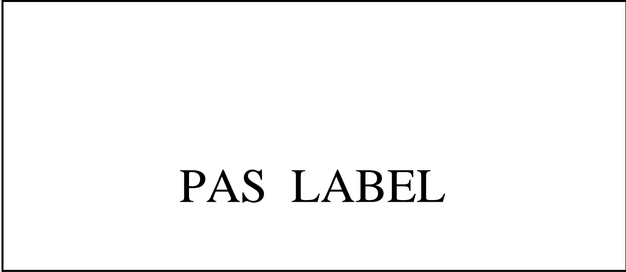
Patients were identified in the Intensive Care Units (ICU) at Cheltenham General and Gloucestershire Royal Hospitals. Patients who had been receiving mechanical ventilation for a minimum of 48 hours were identified. The reason for ventilation was not considered important. The first samples were collected as close to day 3 of ventilation as possible. Samples were then repeated on a Monday, Wednesday and Friday until the patient no longer required ventilation.

The Breathotron was connected to the exhaust arm of the ventilator circuit and a heat and moisture exchange (HME) filter was placed in the circuit between the patient and the Breathotron. This was done to prevent contamination of the Breathotron by respiratory secretions which could have the potential to cross infect study participants. The filters are designed to block micro-organisms but it was not felt that they should trap VOC's and have a detrimental effect on the breath sampling.

A patient data collection proforma was completed for every sampling episode. An example is shown overleaf.

Ventilator Associated Pneumonia: Tandem Breath Analyser Vs. Bronchoalveolar Lavage

Patient Data Collection Sheet



Please complete one of these forms EACH time a patient undergoes BAL.

Study number: **Trap number:**

Date sampling performed:

Current diagnosis (reason for ventilation):

Drugs at time of sampling (inc. sedation etc.):

Antibiotic therapy within last 48h (inc. prophylactic in theatre):

VAP clinical diagnosis criteria

Temp °C

>or equal to 36.5 and <or equal to 38.4	= 0 points
>or equal to 38.5 and <or equal to 38.9	= 1 point
>or equal to 39 and <or equal to 36.0	= 2points

White cell count/mm^3

>or equal to 4000 and <or equal to 11000	= 0 points
<4000 or >11000	= 1 point

Tracheal Secretions

No secretions	= 0 points
Non purulent secretions	= 1 point
Purulent secretions	= 2 points

Oxygenation: PaO_2/FiO_2, KPa

>31.6 or ARDS	= 0 points
< or equal to 31.6 and no ARDS	= 2 points

CXR findings

No infiltrates	= 0 points
Diffuse/patchy infiltrates	= 1 point
Localised infiltrates	= 2 points

Progression of infiltrates

No radiographic progression	= 0 points
Radiographic progression	= 2 points
(after exclusion of CHF and ARDS)	

Culture of tracheal aspirates

Pathogenic bacteria cultured in rare or light growth/no growth	= 0 points
Pathogenic bacteria cultured in moderate/heavy growth	= 1 point
Pathogenic bacteria on Gram stain	= **ADD** 1 point

TOTAL SCORE: /12 (Score 6 or more significant)

Adapted from Pugin et al. 1991

CHF is congestive heart failure.

Chapter 5

Enose Analysis of BAL Samples for the Investigation of VAP

As has been discussed in the introduction to this work, there is no accepted gold standard test for the diagnosis of VAP. All of the investigations discussed have their advantages and disadvantages. This lack of a gold standard clearly has implications when investigating the use of a novel technology for the diagnosis of VAP. In this study we have decided to use quantitative cultures performed on blind BAL samples as our chosen microbiological technique for the diagnosis of VAP. The use of this technique is widespread and accepted within the medical community.

5.1 Bronchoscopic versus Nonbronchoscopic techniques for BAL sampling

Bronchoscopy provides direct access to the lower airways for sampling bronchial and parenchymal tissues directly at the site of inflammation. Proper selection of the correct sampling site can be technically challenging. The majority of intubated patients have purulent looking secretions, and the secretions first seen may represent those aspirated from another site into gravity-dependent airways. Secretions from the upper airways can also be aspirated around the endotracheal tube. Usually the sampling area is selected based on chest x-ray appearances or the segment visualised during bronchoscopy as having purulent secretions[146]. In cases where there are diffuse pulmonary infiltrates or minimal chest x-ray changes in a previously abnormal chest x-ray, selecting the correct site to sample can be challenging. In cases where no clear sampling site exists, and because autopsy studies show that VAP frequently involves the right lower lobe, this area should be sampled[147]. There is no convincing evidence that sampling from multiple sites is more accurate than single specimens for diagnosing VAP.[148]

A variety of nonbronchoscopic sampling techniques have been described in the literature, results have been comparable to those obtained using fibreoptic bronchoscopy [149]. In comparison with protected specimen brush (PSB) and/or bronchoscopic BAL, non bronchoscopic techniques are less invasive, can be performed by clinicians not trained to perform bronchoscopy, have lower initial costs than bronchoscopy, avoid potential contamination via the bronchoscopic channel, are associated with less compromise of gas exchange during the procedure, and can be performed in patients intubated with small endotracheal tubes.

Disadvantages of the blind sampling technique include the potential sampling errors inherent to a blind technique and the inability to visualise the airways directly. Although autopsy studies indicate that pneumonia in ventilated patients has often spread throughout the lungs, several studies on patients with VAP contradict those findings, as some patients had sterile PSB cultures from the non-involved lung [148, 150]. Although the authors of most studies concluded that the sensitivities of nonbronchoscopic and bronchoscopic techniques were similar, the overall concordance was only 80%. This demonstrates that in some patients the diagnosis could be missed by the blind technique.

In our units there was no agreed policy for the diagnosis of VAP in existence. After discussion with senior ICU clinicians and reviewing the literature we decided to use blind BAL sampling as our gold standard with which to compare the Enose. The decision was based on ease of use and low cost.

5.2 BAL Technique

We have adopted the method of blind BAL sampling adopted by Garrard et al.[151]. The patient was ventilated with 100% Oxygen prior to sampling. The patient was positioned 30 degrees head-up and disconnected from the ventilator circuit. A 14 Fr suction catheter was then introduced down the endotracheal or tracheostomy tube into the bronchial tree until for it could be advanced no further. 20 ml of sterile normal saline was then injected through the catheter and immediately aspirated back into the syringe. Care was taken to stop aspiration before the tip of the suction catheter was withdrawn into the endotracheal or tracheostomy tube. The sample was divided in two and placed in sterile sample containers for microbiological and Enose analysis. Quantitative microbiological culture was performed according to the standard hospital protocol.

The sample for Enose analysis was stored at 4°C in a controlled temperature fridge. Samples were collected and then sent via next day delivery post to Cranfield University where they were again stored at 4°C until they were analysed with the Enose. This method of collection and storage may have adversely affected our results and will be discussed later.

The samples for analysis by the microbiology department at Gloucestershire Hospitals NHS Foundation Trust were processed according to the following pre-agreed protocol:

3.8ml sterile saline was pipetted into one universal container, labelled A. 9ml sterile saline was pipetted into a second universal container, labelled B. The BAL sample was vortex mixed. Using a sterile serological pipette 0.2ml of the sample was transferred to container A (1:20 dilution). Container A was vortex mixed. Using a sterile serological pipette 1.0ml from container A was transferred to container B (1:200 dilution). Container B was vortex mixed. 50 microlitres from both containers was inoculated onto the various media shown below, spread with a sterile loop and incubated as shown.

Media	Conditions	Incubation Time
Blood Agar	37°C, 5% CO2	2 days
Chocolate Agar	37°C, 5% CO2	2 days
FAA	37°C, ANO2	2 days
CLED	37°C, Air	1 day
Sabourad	30°C, Air 37°C, Air	5 days 5 days

Table 5.1 Culture media and parameters.

The remaining sample was stored in a - 20°C freezer. After incubation the plates were read and the degree of growth of all significant organisms was recorded. All significant organisms were identified and standard laboratory sensitivity testing was performed. All significant isolates were stored on beads in the -20°C freezer.

5.3 Preparation of samples for headspace analysis

The clinical samples were stored in a 4°C cold room and allowed to warm to room temperature. The samples were then pipetted into 25ml glass vials and allowed to equilibrate at room temperature for 1 hour. The headspace was then analysed in a random order using an NST 3320 Enose (Applied Sensor, Sweden) comprising a hybrid sensor array.

For the microbial species which had been isolated from the clinical samples, the isolates were maintained on nutrient agar plates. One colony per species was inoculated in 10ml sterile nutrient broth (LabM) and incubated for 4 hours at 37°C in a rotary shaker at 100rpm. Thereafter 100 microlitres of each microbial suspension was transferred into a fresh 10ml sterile nutrient broth and incubated for 18hours at 37°C in a rotary shaker at 100rpm. Subsequently, 5ml from each suspension was transferred into 25ml glass vials and left for 1 hour at 37°C for headspace generation. Uninoculated nutrient broth was used as a control and five replicates per treatment were analysed in a random order using the Enose. These studies were repeated at least twice.

The response generated by the Enose sensors in the form of normalised, mean-centred data were analysed using Matlab 7.2(MathWorks Inc., Natick MA, USA). The response is defined as the change in resistance of each of the 24 individual sensors in the detection array to the adsorption and desorption of VOC's in the headspace of the sample being analysed. The Enose used in this study is a NST 3320 manufactured by Applied Sensor of Sweden. It uses a hybrid metal oxide semiconductor sensor array.

5.4 Metal Oxide Sensors

The following description is taken from the Handbook of Machine Olfaction[92].

"Metal oxides such as SnO_2, ZnO, Fe_2O_3 and WO_3 are intrinsically *n*-type semiconductors. At temperatures of 200-500 °C, these respond to reducible gases such as H_2, CH_4, CO, C_2H_5, or H_2S and increase their conductivity. In the atmosphere, some oxygen atoms are adsorbed onto the surface of these semiconductors to trap free electrons from the semiconductor, and consequently a highly resistive layer is produced in the vicinity of the semiconductor surface. The adsorption of oxygen atoms on the semiconductor surface and at grain boundaries of polycrystalline semiconductors creates an electrical-double layer that acts as the scattering centre for conducting electrons. The consequent increase in free electrons and decrease in scattering centres results in an increase in conductivity.

The reaction between gases and surface oxygen will vary depending on the operating temperature of the sensor and the activity of the sensor materials. The increasing sensitivity and selectivity of the sensors for exposure to gases can be realized by

incorporating a small amount of impurities and catalytic metal additives such as palladium or platinum. The impurities act as extrinsic donors (or acceptors) and, consequently, controlling the doped amount of impurities can change the conductivity of the sensors. Doping of the catalytic metal to the sensor or coating with thin catalytic metal film of the sensor surface changes the selectivity of the sensor.

The most widely used semiconducting material as a gas sensor is $Sn0_2$ doped with small amounts of impurities and catalytic metal additives. By changing the choice of impurity and catalyst and operating conditions such as temperature, many types of gas conductors exhibit relatively poor selectivity for gases and remain responsive to many kinds of combustible gases."

5.4.1 AppliedSensor Technologies MOS Sensors (from www.appliedsensor.com)

The following description of the Applied Sensor technologies is taken directly from product information found on the company website:

"AppliedSensor metal oxide semiconductor (MOS) sensors use metal oxide-based sensing thick films deposited onto a Si-micro-machined substrate (micro sensors). The substrate contains electrodes that measure the resistance of the sensing layer, and a heater that heats the sensing layer to 200°C to 400°C.

The sensor responds to changes in the composition of the ambient atmosphere with a change in the resistance of the sensing layer. A large number of toxic and explosive gases can be detected, even at very low concentrations.

Micro sensors operate in wide ambient temperature

MOS sensors detect a wide range of gases, including CO, NO_2, NH_3, H_2S, CH_4, and a wide variety of volatile organic compounds (VOCs). These maintenance-free sensors show high sensitivity, good stability, long lifetime, and short response/ recovery times. They can be operated in a wide ambient temperature range (-40°C to +70°C) and a humidity range of 0 to 100% RH without condensation.

5.4.2 Chemical Principle

The sensing layer is a porous thick film of polycrystalline SnO_2. In normal ambient air, oxygen and water vapour-related species are adsorbed at the surface of the SnO_2 grains. The sensing of target gases takes place as follows:

For reducing gases such as CO or H_2, a reaction takes place with the pre-adsorbed oxygen and water vapour-related species which decreases the resistance of the sensor. For oxidizing gases such as NO_2 and O_3, the resistance increases. The magnitude of the changes depends on the microstructure and composition/doping of the base material, on the morphology and geometrical characteristics of the sensing layer and substrate, as well as on the temperature at which the sensing takes place. Alterations of these parameters allow for the tuning of the sensitivity towards different gases or classes of gases. This is illustrated in the figure below:

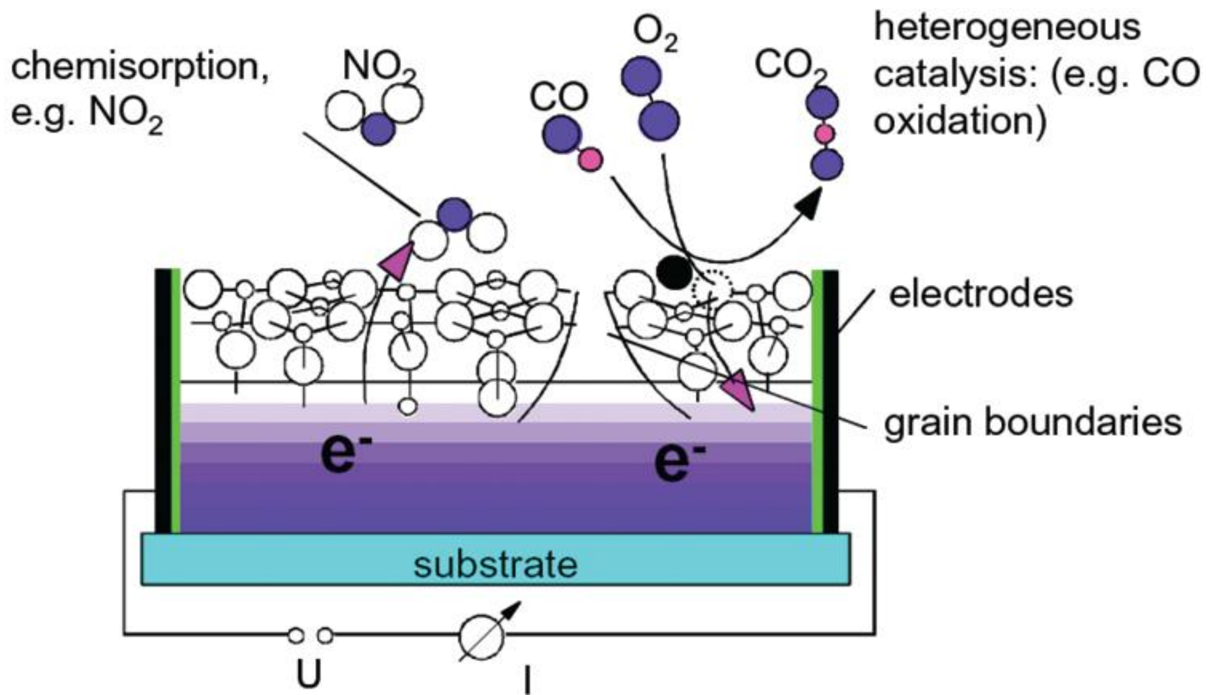


Figure 5.1 Sketch of an MOS sensor illustrating the detection principle. The resistance of the sensing layer changes when molecules react on the surface.

5.4.3 Transducer Principle

The changes in composition of the ambient atmosphere will determine changes in resistance of the sensing layers. In practice, the relationship between sensor resistance and concentration of the target gas usually follows a power law. Over a large range of concentrations, it can be described by:

$$R =\sim K \cdot c\pm n$$

“c” is the concentration of the target gas, “K” is a measurement constant, and “n” has values between 0.3 and 0.8. The positive sign is used for oxidizing gases and the negative sign for reducing gases.

5.4.4 Typical Response Curves

Figures 5.2 and 5.3 show the typical behaviour for a thick-film MOS sensor when exposed to a series of CO pulses. The sensor resistance drops very quickly immediately after CO exposure, and after removal of CO from the ambient atmosphere, the sensor resistance will recover to its original value after a short time. The speed of response and recovery will vary according to the operation temperature, the type of sensing layer, and the gases involved".

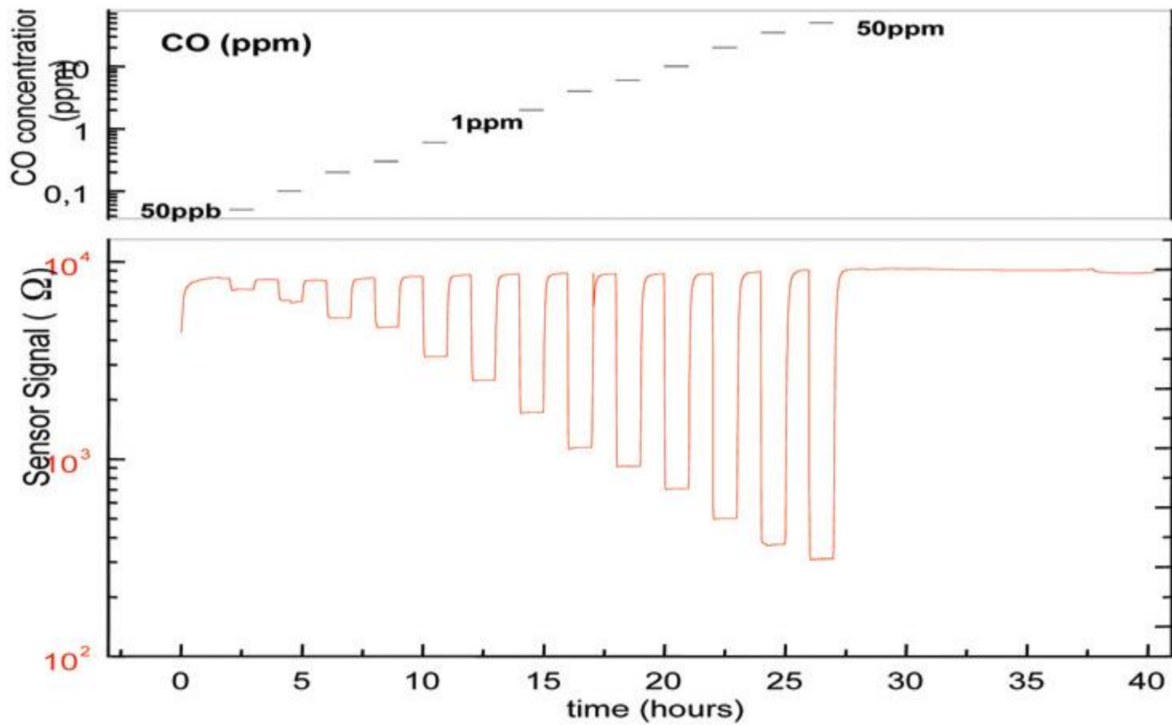


Figure 5.2 Response of a thick-film MOS sensor to CO.

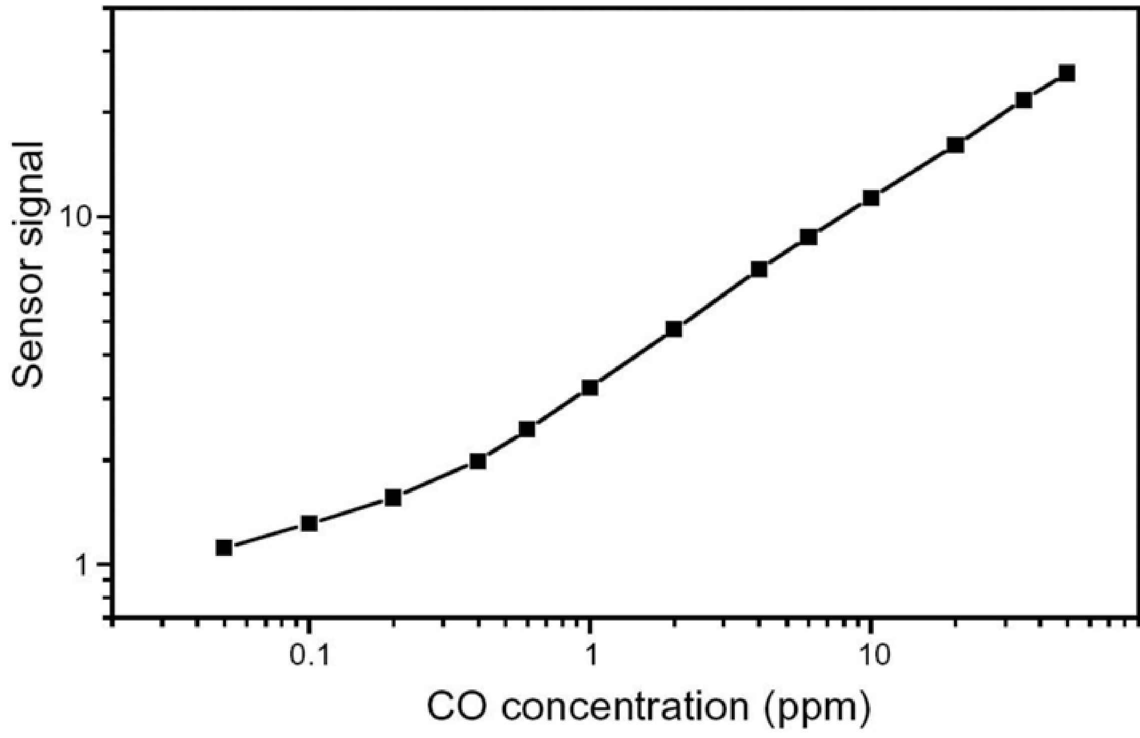


Figure 5.3 CO concentration vs. sensor signal for a thick-film MOS sensor.

5.5 Enose and BAL analysis

The exploration of odour sensing technologies as a method of identifying infections or diseases such as cancer has gathered pace in recent years. It has the potential to offer a rapid, near patient and non invasive method for diagnosis. It has long been known that certain diseases have characteristic odours, from the pear drop smell of acetone on the breath of diabetics to the fishy smell of liver disease. These odours are often difficult for the human nose to detect, especially at low concentrations. We are now turning to modern technology as a way of detecting these odours and measuring them in a scientifically acceptable and reproducible way. The electronic nose is a technology capable of detecting the volatile fingerprints of disease and infection. Enose technology has been successfully used to discriminate between various Mycobacterial isolates in humans[86] and in animals[152]. In vitro studies have also been carried out on bacteria responsible for eye infections [153].

As we have discussed previously, VAP is one of the most common and significant hospital acquired infections. Its high mortality rate and difficult diagnosis combined with the lack of a gold standard diagnostic test, makes it an attractive target for Enose diagnosis. This study explored the potential of volatile fingerprints generated by various pathogenic microbial species not only in vitro but also those present within clinical samples taken from ventilated patients on the Intensive Care Unit. We also attempted to correlate the microbiology culture results with the Enose responses for the clinical samples.

5.6 Materials and Methods

5.6.1 Collection of clinical samples

The patients recruited for this study were mechanically ventilated for more than 72 hours, on the Intensive Care Units at Gloucestershire Royal or Cheltenham General hospitals. The control group were patients ventilated for less than 24 hours for any reason other than pneumonia. All patients underwent a blind bronchoalveolar lavage (BAL) performed by a trained clinician on day three and then every Monday, Wednesday and Friday until ventilation was discontinued. Exclusion criteria were lack of patient consent to sample collection and an inspired oxygen concentration of more than 80%. Patients requiring an inspired oxygen concentration of more than 80% were felt to be too oxygen dependent to tolerate BAL sampling safely. Control samples were collected from patients mechanically ventilated following elective surgery within 24 hours of admission to the ICU with no evidence of pulmonary problems. The control group had one off sampling performed. For a statistical power of 80%, with 5% significance, a sample size of 100 samples was calculated assuming an incidence of VAP of 25% within our patient population. Ethical approval is detailed in Appendix 1.

The blind BAL involved introducing 20ml of sterile normal saline into the lungs via a sterile 14 gauge suction catheter attached to a 20ml syringe. Prior to injection the patient was pre-oxygenated with 100% Oxygen and then disconnected from the ventilator circuit. The catheter was introduced into the bronchial tree via the endotracheal tube until it could be advanced no further. The sterile saline was then injected and immediately aspirated

back, care was taken not to aspirate when the tip of the catheter was inside the endotracheal tube in order to minimise the risk of contamination from microorganisms colonising the tube. The aspirate was collected in sterile containers and divided in two; one sample being sent for standard microbiological culture in the hospital lab and the second sample posted to the University for headspace analysis (blind study). The samples were stored at 4°C prior to analysis. A total of 102 samples were collected over a period of 26 months (April 2006 to June 2008).

5.6.2 Microbial isolates

A total of 16 microbial organisms (including *Staphylococcus, Streptococcus,* MRSA, *Proteus, Pseudomonas, Klebsiella, Enterobacter* and *Candida*) were isolated from the study patients. The isolates were provided by the microbiology lab at Cheltenham General Hospital who had processed the BAL samples for the study. These were maintained on nutrient agar (LabM) at 37°C for this study and stored at 4°C until required.

5.6.3 Headspace analysis

The clinical samples were removed for the cold room and allowed to thaw at room temperature. Following this, the samples were pipette into 25ml glass vials and set aside for one hour at room temperature to equilibrate. The headspace was then analysed in a random order using the NST 3320 Enose (Applied Sensor, Sweden) comprising a hybrid metal oxide semiconductor sensor array of 24 individual sensors.

For the microbial species, the isolates were maintained on nutrient agar plates. One colony per species was initially inoculated in 10ml sterile nutrient broth (LabM) and incubated for four hours at 37°C in a rotary shaker at 100rpm. Thereafter 100 microlitres of each microbial suspension was transferred into fresh 10ml sterile nutrient broth and incubated for 18 hours at 37°C in a rotary shaker at 100rpm. Subsequently, 5ml from each suspension (approximately 10^8 and 10^6 cfu ml^{-1} for the bacterial and yeast species respectively) was transferred into 25ml glass vials, set aside for one hour at 37°C for headspace generation. Uninoculated nutrient broth was used as a control and five replicates per treatment were analysed in a random order using the Enose. These studies were repeated at least twice.

5.6.4 Data analysis

The responses generated by the Enose sensors, in the form of normalised, mean centred data for the response parameter, were analysed by Matlab 7.2 (Mathworks Inc.). PCA was used as a data reduction technique to explore the variance in the dataset, and fed into LDA to correlate Enose response with the findings of BAL sample cultures. The samples are assigned to one of a number of predetermined groups based on observations made about the sample. Due to the sample size, leave one out cross validation was used to evaluate how the results would translate to an independent data set. In the case of the clinical samples, the PCA scores were analysed and the results were then correlated to the findings obtained from microbiological analysis on the patient's BAL samples. Patient anonymity was preserved at all times. All analysis and correlation was performed using patient study numbers. The same analysis technique was applied to the BAL samples

from patients and to the model constructed using species grown in culture media from the bacterial slopes.

5.7 Results

5.7.1 Discrimination of the microbial isolates

One hundred and two samples were obtained from 44 patients and 6 samples from 6 control subjects (1 control subject subsequently became a patient after being ventilated for more than 72 hours). There were 32 males and 18 females. Full details of the patients, control subjects and the organisms isolated can be found in Tables 5.2, 5.3 and 5.4. A summary of the types of microbiological growth is shown in Table 5.3, it can be seen that 12 species were isolated.

Patient	Sample Date	Sex	Age	Growth Type and cfu/ml	Antimicrobial therapy in previous 48h
1	28/04/2006	M	57		Levofloxacin, Vancomycin
1	01/05/2006				
1	03/05/2006				
1	08/05/2006				
1	10/05/2006			Candida sp^3	
2	28/04/2006	M	45	Staphylococcus sp.^3 and Candida^4	Clindamycin, Levofloxacin, Benzylpenicillin
3	28/04/2006	M	76	Streptococcus sp.^3	Flucloxacillin, Clindamycin
4	03/05/2006	M	79	Staphylococcus sp.^3	Vancomycin, Rifampacin
4	05/05/2006				
5	08/05/2006	M	65		Levofloxacin, Teicoplanin
5	10/05/2006				
6	31/05/2006	F	38		Tazocin, Fluconazole
7	02/06/2006	F	72		Teicoplanin, Tazocin
8	09/06/2006	M	41		Fluconazole
8	12/06/2006				

Patient	Sample Date	Sex	Age	Growth Type and cfu/ml	Antimicrobial therapy in previous 48h
9	03/07/2006	M	36		Clarithromycin, Meropenem, Teicoplanin, Fluconazole
9	05/07/2006				
9	08/07/2006			Candida sp.^3	
9	10/07/2006				
10	10/07/2006	F	68	Candida sp.^3 and Pseudomonas sp.^3	Ciprofloxacin, Amoxicillin
11	11/07/2006	M	56		Tazocin
11	13/07/2006			Streptococcus sp (x2)^3, Proteus sp.^3	Tazocin
11	14/07/2006			Streptococcus sp.^3	Amoxicillin
11	17/07/2006				Vancomycin, Levofloxacin
12	28/07/2006	F	61		Levofloxacin, Metronidazole, Fluconazole
13	31/07/2006	F	66		Levofloxacin, Ceftriaxone
14	15/08/2006	F	76		Ceftriaxone
14	16/08/2006				Ceftriaxone
14	18/08/2006			Staphylococcus sp.^3	Ceftriaxone

Patient	Sample Date	Sex	Age	Growth Type and cfu/ml	Antimicrobial therapy in previous 48h
15	16/08/2006	M	52	Klebsiella pneumoniae^3	Tazocin, Clarithromycin
15	18/08/2006			Klebsiella oxytoca^3	Tazocin
15	21/08/2006				Tazocin, Levofloxacin
15	23/08/2006			Klebsiella sp.^3, Staphylococcus sp.^3, Candida sp.^3, Aerococcus sp.^3	Tazocin, Teicoplanin
15	25/08/2006			Candida sp.^3, Enterobacter sp.(x2)^3,	Tazocin, Teicoplanin
16	24/08/2006	F	60	Candida albicans^3	Meropenem, Metronidazole
16	25/08/2006			Candida sp.^3	
16	01/09/2006			Candida albicans^3	Meropenem, Metronidazole, Teicoplanin, Fluconazole
16	04/09/2006				Meropenem, Metronidazole, Teicoplanin, Fluconazole
16	05/09/2006			Stenotrophomonas	Meropenem, Teicoplanin

				maltophilia^5	
Patient	**Sample Date**	**Sex**	**Age**	**Growth Type and cfu/ml**	**Antimicrobial therapy in previous 48h**
17	09/10/2006	M	67		Levofloxacin, Teicoplanin, Metronidazole, Fluconazole
18	09/10/2006	M	42	Staphylococcus sp.^3	Metronidazole, Ciprofloxacin, Fluconazole
18	11/10/2006			Staphylococcus sp.^3	Metronidazole, Ciprofloxacin, Fluconazole
18	13/10/2006			MRSA^5	Metronidazole, Ciprofloxacin, Fluconazole
19	09/10/2006	F	84		Cefuroxime, Metronidazole
19	11/10/2006				
19	13/10/2006				Erythromycin, Tazocin, Teicoplanin, Fluconazole
19	23/10/2006				Teicoplanin
20	11/10/2006	F	76	Candida albicans^3	Vancomycin, Imipenem
20	13/10/2006				Vancomycin, Imipenem
21	06/11/2006	M	39		Vancomycin, Meropenem,Clarithromycin
21	08/11/2006			Staphylococcus sp.^3	Vancomycin, Meropenem,Clarithromycin

Patient	Sample Date	Sex	Age	Growth Type and cfu/ml	Antimicrobial therapy in previous 48h
21	13/11/2006				Vancomycin, Meropenem, Fluconazole
22	15/11/2006	M	65		Levofloxacin, Metronidazole, Benzylpenicillin
23	27/02/2007	F	80	Klebsiella oxytoca^4	Clarithromycin, Tazocin
23	28/02/2007				Clarithromycin, Tazocin, Gentamicin
23	02/03/2007			Klebsiella oxytoca^3	Tazocin, Gentamicin
24	27/02/2007	M	80	Pseudomonas aeruginosa^5, Staphylococcus sp.^3 and Baxteroides sp.^3	
24	28/02/2007			Pseudomonas aeruginosa^5	
25	27/02/2007	M	76	Klebsiella pneumonia^5, Enterococcus sp.^3	Tazocin, Erythromycin

Patient	Sample Date	Sex	Age	Growth Type and cfu/ml	Antimicrobial therapy in previous 48h
26	27/02/2007	M	74		Meropenem, Clarithromycin
26	28/02/2007				Meropenem
26	02/03/2007				Amoxicillin
27	05/03/2007	M	82	Pseudomonas aeruginosa^3, Candida sp.^5	Imipenem, Clindamycin
28	05/03/2007	F	78		Clarithromycin, Tazocin, Nystatin
29	02/04/2007	M	58		Ceftriaxone
30	08/05/2007	F	43		Meropenem, Vancomycin, Fluconazole
31	25/06/2007	F	69		Tazocin, Rifampacin, Teicoplanin
31	26/06/2007				Teicoplanin, Meropenem
31	27/06/2008				Teicoplanin, Meropenem, Erythromycin
32	26/06/2007	F	54	Candida sp.^5	Gentamicin, Meropenem, Fluconazole
33	15/01/2008	M	62		
33	16/01/2008				

Patient	Sample Date	Sex	Age	Growth Type and cfu/ml	Antimicrobial therapy in previous 48h
34	17/01/2008	F	82		Tazocin
35	06/02/2008	M	65	Acinetobacter^5, MRSA^5	Gentamicin, Benzylpenicillin, Clarithromycin
35	08/02/2008			Acinetobacter^3, MRSA^3, Streptococcus sp.^4	Benzylpenicillin, Clarithromycin, Linezolid
36	27/02/2008	M	71	Candida sp.^4	Flucloxacillin, Rifampacin
36	29/02/2008			Candida sp.^4	Flucloxacillin, Meropenem, Rifampacin
37	29/02/2008	F	76		Meropenem, Tazocin
37	03/03/2008				Meropenem, Tazocin, Teicoplanin
37	07/03/2008				Erythromicin, Ciprofloxacin, Gentamicin
37	10/03/2008				Erythromicin, Ciprofloxacin, Gentamicin
37	12/03/2008				Ciprofloxacin
37	14/03/2008				Ciprofloxacin
37	17/03/2008			Staphylococcus sp.^3	

Patient	Sample Date	Sex	Age	Growth Type and cfu/ml	Antimicrobial therapy in previous 48h
38	07/03/2008	M	77	MRSA^5	
39	14/03/2008	M	69	Enterobacter sp.^3	Ciprofloxacin
40	02/04/2008	M	77		Ceftriaxone, Aciclovir
41	07/04/2008	M	79	Candida albicans^3	Tazocin, Metronidazole
41	09/04/2008			Candida albicans^3	Tazocin
41	11/04/2008				Tazocin
41	15/04/2008			Candida albicans^3	Meropenem
42	08/04/2008	M	59		
43	10/04/2008	F	69	Staphylococcus sp.^3	Gentamicin, Amoxicillin, Metronidazole
44	16/04/2008	M	79	Candida sp.^3	Meropenem, Flucloxacillin
Control1	12/03/2008	M	69		Ciprofloxacin
Control2	19/03/2008	F	35		Erythromycin, Meropenem, Fluconazole
Control3	20/03/2008	M	19	Neisseria sp.(x2)^3	Cefuroxime, Metronidazole, Flucloxacillin
Control4	19/05/2008	M	60		Ciprofloxacin
Control5	11/06/2008	M	46		Co-Amoxiclav
Control6	25/06/2008	M	61		

Table 5.2 Microbiological growth and antimicrobial therapy by individual BAL sample.

Organism Isolated	Number of Isolates
Gram Positive	
Staphylococcus aureus (all MRSA)	5
Coagulase negative Staphylococci	13
Streptococcus species	6
Enterococcus species	1
Aerococcus species	1
Gram negative	
Klebsiella species	6
Enterobacter species	4
Pseudomonas aeruginosa	4
Proteus species	1
Stenotrophomonas species	1
Acinetobacter species	2
Neisseria species	1
Bacteroides thetaiotaomicron	1
Fungi	
Candida species	20

Table 5.3 Number of isolates of the species grown from the bronchoalveolar lavage samples, in order of pathogenic potential. The total includes those found in mixed growth samples.

Patient Number	Age	Sex	Reason for Ventilation
1	57	M	Community Acquired Pneumonia/Pulmonary Oedema
2	45	M	VF arrest
3	76	M	Post op for scrotal sepsis
4	79	M	Community Acquired Pneumonia
5	65	M	Community Acquired Pneumonia
6	38	F	Overdose
7	72	F	Septic Arthritis, Acute Respiratory Distress Syndrome
8	41	M	Pelvic Trauma
9	36	M	Aspiration Pneumonia, Fitting
10	68	F	Metabolic acidosis and Acute Renal Failure
11	56	M	Head Injury
12	61	F	Out of hospital cardiac arrest
13	66	F	Acute hepatitis, Acute Respiratory Distress Syndrome
14	76	F	Sepsis
15	52	M	Pneumonia
16	60	F	Biliary Sepsis, VF arrest
17	67	M	Post op total colectomy
18	42	M	Biliary Sepsis
19	84	F	Pancreatitis, Pleural effusions
20	76	F	Not documented

21	39	M	Community Acquired Pneumonia, Empyema
22	65	M	Out of hospital arrest
23	80	F	Community Acquired Pneumonia
24	80	M	Perforated DU, Fast Atrial Fibrillation
25	76	M	Post op total colectomy
26	74	M	Respiratory failure, sepsis
27	82	M	Necrotising fascitits
28	78	F	CVA, Pneumonia
29	58	M	Community Acquired Pneumonia, COPD
30	43	F	Pelvic sepsis
31	69	F	Community Acquired Pneumonia
32	54	F	Anastamotic leak, Cardiac arrest
33	62	M	Post Oral Surgery
34	82	F	Sepsis
35	65	M	COPD
36	71	M	Sepsis
37	76	M	Post colonic resection
38	77	M	Post Cardiac Bypass surgery
39	69	M	Acute Pancreatitis
40	77	M	Encephalitis
41	79	M	Post colonic surgery
42	59	M	CVA

43	69	F	Post Total Hip Replacement
44	79	M	Sepsis post Total Knee Replacement
Control 1	69	M	Acute Pancreatitis
Control 2	35	F	Acute Pancreatitis
Control 3	19	M	Dental Abcess
Control 4	60	M	Post Oral surgery
Control 5	46	M	Post Maxillectomy
Control 6	61	M	Post Maxillectomy

Table 5.4 Summary of study participant demographics and reasons for ventilation.

5.7.1 Analysis of clinical samples

Following headspace analysis of the clinical samples, the Enose data was correlated with growth information from the hospital microbiology lab using PC fed LDA. Of the 102 clinical samples collected 88 were used for further analysis; 4 samples had no Enose data, and 10 samples all measured on the same day were excluded as outliers (Figure 5.4).

Of the 88 samples used for further analysis, 5 had Gram +ve growth, 7 Gram –ve, 8 Fungi, 46 No growth, 10 Mixed growth, 12 No growth & no antibiotics.

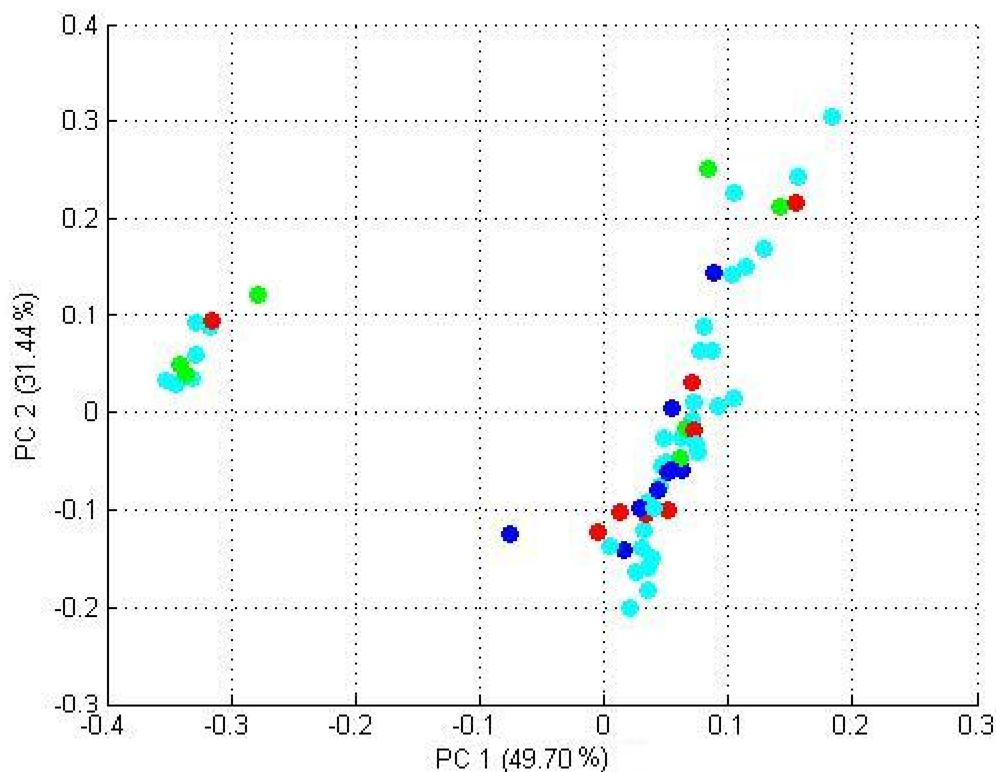


Figure 5.4 Demonstration of outlier data that was excluded from the dataset for subsequent analysis.

A rudimentary classification differentiating samples with and without microbiological growth would allow the clinician to introduce empirical antimicrobial therapy earlier than would otherwise be possible. Therefore the performance of a two group clinical model was evaluated. Of the 88 samples,30 showed microbiological growth & PC fed LDA classification model demonstrated a training performance of 68.2%, which is shown in Figure 5.5. The classification performance is shown in Table 5.5.

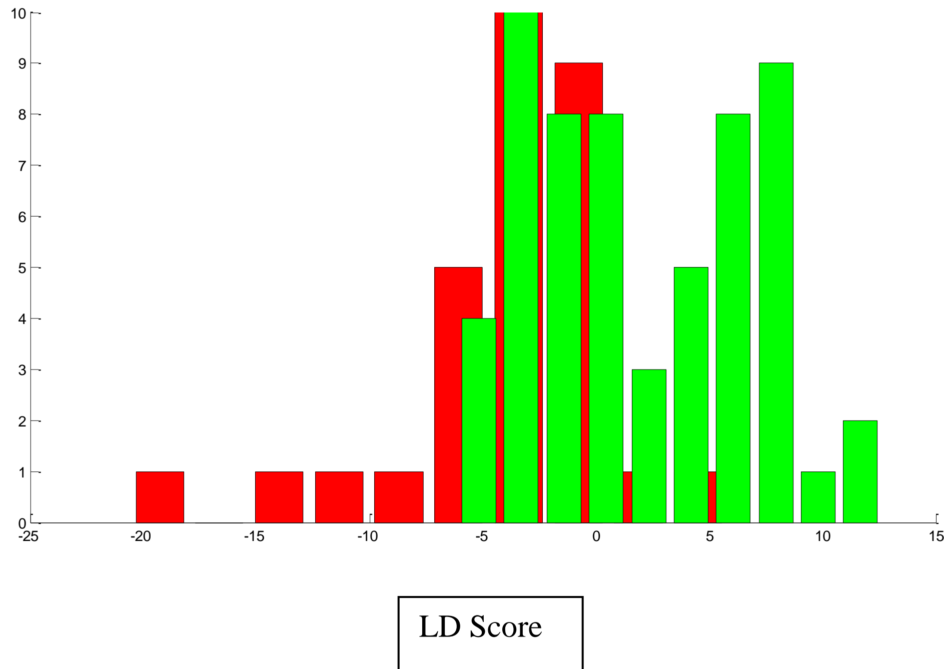


Figure 5.5 Histogram of linear discriminant scores shows misclassification of a significant number of Enose fingerprints from samples with no microbiological growth (green bars) and samples with microbiological growth (red bars). In particular, misclassified no growth samples are seen, to the left of zero, overlapping the samples with microbiological growth.

		Enose prediction	
		Growth	No Growth
Microbiology Gold Standard	Growth	24	6
	No Growth	22	36

Table 5.5 Classification performance of the two group clinical model

12 samples were collected from patients who had not received antimicrobial therapy within 48 hours of sampling. 9 of these samples were culture negative and 3 grew organisms. Therefore a further two group PC fed LDA classification model was developed with patients with no growth samples on antibiotics removed from the analysis. A classification model of the Enose fingerprints of the 9 no growth samples, from patients not on antibiotics, and 30 samples with microbiological growth, which had a training performance of 89.7% correct prediction (Table 5.6 & Figure 5.6). Further, 77% of samples were correctly classified by the leave one out cross validated model, sensitivity and specificity of 56-83%. (Table 5.7). The decrease in the performance on cross validation indicates insufficient numbers of samples & patients are included in the model.

		Enose prediction	
		Growth	No Growth
Microbiology Gold Standard	Growth	28	2
	No Growth	2	7

Table 5.6 Classification performance of the two group clinical model of the 9 no growth samples, from patients not on antibiotics, and 30 samples with microbiological growth.

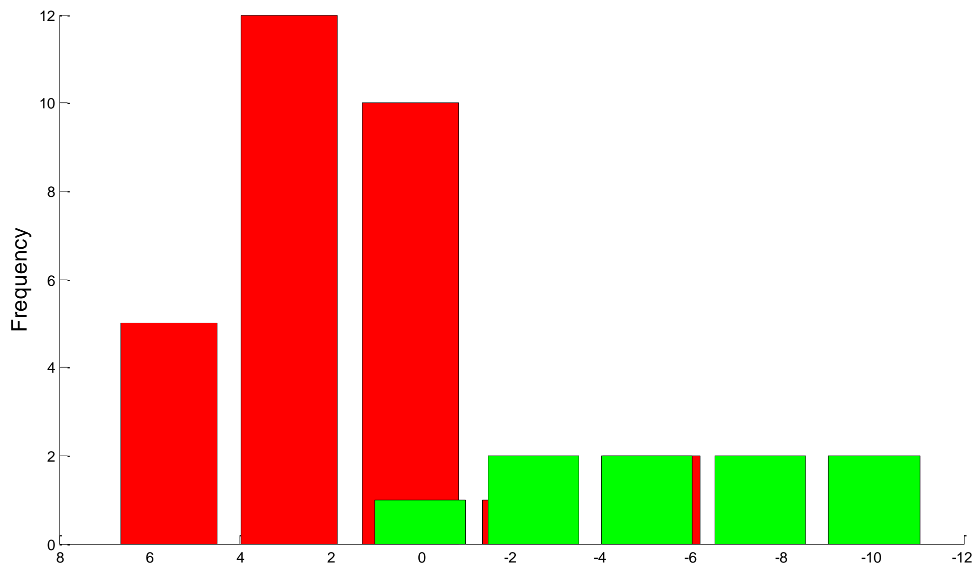


Figure 5.6. Histogram of linear discriminant scores shows clear discrimination of the Enose fingerprint from samples without microbiological growth from patients not on antibiotics (green bars) and with microbiological growth (red bars).

		Enose prediction	
		Growth	No Growth
Microbiology Gold Standard	Growth	25	5
	No Growth	6	6

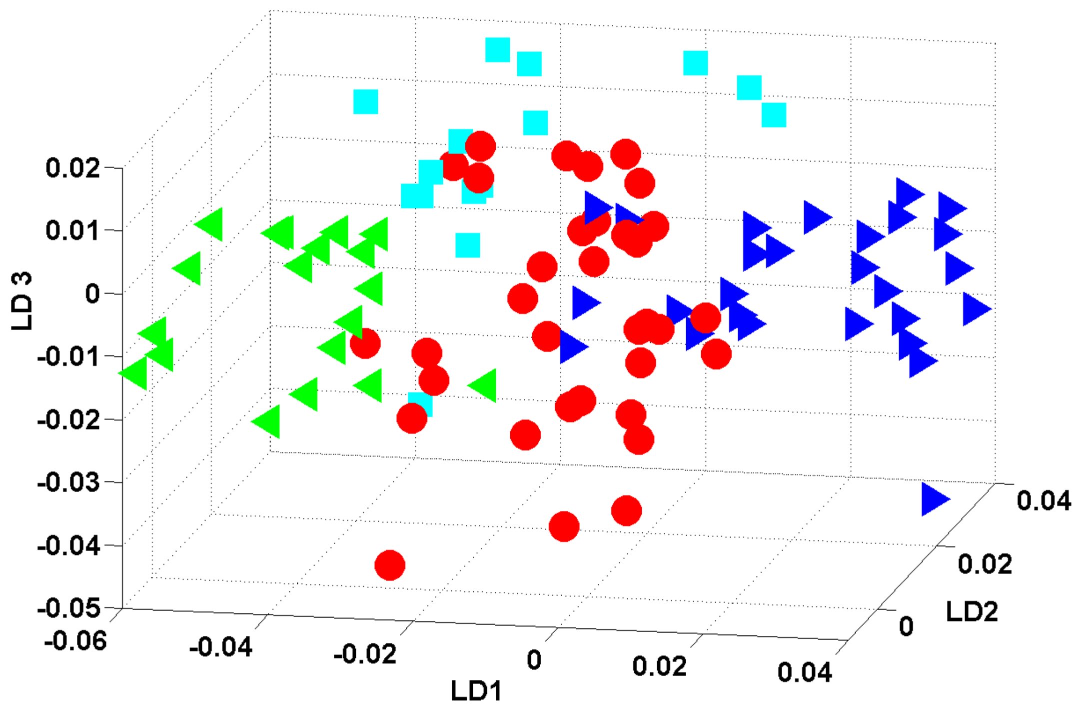
Table 5.7 Classification performance of the leave one sample out cross validated two group clinical model of the 9 no growth samples, from patients not on antibiotics, and 30 samples with microbiological growth.

A clinically significant 4 group classification (Gram-positive, Gram-negative, Fungi and no growth) was then developed, to evaluate the Enose fingerprints from 29 samples; 9 with no microbiological growth from patients not on antibiotics, 5 samples with Gram-positive growth, 7 samples with Gram-negative growth and 8 with fungal growth. Samples with mixed growth were again excluded from this analysis. Seventy six percent of samples were correctly classified by the model, with sensitivity 60-100% and specificity of 81-100% (Table 5.8 & Figure 5.7).

		Enose prediction			
		Gram +ve	Gram –ve	Fungi	No Growth
Microbiology Gold Standard	Gram +ve	3	1	1	0
	Gram –ve	0	7	0	0
	Fungi	0	3	5	0
	No Growth	0	1	1	7

Table 5.8 Classification performance of a four group classification model developed with 29 samples; 9 with no microbiological growth from patients not on antibiotics, 5 samples with Gram-positive growth, 7 samples with Gram-negative growth and 8 with fungal growth.

Figure 5.7 Scatter plot of linear discriminant scores of the electronic nose response to significant groups, Gram positive (◀), Gram negative (▶), Fungi (●) and no microbiological growth (■).



The results of our Enose analysis of the BAL samples gave a significantly lower discrimination ability compared to what we had expected from other published work. We postulated that this was due to patient related factors and the use of antimicrobial therapry prior to sampling. In order to investigate this theory we constructed a "lab based model" using organisms isolated from the BAL samples and then grown on a culture medium. This allowed us to use exactly the same organisms but without the presence of the BAL fluid which we theorised contained elements of the host response to infection. It also negated the problem of prior antimicrobial therapy.

We accept that this is an artificial situation without direct clinical relevance, however it does allow us to demonstrate improved discrimination of the microorganisms seen. It has also provided the opportunity for discussion regarding the use of antimicrobial therapy and which host factors may be involved.

For clarification of the following results, the first section (*5.7.1)* relates to the clinical BAL samples and the second section (*5.7.2*) relates to the "lab based model" described above.

5.7.2 The lab based model

This section refers to the microorganisms isolated from the BAL samples and then grown in a culture broth before analysis. The broth was used as a control.

The measured Enose data was used in the development of multivariate classification models. PCA fed LDA showed discrimination of growth samples (bacterial & fungi) from the controls (Figure 5.8).

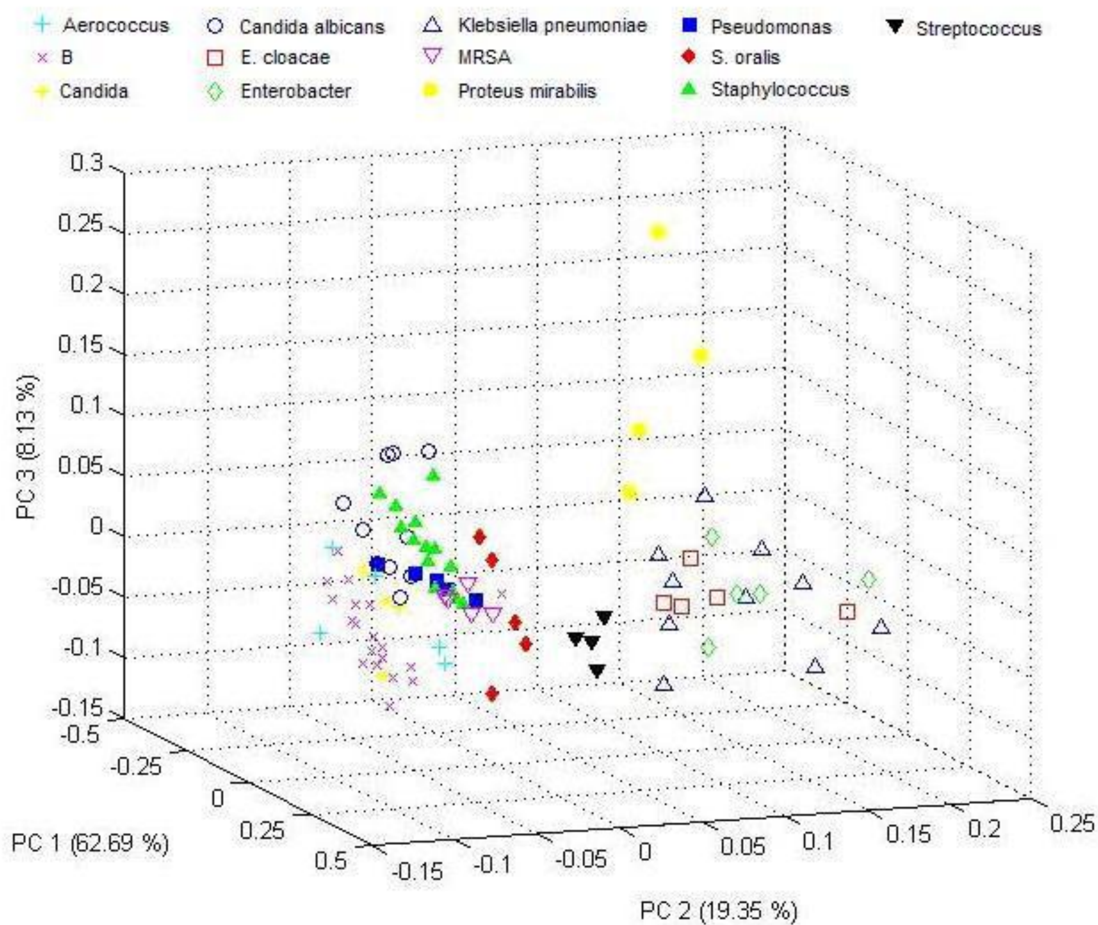


Fig 5.8 PCA model demonstrating separation of the 12 microbial species and broth control samples on the basis of Enose data. From Sahgal, N 2008 [154]

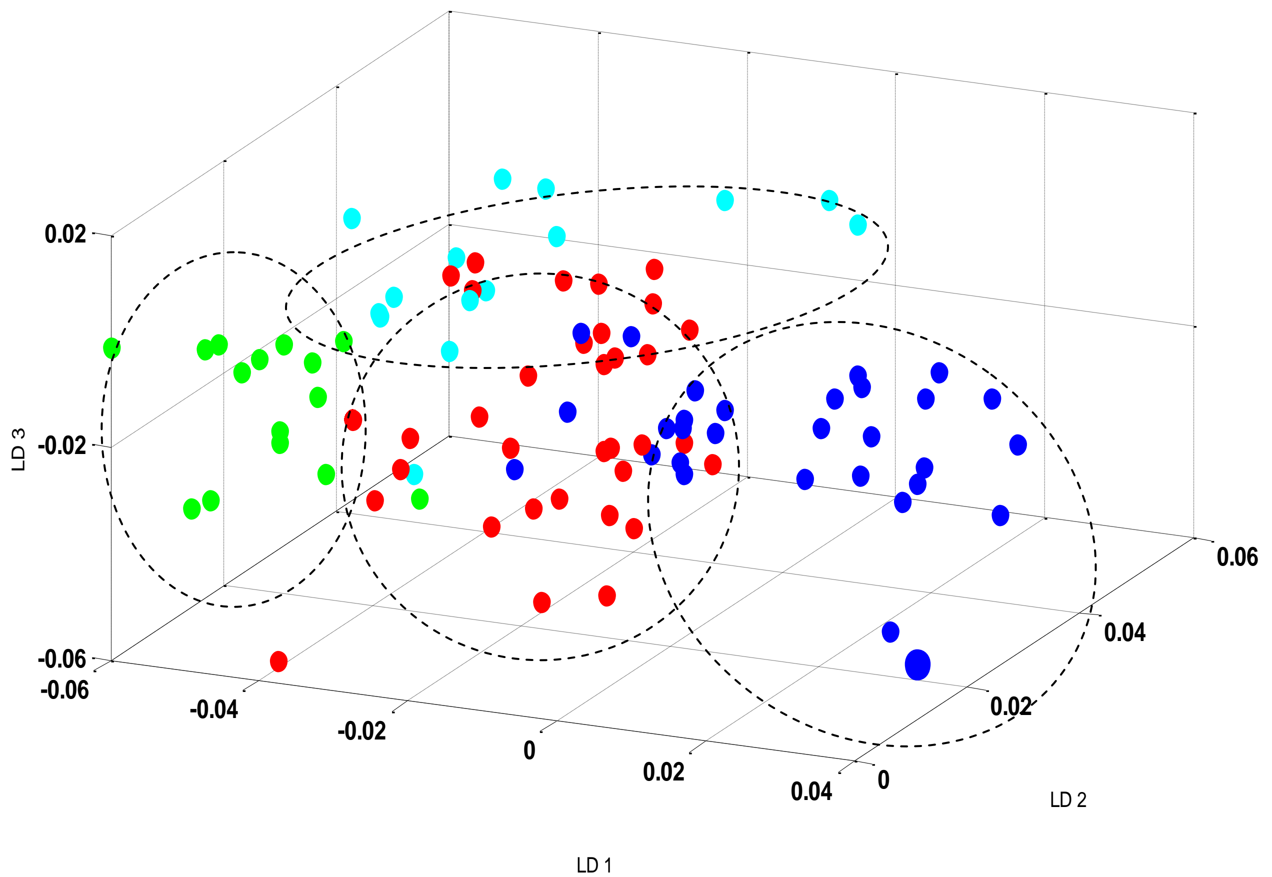


Figure 5.9 Scatter plot of linear discriminant scores of the electronic nose response to microorganisms isolated from clinical bronchoalveolar lavage samples. Each measurement is coded according to the microbiological classification into four clinically significant groups, Gram positive (green), Gram negative (blue), Fungi (red) and controls (cyan). These are the same species as shown in Figure 5.8 but grouped as described above.

The performance of the classification model was tested with leave one out cross validation demonstrated correct classification of 70% of samples with sensitivity 56-84% and specificity 81-97%. The classification breakdown is shown in Table 5.9 & Figure 5.9. The decrease in the performance on cross validation indicates insufficient numbers of samples & patients are included in the model.

		Enose prediction			
		Gram +ve	Gram –ve	Fungi	No Growth
Microbiology Gold Standard	Gram +ve	19	2	9	4
	Gram –ve	6	24	0	0
	Fungi	4	0	10	1
	No Growth	2	0	1	16

Table 5.9 Classification performance of a four group classification model with a leave one out cross validation of the electronic nose response to microorganisms isolated from clinical bronchoalveolar lavage samples.

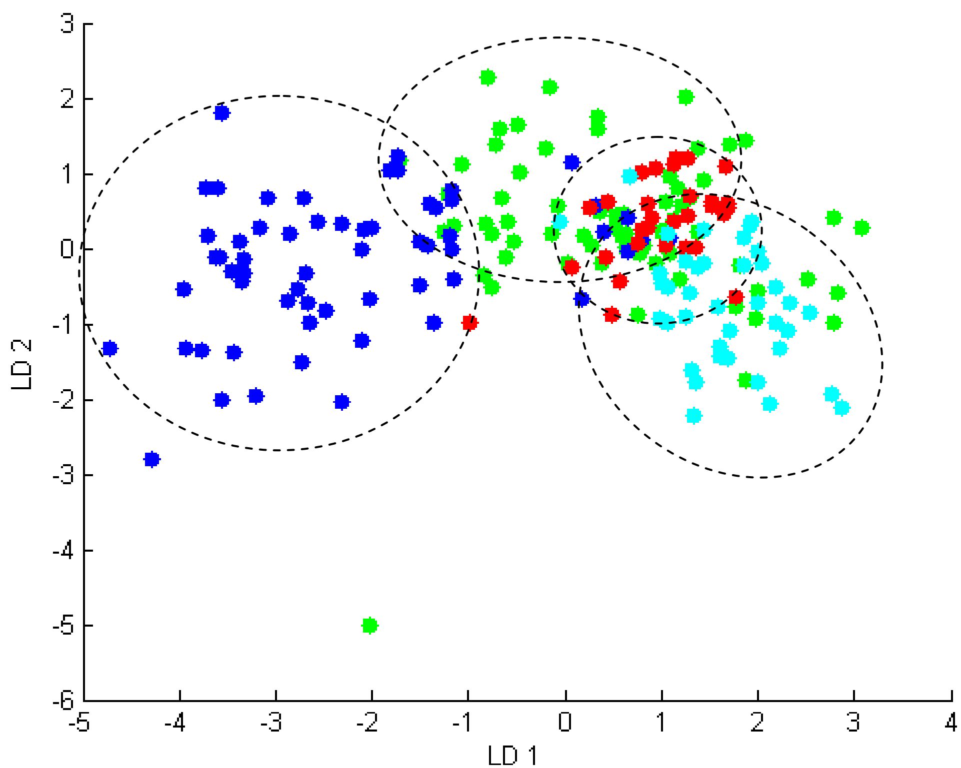


Figure 5.10 Scatter plot of cross validated LDA classification model of the electronic nose response to microorganisms isolated from clinical bronchoalveolar lavage samples. Each measurement is coded according to the microbiological classification into four clinically significant groups, Gram positive (green), Gram negative (blue), Fungi (red) and no microbiological growth (cyan).

5.8 Discussion

Thepurpose of this study was to assess the potential of the Enose in the clinical setting, therefore we looked at a clinically relevant four group model of Gram-positive, Gram-negative, Fungi and no growth. Whilst not as useful as knowing the exact organism present in the fluid, being able to identify which of these groups the infectious organism belongs to is clinically relevant and would allow the institution of narrower spectrum empiric antibiotic therapy whilst awaiting traditional culture results. This would potentially mean reduced antimicrobial resistance and side effects, which are major problems as has already been discussed.

This approach resulted in 83% classification accuracy when PCA fed LD analysis was used. Not all the microbes grew well in the culture medium. In particular this was an issue for some Gram-positive organisms and the *Candida* species, which may be a reason why these samples group tightly to the uninoculated broth controls. A different culture medium may suit these groups better, however it may be difficult to find a culture medium which provides optimal growth for all the different microbial species encountered.

Dutta et al.[153] investigated the possibility of separating six species of bacteria responsible for eye infections in saline. This was done using lab cultures and a conducting polymer Enose in conjunction with a neural network. The bacterial

organisms were investigated in different concentrations. Discrimination was not possible between the three different concentrations of individual bacteria. However it was possible to discriminate between the different bacterial species when they were analysed at the same concentration. The authors were also able to distinguish between 2 closely related *Staphylococcus* species when analysing 4 bacteria known to cause ENT infections. The clinical samples were cultured on agar media overnight prior to analysis. This infers that bacterial agar cultures produce more volatiles when compared to culture broth. This has also been suggested by Casalinuovo et al. [155] for bacteria but not yeasts. No mention is made of the use of controls in either of these studies. This may have had an impact on the ability to discriminate between species.

Analysis of the clinical BAL samples was first performed on the most basic level of a 2 group model (growth vs. no growth). A basic model like this would at least alert the clinician to the possibility of needing to commence empirical antimicrobial therapy if the clinical status of the patient gave cause for concern. The model was constructed again using PCA fed LDA classification. 88 samples were included in the model, 58 with no microbiological growth and 30 with growth. 68% of samples were correctly classified using the leave one out cross validated model, with sensitivity and specificity of 67-69%.

The aim of this study was to assess the ability of the Enose to discriminate between microbial isolates from patients and actual BAL samples from patients with or at risk from VAP. For the *in vitro* studies on the isolates, twelve bacterial and yeast species were analysed together. Only the *Enterobacteriaceae* could be clearly distinguished form

the other samples. There also appeared to be a small distinction between the *Streptococcus, Staphylococcus* and *Candida* species. It was impossible to discriminate between all 12 individual species. This may be in part due to the small number of samples from each species.

We have been concerned by the fact that the vast majority of our clinical samples were taken from patients who had been receiving antibiotic therapy in the 48 hours prior to sample collection. As previously discussed this can have a significant effect on bacterial culture results. This may be causing a false negative effect i.e. the Enose may be correctly classifying samples into the "growth" group but cultures are failing due to the use of antibiotics. This could explain the reduced accuracy of the Enose on the clinical BAL samples when compared to the lab isolate samples.

It is also unclear as to the basis on which Enose discrimination is taking place. It may be that the Enose is capable of detecting the volatile fingerprint of microorganisms detected in the BAL fluid but his hypothesis is likely to be too simplistic. The host response to infection is a complex and dynamic series of events that is under increasing investigation as a major contribution to death from sepsis. The initial reaction relies on cellular and molecular components of the innate immune system. Cytokines with proinflammatory roles such as tumour necrosis factor α (TNFα) are released along with components of the complement cascade. The inability to regulate this inflammatory response in the presence of severe sepsis is characterised by widespread microvascular injury and thrombosis as well as multi organ dysfunction.

Bergeron et al.[156]studied the correlation between cytokine levels in lung tissue, BAL fluid and serum in relation to the time course and outcome of *Streptococcus pneumoniae* pneumonia in mice. Samples were taken at time zero (pre-infection) and at 1, 2, 4, 12, 24, 48, 72 and 96 hours post-infection. They reported that TNFα was the first cytokine recovered from BAL fluid. Levels showed a significant increase from 1-12 hours post infection when compared with pre-infection levels. Levels rapidly dropped to normal after 12 hours indicating the TNFα rise in BAL fluid to be transient despite progression of the pneumonia. TNFα levels in the lung tissue itself rose to very high levels at 12 hours; this may indicate the involvement of interstitial inflammatory cells. TNFα levels in serum were absent or at a very low level until 48 and 72 hours; at this time a very marked and rapid increase was noted. This coincided with migration of bacteria to the bloodstream. The authors state that these findings clearly demonstrate compartmentalization of TNFα secretion to the site of infection, with successive appearances in BAL, lung tissue and finally blood.

Interleukin-1 (IL-1) release was seen in lung tissue throughout the timecourse of the experiment combined with a very transient appearance of this cytokine in BAL and serum at 12 hours. Both TNFα and IL-1 levels peaked at 12 hours in BAL and lungs. This is prior to bacterial dissemination to blood and may implicate these cytokines in the breaching of the alveolar-capillary barrier.

Interleukin-6 (IL-6) levels were significantly increased very early in BAL fluid and lung tissue after infection (2 hours), with a peak at 4 hours that also corresponded with partial release of IL-6 in serum. Levels remained elevated throughout the experiment. IL-6 serum levels showed 2 time points for elevation; early at 4 hours and then later at 48 hours until death. This late rise correlated with bacteraemia.

Leukotriene B_4 (LTB_4) levels, which were higher in BAL fluid than in the lung, increased in BAL fluid from 12-72 hours and in lung tissue at 24 hours, which accompanied and followed polymorphoneuclear cell (PMN) recruitment. The amount of LTB_4 recovered decreased with time, as did the number of monocytes and other secretory inflammatory cells in whole blood.

Nitric oxide (NO) release was seen in the serum of infected mice throughout the experiment. In contrast to this, a brief spontaneous release of NO was observed in BAL fluid 1 hour after infection, at a time when only alveolar macrophages are recovered from BAL fluid. A second sustained phase (48-72 hours) of high NO secretion in lung tissue corresponded with the massive monocyte/macrophage recruitment period. Tissue injuries attributable to high NO levels were seen during the same period.

The presepticaemic and septicaemic stages of infection described in this study were demonstrated by the successive localisation of TNFα secretion to BAL fluid and blood respectively. In the context of pneumococcal pneumonia, TNFα measurement could

prove useful for monitoring the presepticaemic and septicaemic stages of pneumonia. Monitoring of inflammatory mediators in BAL fluid could also generate markers of disease evolution, including cytokines, LTB_4 and NO. Whilst IL-6 in BAL fluid may be a good indicator of early infection, it did not reflect the evolution of infection and therefore cannot be measured in BAL fluid as a way of charting disease progression. TNFα secretion, in contrast to this, correlated with the initial inflammatory response localised to the lungs in the absence of systemic involvement. There seems to be a spillover of IL-1 production from cells to fluids when overproduction occurs. Detectable levels of IL-1 in serum or BAL fluid may therefore indicate a very active inflammatory response. A combination of the profiles of TNFα in blood and NO in BAL provided an accurate estimation of disease state which chronologically correlated to a worsening of the pathological scoring of lung tissue.

This paper demonstrates that a multitude of factors involved in the host response to infection can be observed in BAL fluid. It is possible that these mediators of the inflammatory response may be contributing to the volatile fingerprints of BAL fluid from those patients with VAP. Further work needs to be done to determine whether the Enose is capable of detecting the presence of these inflammatory mediators in BAL fluid. It would also be interesting to see how the volatile fingerprint changes with time reflecting the changes in levels of individual compounds reported in the study by Bergeron et al.

5.9 Conclusions

This study has demonstrated that the Enose is capable of accurately discriminating between samples in the lab based model. The accuracy of this discrimination decreases when the clinical samples are analysed. The reasons for this are unclear but there are several factors that are likely to be of importance. The first of these is the use of antimicrobial drugs prior to sampling taking place. These drugs are likely to be responsible for the reduced classification accuracy in the clinical sample model. Individual host response may also be playing a role. We have discussed the fact that multiple inflammatory mediators are present in BAL fluid from mouse studies. How the presence of these mediators affects the ability of the Enose to discriminate between samples is unknown.

This method of BAL analysis using an Enose has shown promise as a near patient diagnostic tool but further work needs to be done. In particular it would be interesting to repeat the study in a centre where the use of surveillance BAL sampling is standard. This practice involves BAL samples being taken routinely from all ventilated patients on the first day of ventilation and subsequent days. This design would yield large numbers of BAL samples from patients who are not on antibiotics and do not have VAP. This would increase our number of no growth, no antibiotic samples. Multiple samples like this would also allow us to develop a "BAL timeline" from individual patients who do not have VAP but subsequently develop it. This would allow us to investigate changes in BAL fluid through the time course of the disease. Further studies should be performed to

investigate the constituents of the inflammatory host response that may be detected in BAL fluid and how these affect Enose analysis.

Chapter 6

GC-MS Analysis of Breath

Modern breath analysis began in the 1970s when Pauling et al.[3] determined in excess of 200 compounds in human breath using gas chromatography. Gas Chromatography-Mass Spectrometry (GC-MS) has been widely used in the field of breath analysis ever since. In this chapter we will focus on GC-MS analysis of breath samples collected from the same patients described in the previous chapter.

6.1 Materials and Methods

Breath samples were collected from patients immediately prior to them undergoing BAL sampling as described earlier. Breath was collected using the "Breathotron" which was designed and built at Cranfield University. A detailed description of the Breathotron can be found in the next chapter. The ethical approval and consent process is the same as described previously.

The Breathotron was connected into the closed circuit between the patient and the mechanical ventilator. The sampling arm was connected as close as possible to the patient in the circuit to limit the number of VOCs detected from the various materials used to manufacture the breathing circuit and endotracheal tubes. A Heat and Moisture Exchange bacterial filter was connected between the patient and the sampling apparatus. This was to prevent sputum and other potentially hazardous material contaminating the Breathotron. This filter is designed to trap bacteria and does not impede the flow of VOCs from the expired breath into the sampling apparatus.

Breath is passed through standard stainless-steel automated thermal desorption (ATD) cartridges, containing dual packing comprising 50% Tenax TA and 50% Carbotrap (Markes International Limited, Llantrisant, UK). These materials trap the compounds of interest for later analysis in the laboratory. The Breathotron samples a small amount of air from each expired breath until a total volume of 500ml of expired breath has been passed through the sorbent trap. This trap acts as a pre-concentrator of the VOCs prior to GC-MS analysis. They also provide a stable transport and storage medium for the VOCs. The software on the Breathotron is set to sample the alveolar component of expired breath. The sorbent traps were then sent to Cranfield University for analysis.

ATD cartridges were conditioned before use by purging with helium carrier gas for 2 min at room temperature followed by 30 min at 335 ºC. Conditioned cartridges were then analysed by GC-MS using the protocol described below in order to confirm the effectiveness of the conditioning procedure. Conditioned cartridges were sealed with locking caps until required for use, a clean cartridge being used for each procedure.

Captured volatiles were analysed using an AutoSystem XL gas chromatograph equipped with an ATD 400 thermal desorption system and TurboMass mass spectrometer (Perkin Elmer, Wellesley, MA). The heated valve of the ATD 400 was maintained at a constant 180 ºC. CP grade helium (BOC gases, Guildford, UK) was used as the carrier gas throughout, after passing though a combined trap for the removal of hydrocarbons, oxygen and water vapour. The trap was renewed regularly following the manufacturer's recommendations.

Cartridges were desorbed by purging for 2 min at ambient temperature then for 5 min at 300 ºC. Volatiles purged from the cartridge were captured on a cold trap which was initially maintained at 30 ºC. Once desorption of the cartridge was complete, the trap was heated to 320 ºC using the fastest available heating rate and maintained at that temperature for 5 minutes whilst the effluent was transferred to the gas chromatograph via a heated (210 ºC) transfer line coupled directly to the chromatographic column.

A Zebron ZB624 chromatographic column was used (Phenomenex, Torrance, CA). This is a wall-coated open tubular column (dimensions 60m×0.4mm×0.25mm ID), the liquid phase comprising a 0.25 μm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane. The gas chromatograph oven was maintained at 50 ºC for 4 min following injection and was then raised at 10 ºC.min-1 to 220 ºC for 9 min. Separated products were transferred by heated line, maintained at 240 ºC, to the mass spectrometer and ionised by electron bombardment. The spectrometer was set to carry out a full scan from mass/charge ratios (m/z) 33 to 350 using a scan time of 0.3s with a 0.1s scan delay. The resulting mass spectra were combined to form a total ion chromatogram (TIC) by the GC-MS integral software (TuboMass ver 4.1).

6.2 The structure of the GC-MS data

The format of the GC-MS data is as follows: It is essentially a 3 dimensional array, where the data are individual time segments (typically 100 per second) of ion spectra and individual ion intensities. As compounds pass through the GC column, they are separated according to their physico-chemical properties, so small compounds, for example, pass through the column faster than large compounds and hence come off earlier. When they leave the column, they pass into the mass detector which bombards the compound with electrons, fragmenting them. The fragmentation pattern is characteristic of the compound and detector and can be used to identify it.

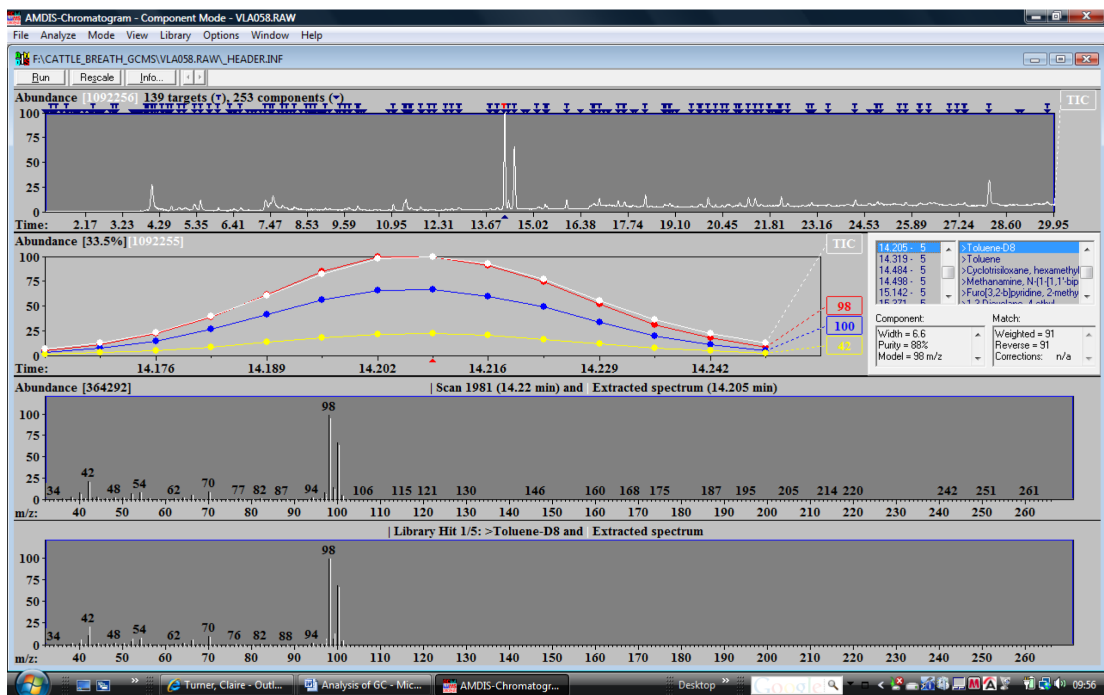


Fig 6.1 Format of GC-MS data

The figure above shows how the data are formatted. The top of 4 panels shows a total ion chromatogram. This shows the total ion count as detected by the mass detector as the compounds leave the gas chromatography column and is fragmented. A typical chromatography run will last for 30 minutes and resolve up to several hundred compounds. The detector produces a spectrum (3rd panel down) every 0.001 second (this is set by the user) of m/z value (essentially, this is the ion fragment mass) against intensity. GC-MS data can be examined by other programs, and in conjunction with commercially available libraries, unknown compounds may be identified by comparing fragmentation patterns obtained with those in the library. An example of such a program (used in this study) is AMDIS, Automated Mass Spectral Deconvolution and Identification System, developed by NIST, the National Institute of Standards and Technology.

AMDIS uses the commercially available NIST library. It works by matching a spectrum obtained at a particular scan number and finds the best match between that and spectral database entries in the library. The difficulty is that in many cases, the library match does not give the correct compound, either because of noise, column bleed, or the co-elution of more than one compound, so it is often necessary to manually go through each resolved compound in the spectrum. This is very time consuming where there are a large number of data sets to look at. The AMDIS data can be imported into an Excel file and used to check compounds. Information about the retention time of known compounds also aids identification.

Analysis of GC-MS data ideally will enable unique biomarkers to be found for a particular disease. An example: suppose there are samples from people who have one disease, samples from people who have another disease and samples from people who have neither disease, i.e. there are 3 categories and hence 3 sets of sample data. The aim would then be to find compounds that occur uniquely in samples of one disease or the other disease group, but not the non-diseased group. In practice, this will mean finding the retention, or scan time where these differences occurs to enable specialists identify the specific compounds.

6.3 Results

A total of 101 breath and 1 ambient air samples were collected over the study period. 3 breath samples were lost due to sampling errors. This left a total of 98 breath samples collected from 44 patients and 6 controls.

60 samples were scrutinised and the compounds present discerned. Of these 60 samples, 35 samples were from patients with microbiological growth (this represents this entire patient group), 12 samples from patients with no growth and 13 samples from healthy volunteers. The healthy volunteers' breath samples were collected for another study run by Cranfield University. As we have no healthy patients in our study these were used for comparison. 2 samples from the growth group were discarded due to problems during the GC run. This left a total of 58 samples.

The remaining 51 samples from patients with no microbiological growth were not included due to financial constraints with the analysis.

The figures below show chromatograms from a "no growth" sample and a "growth" sample.

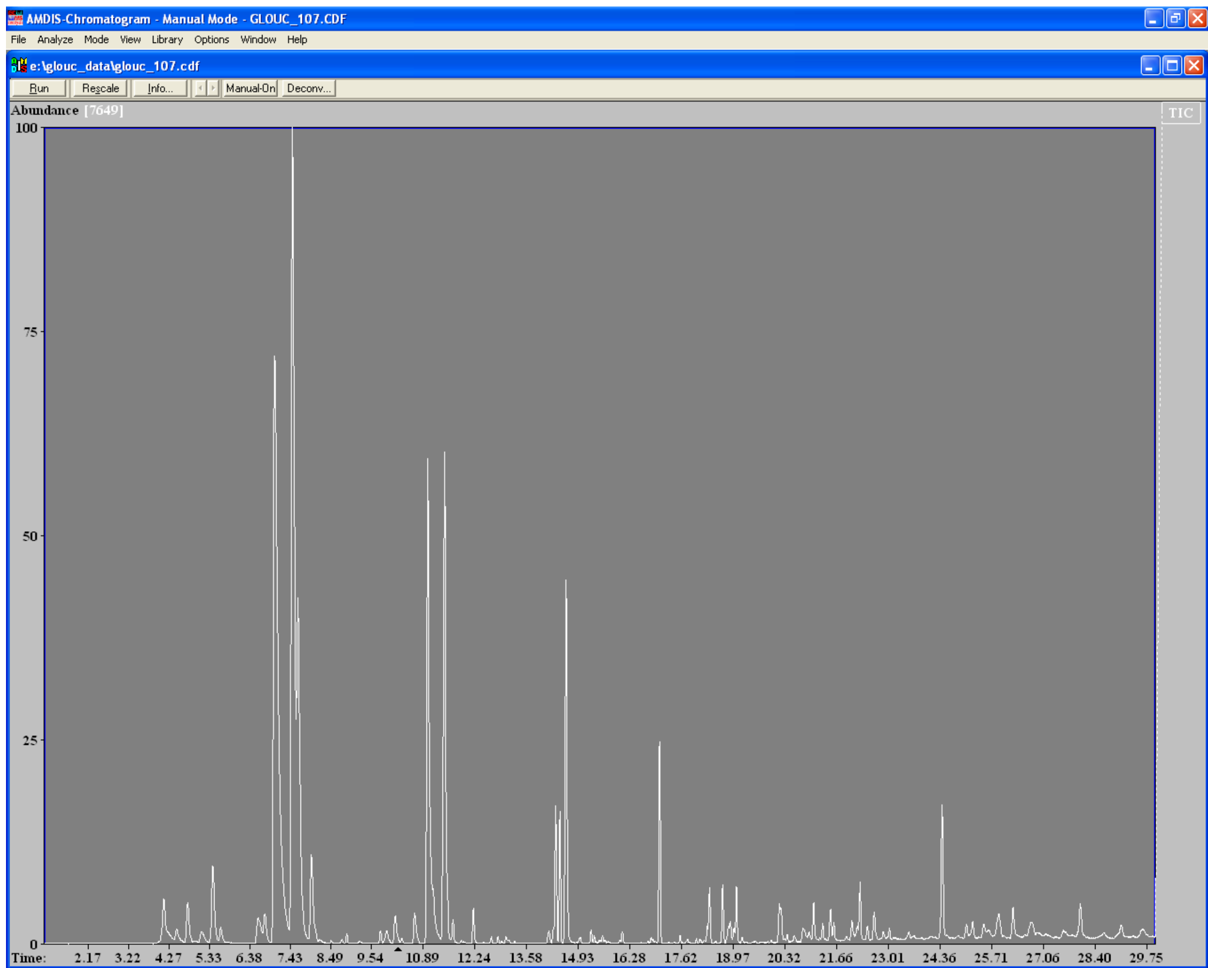


Fig 6.2 Chromatogram of sample G107 as an example of a "no growth" sample.

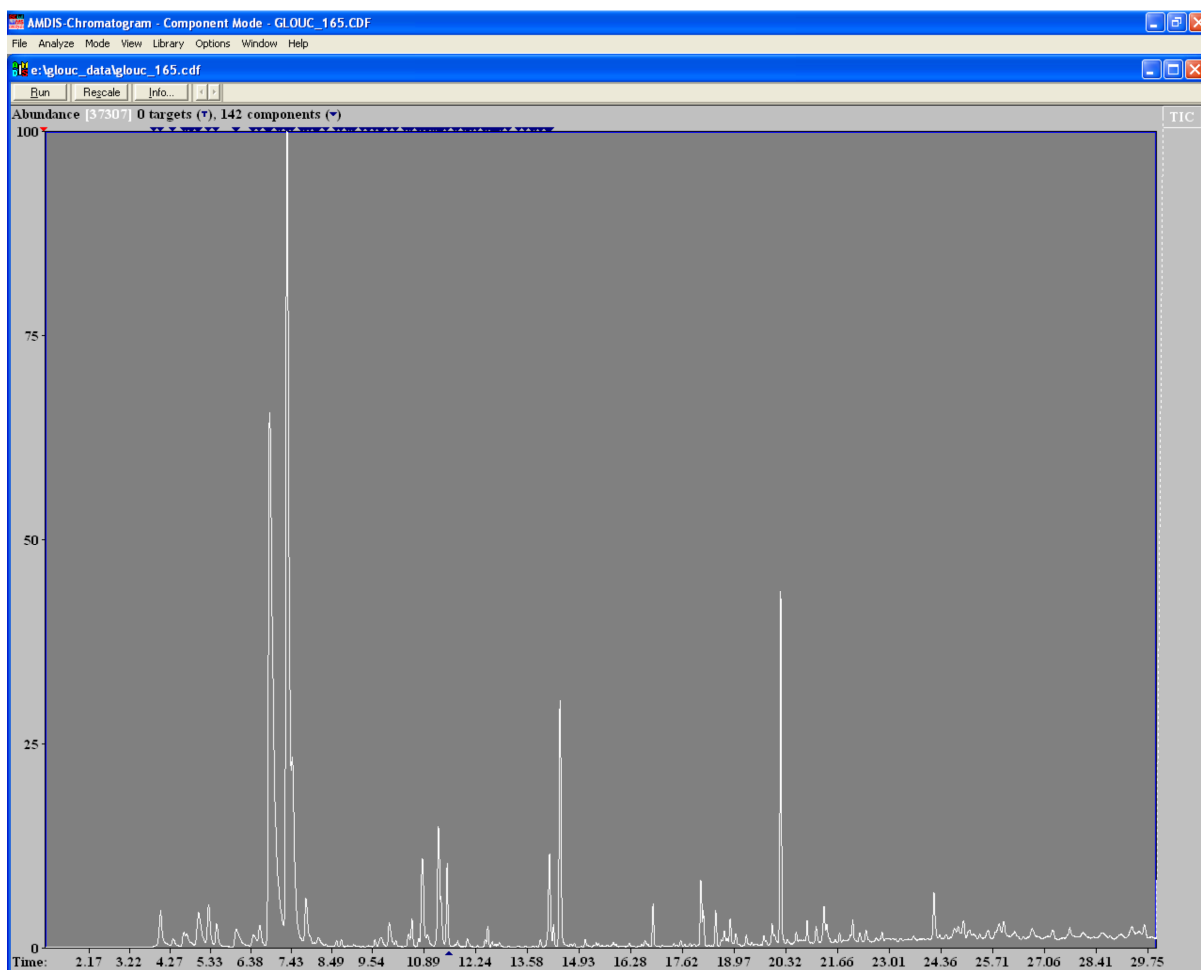


Fig 6.3 Chromatogram of sample G165 as an example of a "growth" sample.

Using AMDIS and the NIST database a list of the most likely compounds relating to retention times was compiled for each of the breath samples analyse. Each individual breath sample contained between 223 and 525 different peaks which needed to be identified.

Toluene D8 was used as an internal standard in the analysis of each breath sample. Fifty nanograms of Toluene D8 was introduced into the ATD tube prior to analysis. By knowing the percentage of Toluene D8 detected by GC-MS analysis that equates to 50ng it is possible to approximate the amount in nanograms of the other compounds detected. This is an approximation only but is a valid calculation when comparing similar samples.

Using this method the 30 most abundant compounds in each sample were identified and quantified. These 30 compounds from each sample were then combined to make a list of 253 different compounds that appeared in the 30 most abundant compounds across all the samples. Anaesthetic gases were excluded from the top 30 list. The amount in nanograms in compounds appearing in each sample was combined to allow PCA analysis in an attempt to differentiate between the healthy volunteers, growth and no growth groups. This table is too large to be displayed in the text but is included on the CD ROM (Table 6.1).

Initially, 10 of the breath samples that were collected from patients who had microbiological growth at the time of collection and 10 samples from patients without microbiological growth were analysed. By comparing the samples we found that the following compounds were present in the breath of all patients with microbiological growth but not present at all in the breath of those without microbiological growth.

These compounds were:

Ethanol 2-phenoxy

Ethyl Alcohol

Furan tetrahydrochloride

Heptane

Hexane

Isopropyl alcohol

Propene

To investigate whether any of these compounds represented potential breath markers for VAP, the remaining samples from patients with microbiological growth were analysed. From these results it was clear that there was no subset of compounds that were present in all the growth samples exclusively. The following compounds were however present in the majority of the samples from patients with microbiological growth:

Methyl alcohol	Acetic acid	Cyclohexane
Nonanal	Acetaldehyde	Cyclohexanone
Pentane	Acetone	Ethyl alcohol
1,3 Butadene 2 methyl	Acetonitrile	Heptane
Toluene	Acetophenone	Isopropyl alcohol
p-xylene	Benzaldehyde	Heptane
Phenol	Benzene	

Unfortunately as none of these compounds are present exclusively in the microbiological growth samples, none of these compounds can individually be considered breath markers of VAP merely by their presence or absence in a breath sample.

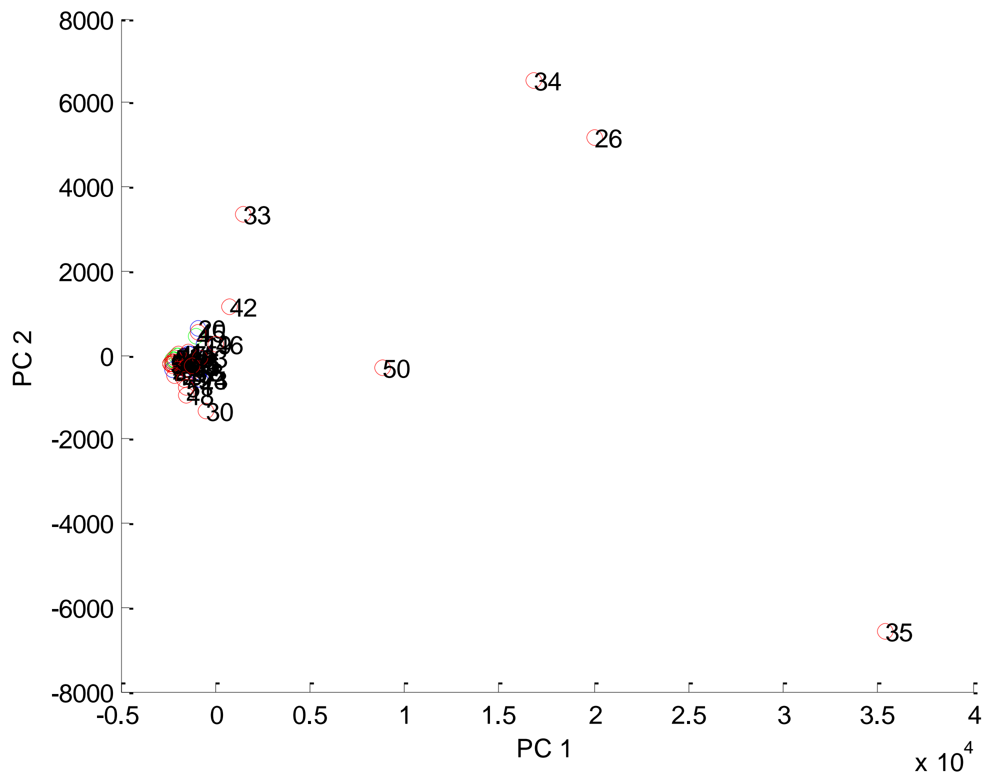


Fig 6.4 LD score plot showing all samples.

Samples 26, 33-35 and 50 seen to be clear outliers on PCA (v high level of counts) therefore excluded from analysis

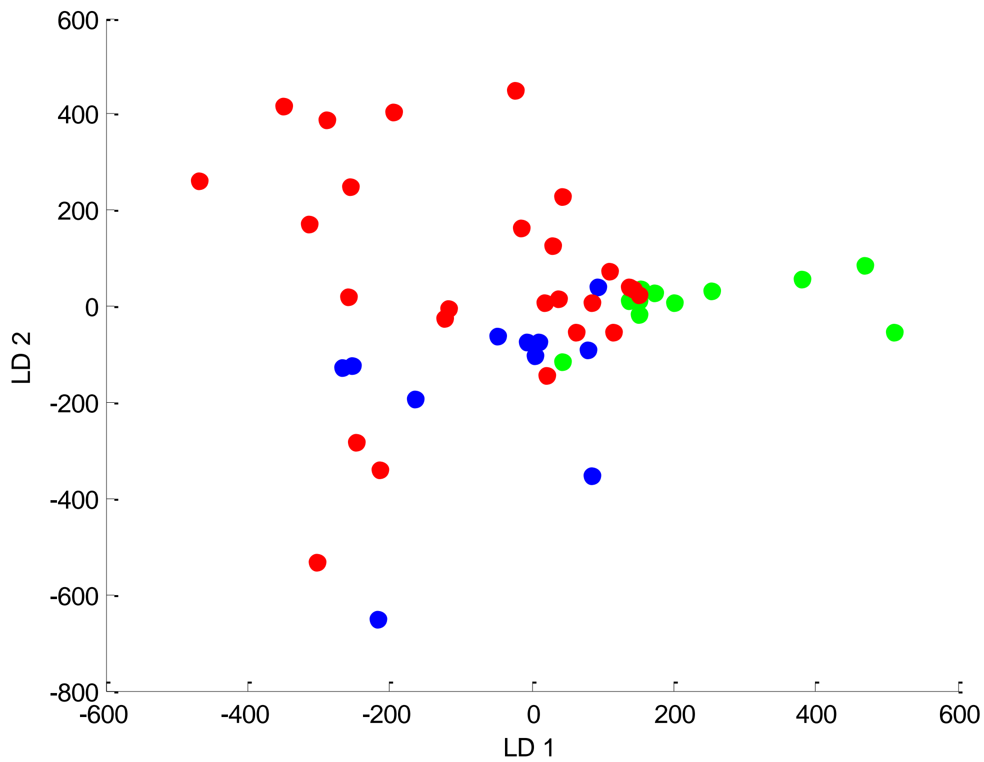


Figure 6.5 LD score plot showing differentiation of samples from healthy individuals (green), no growth BAL (blue) & growth (red).

PCA fed LDA was performed on the samples which were split into 3 groups:

1. Healthy Volunteers
2. No growth
3. Growth

The results are displayed in figure 6.5 above.

Training Classification Performance

Class 1 Percentage Correct 92.3077

Class 2 Percentage Correct 90.9091

Class 3 Percentage Correct 57.6923

answer =

```
    12     1     0
     1    10     0
     6     5    15
```

Training performance 74.0000

Prediction performance 0.0000

LEAVE ONE OUT CROSS VALIDATION:

answer =

```
     9     0     4
     0     8     3
     0     1    25
```

groupcorr =

```
   69.2308   72.7273   96.1538
```

Performance 84.0000 CORRECT

missed =

2

11

12

13

22

23

24

The above are the samples misclassified by the model.

Sensitivity 69-96%

Specificity 71-100%

A review of the PC loads was undertaken in order to ascertain which compounds were contributing most to the differentiation between groups. This was done by marking the peaks in the loads. This is illustrated in figure 6.6 below.

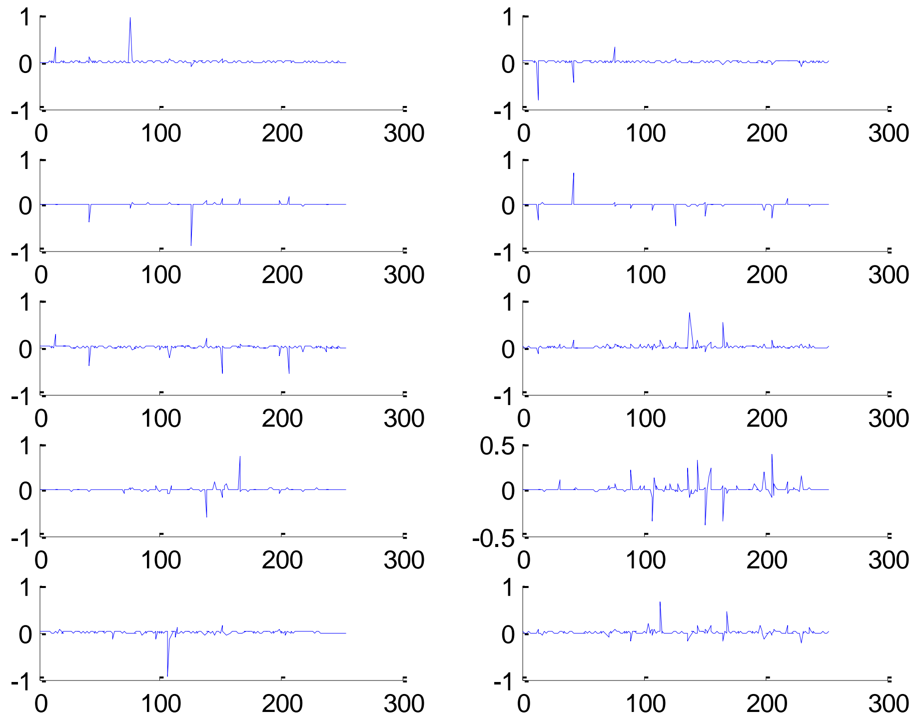


Figure 6.6 PC loads 1 to 10.

Use ANOVA to identify significant PCs

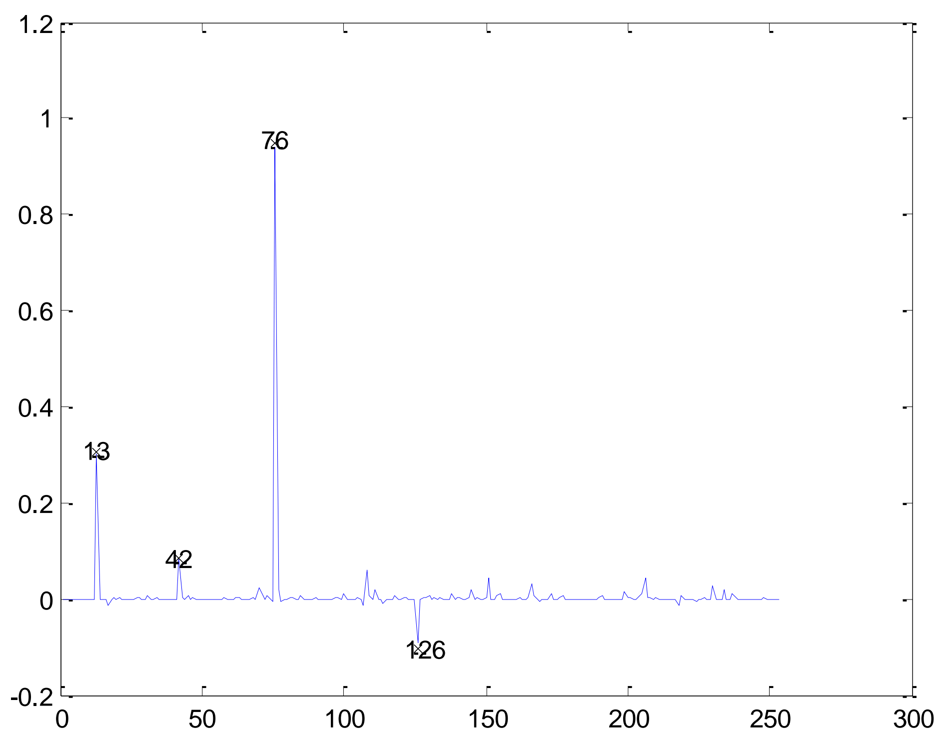


Figure 6.7 PC 1 with significant peaks marked.

13 - 1,3-Butadiene, 2-methyl- (isoprene)

42 - 2-Propanol, 1,1,1,3,3,3-hexafluoro-

76 -Acetaldehyde

126 - Ethane, pentafluoro-

Mean values of Isoprene in ng (Peak #13)

Healthy volunteers	0
No growth	469.76
Growth	1508.28

Mean values of Acetone in ng (Peak #76)

Healthy volunteers	264.10
No growth	927.63
Growth	3318.34

Mean values of Acetaldehyde in ng (Peak #76)

Healthy volunteers	9.6
No growth	53.6
Growth	122.4

Mean values of Ethane,pentafluro- in ng (Peak #126)

Healthy volunteers	0.86
No growth	8.24
Growth	98.25

6.4 Discussion

The majority components of human breath are nitrogen, oxygen, carbon dioxide, water and inert gases. The remaining small fraction of human breath comprises trace components occurring in concentrations in the nmol/l – pmol/l (ppbv-pptv) range. More than 500 of these compounds have been described. These volatile substances may be formed in the body or may be ingested or inhaled from the environment. Exogenous molecules, especially the halogenated organic compounds may be analysed for environmental or expositional issues to assess compound specific uptake into and elimination from the body[157]. In order to establish which compounds are exogenous or may represent pathological processes within the body, it is important to establish which substances are endogenous.

These endogenous compounds include inorganic gases such as NO and CO; VOCs such as ethane, pentane, acetone, isoprene; and other normally non-volatile substances such as isoprostanes, peroxynitrite or cytokines that can be determined in breath condensate.

Quantitative analysis of breath condensate is impeded by several factors. There is no clear relationship between assumed alveolar or airway concentrations and substance concentration in the condensate [158]; in addition some of these compounds are of limited stability.

Volatile organic substances such as ethane, pentane, isoprene or acetone can provide information regarding different biochemical processes in the healthy and diseased human body. Kinetics of VOCs can be approximated according to substance solubilities. In addition, for most of the exhaled organic compounds there are no problems with stability.

6.4.1 What constitutes normal breath?

The makeup of alveolar breath is distinctly different from that of inspired air. VOCs present in alveolar breath are either extracted from the inspired ambient air or added as a result of metabolic processes within the body. Some of these processes have been understood for many years, the most obvious being that oxygen is extracted from inspired air and carbon dioxide is added to alveolar breath via the oxidative metabolism of glucose[159].

In 1971 Pauling et al, demonstrated that several hundred VOC's could be demonstrated in normal human breath [3]. This number has now increased to several thousand compounds detectable in low concentrations [113]. This large number of compounds and the wide variation between individuals is a problem for the investigator. In order to determine which breath VOC's are "abnormal" and therefore may be associated with a pathological process or disease, it is necessary to understand what can be considered normal.

In 1999 Phillips et al.[160] examined the breath of 50 healthy volunteers. All subjects were fasted from midnight and samples were collected between 07:00 and 12:00 midday. The group studied comprised 27 males and 23 females. The number of VOCs detected in individual breath samples varied from 157 to 241 with a mean of 204.2. 3481 different VOCs were observed at least once and only 27 VOC's were observed in all subjects. The 27 VOC's observed in all 50 subjects are shown in table 6.2 overleaf

VOC	Positive/Negative Alveolar Gradient
Isoprene	Positive
Benzene, (1-methylethenyl)	Positive
Napthalene	Positive
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1dimethylethyl)	Positive
Napthalene, 1-methyl-	Positive
Butane, 2-methyl-	Positive
Tetradecane	Positive
Pentodecane	Positive
Dodecane	Positive
Benzene	Negative
Benzene, 1-ethyl-2-methyl-	Negative
Benzene, ethyl	Negative
Benzene, methyl	Negative
Benzene, propyl	Negative
Cyclohexane, methyl	Negative
Decane	Negative
Heptane	Negative
Heptane, 2-methyl	Negative
Heptane, 3-methyl	Negative

Hexane	Negative
Hexane, 3-methyl	Negative
Nonane	Negative
Pentane, 2,3,4-trimethyl	Negative
Pentane, 2-methyl	Negative
Pentane, 3-methyl	Negative
Propane, 2-methoxy-2-methyl-	Negative
Undecane	Negative

Table 6.2 27 VOC's found in the breath of all 50 healthy volunteers (most abundant first). Adapted from Phillips et al. 1999[160].

Phillips et al. use the concept of alveolar gradient as a method for compensating for VOCs that may be entering the body from the ambient air. The amount of VOC in the ambient air is subtracted from the amount of the same VOC present in breath. This gives the "alveolar gradient". A positive gradient indicates the VOC is endogenous and a negative gradient indicates the VOC has come from the ambient air. This is a recognised attempt to compensate for environmental VOCs; however it has problems. VOC's may be detectable in the breath for days or weeks after ingestion depending on the individuals' environment and other factors such as age and health. Subtracting VOCs detected in the ambient air at the time of sampling does not correct for this phenomenon. It is impossible to accurately account for this as the pathways by which many of these

compounds arise in the breath are unknown. It also requires that two samples are collected and analysed for each patient studied.

The alveolar gradient concept does give an indication of the kinetics of the VOC concerned within the body; this is because it varies with the rate of production and the rate of clearance of the VOC. The polarity of the gradient shows whether production or clearance is the predominant process.

An alternative method to compensate for the ambient air is to provide the patient with VOC-free air to breathe prior to collection of the breath sample. This is a logical approach but very difficult to achieve practically; almost all “pure” air contains VOC’s in picomolar concentrations. In practice it would take days or weeks of breathing pure air in order to eliminate the exogenous compounds found in ambient air.

The body pool of VOCs is populated form two sources: the lungs and extra pulmonary sources. The latter is usually from metabolic pathways but food, drugs and absorption via the skin may also contribute. The movement of VOCs through the body is illustrated in figure 6.8 below:

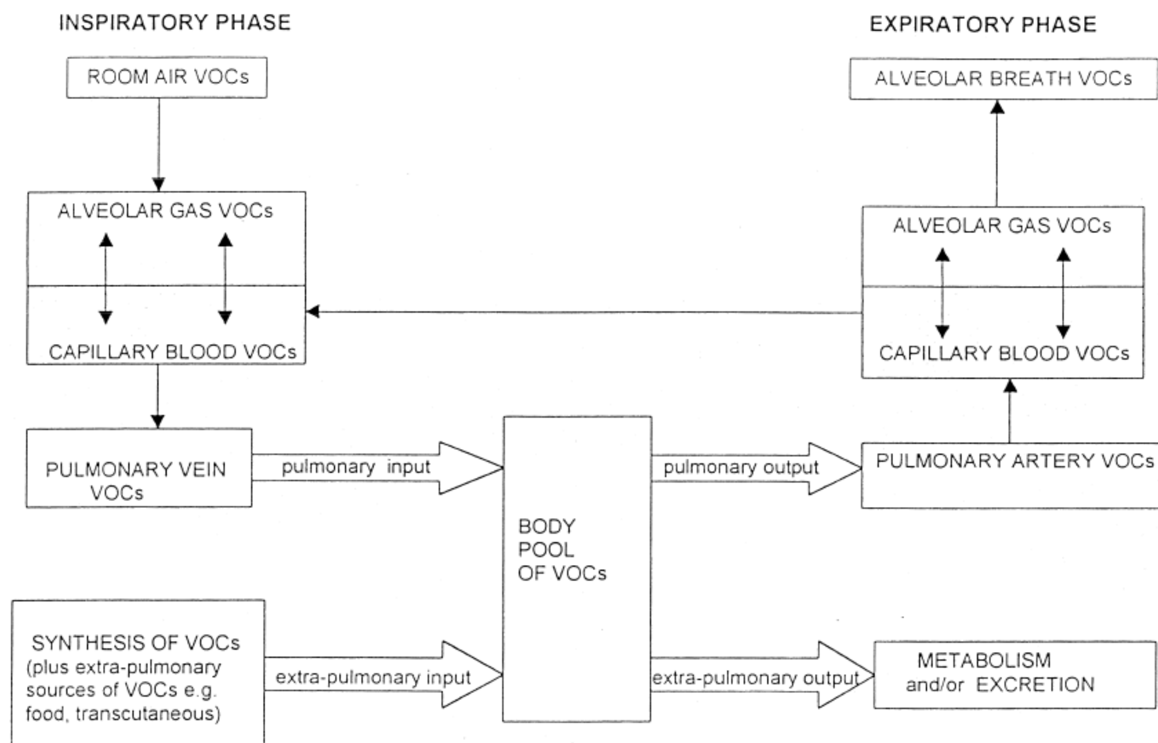


Fig 6.8 Pathways of VOCs through body compartments. Phillips et al 1999.[160]

VOCs in the inspired air and the capillaries rapidly equilibrate in the alveoli. The predominant process is dependent on the phase of respiration. Room air VOCs equilibrate with lung venous blood during the inspiratory phase, whilst lung arterial blood equilibrates with VOCs in alveolar breath during the expiratory phase.

Our results have shown no Isoprene in the breath of the healthy volunteers group and an increasing mean amount in the no growth and growth groups. These findings are interesting for several reasons. Firstly, Isoprene is ubiquitous in human breath and therefore should be present in all the samples analysed. Isoprene has been identified as a marker of oxidative damage as we have already discussed. The fact that Isoprene levels are elevated in all our study patients when compared to healthy volunteers can be

considered an indicator of their physiological state. All of our study subjects are in the ICU setting receiving mechanical ventilation, which is clearly an abnormal physiological state. If the patients in whom we have identified microbiological growth can be considered “sicker” than those in whom we did not culture any organisms then further elevations in breath Isoprene levels could be considered evidence that these patients are more “physiologically stressed”.

Foster et al.[102] have postulated that an increased breath Isoprene level is an indicator of free radical mediated damage to lung epithelium. We would expect damage to lung epithelium in the setting of VAP, but the use of mechanical ventilation itself may also be a cause of lung injury in our patient group.

Breath Acetone is a marker of ketosis which is raised in starvation or in people with diabetes mellitus. Our patient group showed an elevated mean acetone level in the breath when compared to the healthy volunteers. Mechanically ventilated patients cannot consume what can be considered a “normal diet”. Our subjects would either be in a state of temporary starvation or being fed either by a nasogastric tube or intravenously. This may explain the elevated acetone levels seen in the study group. We did also see a higher mean acetone level in the growth group compared to the no growth group.

Acetaldehyde is formed via the metabolism of ethanol in the liver. Most of the literature on the presence of acetaldehyde in breath relates to its relationship with ethanol. As our patient group are not in a position to be consuming ethanol we must look for another source. It has been reported that oropharyngeal flora can produce acetaldehyde locally[161]. The presence of an endotracheal tube and critical illness can dramatically alter normal body flora including that in the upper respiratory tract. Gas from here should be excluded when sampling via an endotracheal tube however it is well known that microorganisms colonise these devices and these could be contributing to the elevated acetaldehyde levels seen. Further work is needed to investigate whether pathogens deep in the lungs as would be seen in VAP could be a potential source of increased breath acetaldehyde levels.

Ethane,pentafluro is a refrigerant gas that is also used in fire suppression systems, it does not damage the ozone layer. Its presence in the breath of our study subjects must be regarded as an exogenous compound. The source of this and the reason its levels are higher in the growth patients then the no growth patients is unclear.

6.4.2 Volatile Inhalational Anaesthetic Gases

22 samples analysed had Isoflurane, Desflurane or Sevoflurane (or a combination thereof) present. These 22 samples were from 17 patients. This finding is interesting as all patients were sampled at least 72 hours after any anaesthetic gases would have been given. Isoflurane is 93% excreted via the lungs[162]. The finding of detectable levels of these gases in the breath of patients several days after administration is unexpected.

A summary of samples with volatile anaesthetic gases detected by GC-MS analysis is included on the accompanying CD-ROM (Table 6.2).

This prompted a review of the casenotes of the patients concerned. The following points need explanation:

1. Some patients have more than one type of anaesthetic gas detected in the breath when only one agent was given.
2. Some patients have a different agent detected to the one given.
3. We were unable to find any evidence of some patients being given any anaesthetic agent at all prior to sampling.
4. The presence of significant amounts of these agents in the breath of study patients may be masking the presence of smaller quantities of significant VOCs of interest.

It is difficult to explain these anomalies satisfactorily. The presence of anaesthetic agents in the breath when no agent has been administered, points to contamination. This could either be from the gases the patient is breathing in or from the Breathotron machine itself. The fact that the patients are breathing piped gases from a closed circuit on a mechanical ventilator makes this extremely unlikely as a source of contamination. GC-MS analysis of the inhaled gas mix for every sampled patient would have to be performed to conclusively exclude this as a source.

It is possible that the residual breath in the sampling loop of the Breathotron could have been contaminated by samples of other patients. This is only likely if patients had been sampled in quick succession in a "back to back" fashion. As can be seen from the table above, none of the samples in which anaesthetic gases were detected were taken from different patients on the same day.

A third source of potential contamination is the anaesthetic apparatus itself. Prior to surgery the patient is anaesthetised in the anaesthetic room adjacent to the operating theatre. After the patient is asleep they are disconnected from the anaesthetic machine and moved into the operating theatre. Here they are connected to a second anaesthetic machine. In the anaesthetic room the volatile agent of choice is commonly Isoflurane. When moved into the operating theatre, the patient could be continued on Isoflurane or switched to Desflurane or Sevoflurane depending on the Anaesthetist in charge of the case.

Another factor is that the breathing circuit (the tubing connecting the patient to the anaesthetic machine) is changed daily and can therefore be used for multiple patients in the same day. Within these patients different anaesthetic agents could be used. The breathing circuit does contain a single use filter which protects patients from infection. This filter would not however exclude volatile anaesthetic gases. The anaesthetic machines and breathing circuits are the most likely source of contamination with volatile anaesthetic gases.

However, none of the scenarios described above explain the presence of volatile anaesthetic gases in the breath of patients which have not been anaesthetised prior to their ICU admission. Another finding that is difficult to explain is that in the 2 patients from whom we have more than one sample of breath containing anaesthetic gases, the amount of Isoflurane detected in the last sample is greater than that in the first sample. Over time and without further exposure to Isoflurane the amount of Isoflurane detected in the breath should be decreasing.

It is possible that the hospital notes are incomplete and anaesthetic charts or operation notes have been lost or misfiled. However, the amounts of anaesthetic gases detected in the breath of the patients who do not appear to have been given any volatile anaesthetic gases are very small indeed. This suggests contamination of the sample.

A literature research reveals no studies that have looked at the amounts of volatile anaesthetic gases in exhaled breath over time using GC-MS in critically ill patients. Carpenter et al.[162] looked at the metabolism of anaesthetic gases in healthy patients. They used GC-MS analysis of breath samples over a period of up to nine days in an attempt to quantify how much of these agents are excreted unchanged in breath and how much is altered by metabolism prior to elimination. They measured the total amount of Isoflurane (and also enflurane, halothane and methoxyflurane) taken up during a 2 hour "wash in" period and then the total amount recovered unchanged in exhaled breath over a 5-9 day "washout" period. Using this method they concluded that 93% of the total amount of inhaled Isoflurane is excreted unchanged in breath over the washout period. A total of nine patients were studied. It should be noted that all nine patients received a mixture of all four anaesthetic agents in conjunction with nitrous oxide for 2 hours. The study describes the total amounts of gases recovered in the breath, but the individual amounts detected at each of the sampling times throughout the washout period are not reported. This does not give a picture of breath excretion over time, merely the total amount recovered.

These findings are very different to details quoted in the product data sheet (Rhodia, New Zealand. 1999):

"In man about 0.2% administered is evident as recoverable
metabolites (fluoride and organic fluorine), with approximately 50% of these
excreted in the urine, the principal metabolite being trifluoracetic acid.
Enzyme induction associated with pre-existing drug therapy would not appear to
be an important factor in the metabolism of ISOFLURANE in man, mainly because
the overall metabolism of ISOFLURANE is so low"

Further work is necessary to establish over what time period volatile anaesthetic agents can be detected in the exhaled breath of critically ill mechanically ventilated patients. The effects of altered vital organ function and multiple drug interactions in this patient group may well have a significant impact. It would also be interesting to measure levels of these compounds in the ambient air of the ICU environment as this could have implications for ICU staff with chronic, low level exposure to these compounds. Summer et al.[163] published a study looking at Sevoflurane concentrations in expired breath of operating room staff, using Proton Transfer Reaction Mass Spectrometry. They sampled the breath of 40 staff members before commencing duty and at 0, 1, 2 and 3 hours after finishing duty and again before commencing duty the next day. Sevoflurane levels in exhaled breath of OR staff was significantly elevated at all time compared to control subjects who did not work in the OR.

The level of exposure in the ICU should however be far less as except on rare occasions patients do not receive volatile anaesthetic gases in the ICU.

The results could also have implications for any further work on VAP and exhaled breath as the high levels of these compounds detected by GC-MS in the initial few days after anaesthesia could mask compounds of interest that may be present at much lower concentrations.

It may be that such a study is unlikely to yield meaningful results as the ICU patient population is a heterogenous group with multiple pathologies and varying degrees of organ dysfunction as well as a wide variation of drug therapies.

Chapter 7

The Breathotron

7.1 Background

The Breathotron is an evolution of the eNostril developed by Lee-Davey at Cranfield University in 2004[164]. The Breathotron is a novel breath analyser conceived and built by Cranfield Health. It was initially intended for use in chronic health conditions such as cystic fibrosis and diabetes. The Breathotron contains a single Mixed Metal Oxide Sensor as opposed to the traditional multiple sensor arrays employed in most electronic noses. The sensor of choice is the CAP25, manufactured by Capteur (City Technology Ltd, Portsmouth, England). A single sensor has been used in an attempt to increase portability and affordability when compared to commercially available Enoses.

The CAP25 is a general air quality sensor, originally intended for automotive and aerospace applications. It was selected due to its broad band response and low cost. The Breathotron is a stand alone, mains powered portable device which can be controlled either by the built in portable digital assistant (PDA) or by connection to a PC. The PDA is controlled by eNostril software which was written in house at Cranfield Health. Operating instructions can be found in Appendix 2. A photograph of the Breathotron and a schematic are shown below.

Fig 7.1 The Breathotron.

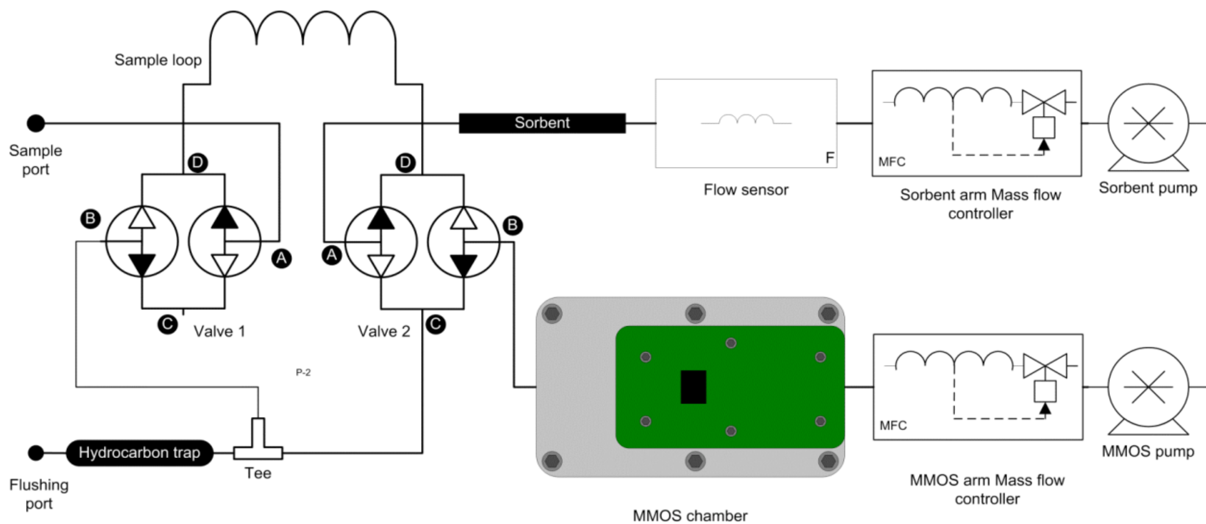


Fig 7.2 Breathotron Schematic.

The Breathotron also incorporates a sorbent trap to allow the collection of breath samples for GC-MS analysis as described in the previous chapter. It therefore has the potential to incorporate both Enose analysis and collection of alveolar breath samples for GC-MS analysis in one portable unit. In this study the Mk IIa version of the Breathotron was used.

The following is taken from the operating instructions in Appendix 2:

The version of the eNostril software used in this study is designed to enable the Breathotron to sample alveolar breath. The signal from the flow rate sensor is integrated so that the cumulative exhaled volume for any given breath is known, and sampling is initiated when this exceeds the estimated volume of the upper respiratory tract and the dead space of the face mask assembly.

The sampler incorporates two distinct methods for measuring compounds contained in the breath. In the first method, breath is passed through a cartridge containing granules of special polymers and/or activated carbon which trap the compounds of interest for later analysis in the laboratory. The second method uses a semiconductor sensor (mixed metal oxide sensor, MMOS) which produces an electrical signal in response to a broad range of compounds typically found in breath. This signal is digitised and stored on the PDA for subsequent numerical analysis. After sampling, breath is vented to the atmosphere and no gaseous samples are retained.

In order to present a defined sample to the MMOS sensor, the Breathotron incorporates a sampling loop of fixed known volume. To pass a sample across the sensor, the loop is first switched into the sampling position so that it becomes charged with air or breath drawn in via the sampling port. When fully charged the loop is switched into the flush position, in which its contents are driven across the sensor using cleaned air at a fixed flow rate. Appropriate pumping and switching arrangements are provided so that a constant flow rate (required for correct operation) is maintained across the sensor while the instrument is in operation.

The Breathotron was originally intended to be used by awake, spontaneously breathing patients via a disposable facemask. However, for the purposes of this study it was modified to allow connection into the breathing circuit of a mechanical ventilator

7.2 Data Analysis

The raw data gathered from the MMOS sensor response profile is digital in nature. To allow further analysis this information must be converted via an analogue digital converter (ADC) stage[165]. The resulting output data can then be subjected to processing using a variety of techniques such as principal components analysis (PCA), discriminant function analysis (DFA), cluster analysis or artificial neural network (ANN).

Following feature extraction as described below, the MMOS sensor data was analysed using Matlab 7.2.

7.3 Feature Extraction

The figure below shows a typical response curve from a mixed metal oxide sensor.

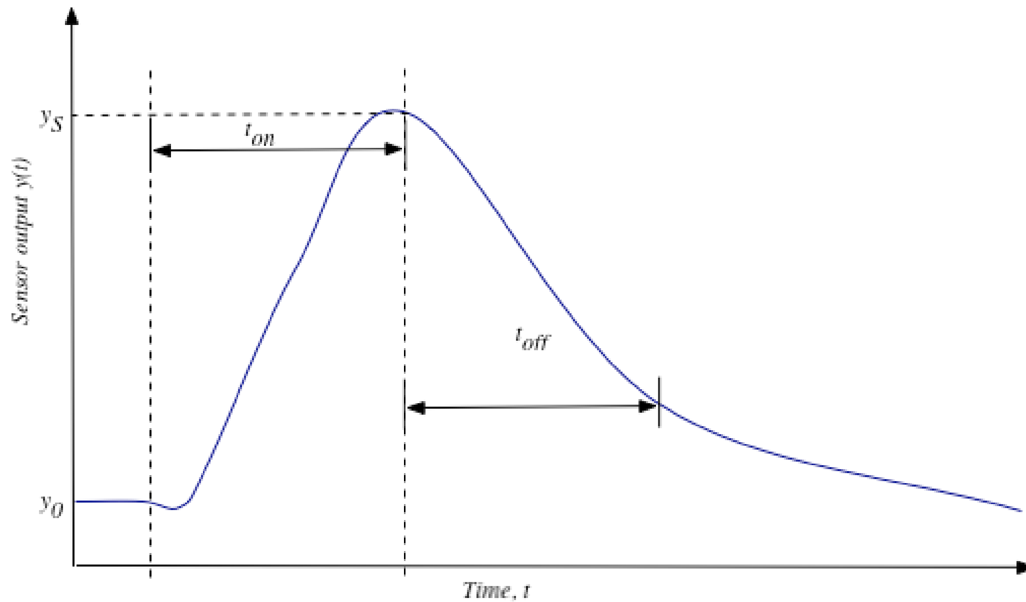


Fig 7.3 Dynamic response of MMOS sensor. (Adapted from Gardner and Bartlett 1999)[166]

Feature extraction is a technique for reducing the number of variables needed to describe a large set of data effectively. Utilising this technique means that the entire response curve is not necessary. Key features such as maximum signal height, baseline and the area under the curve as well as a variety of others can be used to describe the data. Analysing large sets of variables is time consuming; feature extraction can be employed to determine the most accurate combination of variables that describe the data.

Four commonly extracted features are illustrated in the figure below. (a) shows the difference between peak resistance and baseline, (b) shows the total area under the curve, (c) the area under the curve from start of the signal to maximum response and (d) is the time from beginning of the signal to maximum response.

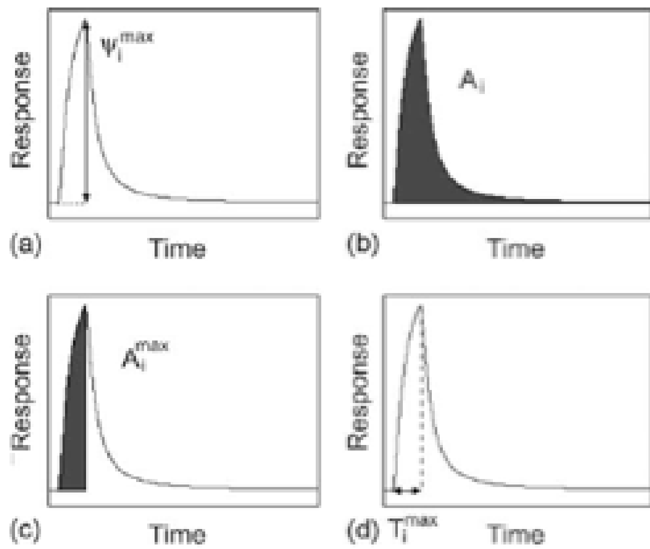


Figure 7.4 An example of feature extraction. Carmel et al. 2003[167]

7.4 Breath Sampling

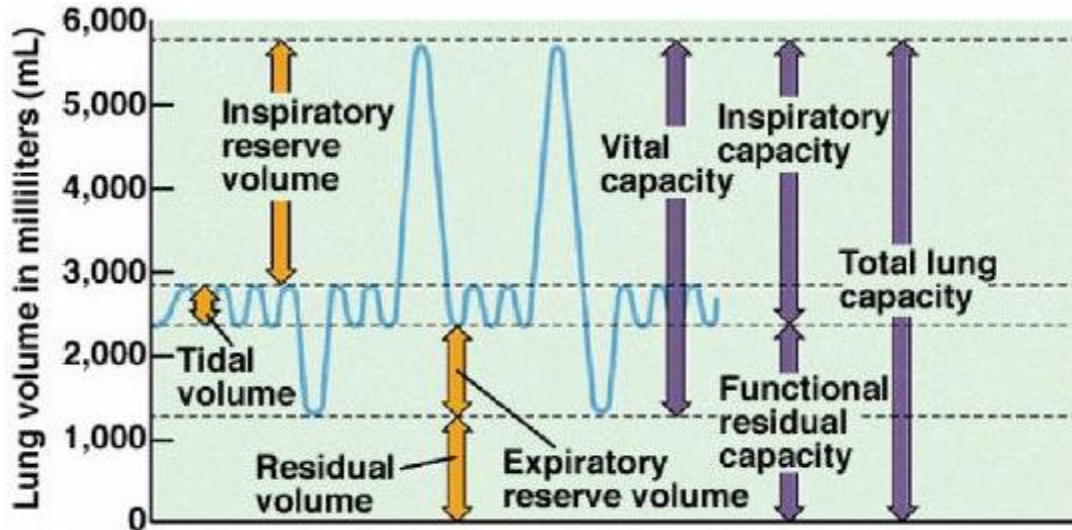


Fig 7.5. Respiratory Volumes and Capacities from Tamarkin[168]

The figure above is a schematic representation of the constituents of lung volumes. It is necessary to have a basic understanding of lung physiology and breathing in order to understand breath sampling.

An average 70kg human at rest inhales and exhales around 500ml each breath, this is known as the tidal volume. As can be seen from the above figure, the total lung capacity is about 6000ml. The vital capacity is the maximum amount of air that can be inhaled with a deep breath and then exhaled, this is approximately 4800ml. The remaining 1200ml (residual volume) remains in the lung and bronchial tree. Dead space gas is air within areas of the respiratory tract in which gas exchange does not take place and is about 150ml or 30% of tidal volume. As has already been discussed in the previous chapter alveolar air and not dead space gas contains the VOC's which are of interest, so it

is important to avoid sampling dead space gas if possible. As part of this study is to evaluate the Breathotron as a breath collection device, there follows a description of some other alveolar breath collection devices.

7.4.1 Breath Collecting Apparatus (BCA)

This was developed by Phillips in 1997 in order to facilitate the collection of alveolar breath for GC-MS analysis. This is a research tool and not commercially available.

Advantages of the device are:

1. Minimal resistance during exhalation which enhances patient comfort.
2. Sterile, single use mouthpieces prevent the risk of cross infection.
3. Alveolar breath sampling avoids dead space gas.
4. Collects breath onto a sorbent trap allowing preconcentration.

Operation

The patient is fitted with a nose clip and breathes through the disposable mouthpiece into the device. Breath is exhaled into a reservoir which is open to the room at one end. As the patient exhales the residual air in the reservoir is forced out into the atmosphere. The sampling port is as close to the subjects' mouth as possible, this means the deadspace gas exhaled will be at the furthest end of the reservoir. This allows sampling of alveolar air. This method of collecting exhaled breath in a long, tubular reservoir; known as a Haldane-Priestly tube, is used commonly. Reservoir tubing is heated to 40°C to prevent condensation of breath vapour on the internal surfaces of the device. It takes approximately 5 minutes to collect the 10l of breath required for sampling.

A schematic of the device is shown in the figure below.

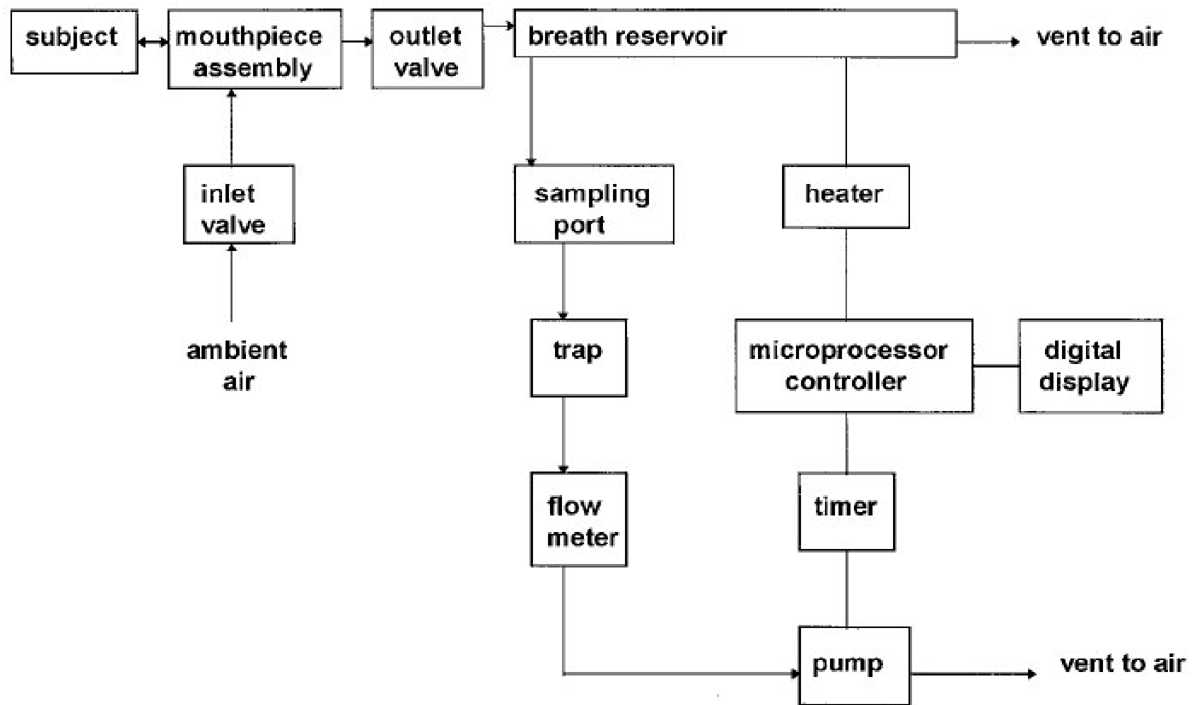


Fig 7.6 Breath Collecting Apparatus from Phillips 1997[113]

7.4.2 Portable Spirometer/Alveolar Breath Collection system(Raymer et al 1990)[169]

This is again an experimental tool and is not commercially available. The device samples alveolar breath into evacuated stainless steel containers for GC-MS analysis. Advantages of the device include its portability and its components can be easily sterilised and therefore reused.

Operation

The principle of operation is also based on the Haldane-Priestly tube. The reservoir in this case is a Teflon tube with a capacity of one litre; samples are collected from the reservoir and stored in an evacuated stainless steel canister. Volunteers inhale filtered VOC free air to prevent contamination from VOC's in the ambient air.

The device is illustrated below:

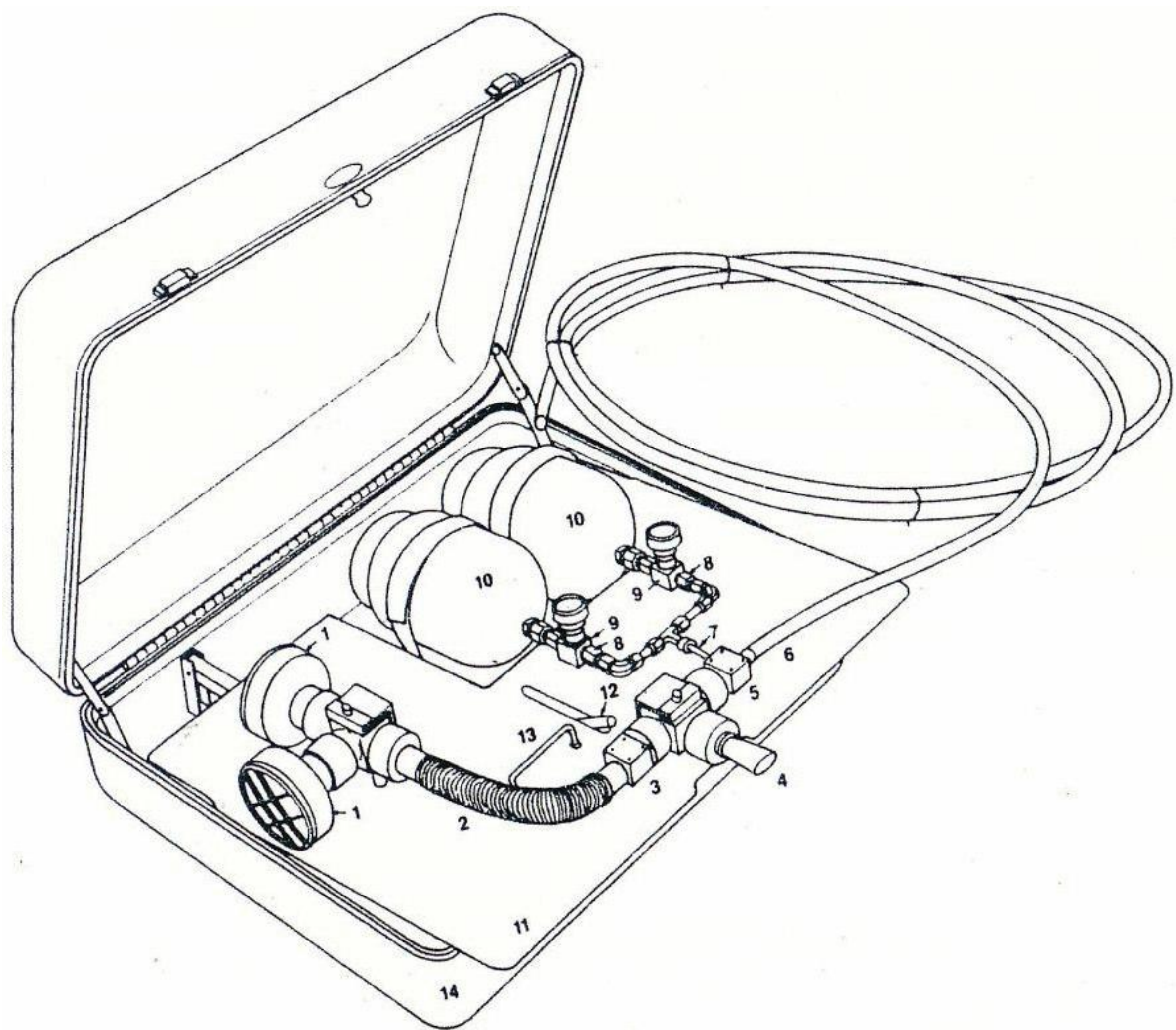


Fig 7.7. Portable breath collection system (Raymer et al., 1990). Numbered parts as follows: (1) organic vapour respirator cartridge; (2) flexible disposable polyethylene tubing; (3) one way Teflon inhale valve; (4) mouth piece; (5) one-way Teflon exhale valve; (6) Teflon tube to contain exhaled breath; (7) sampling port; (8) flow restricting orifice; (9) on-off valve; (10) polished canisters; (11) aluminium platform; (12) handle; (13) handle; (14) aluminium case.

7.4.3 End-Expiratory Air Sampling Device (Yeung et al., 1991)[170]

This device was developed to monitor breath hydrogen concentrations in infants to aid in the clinical diagnosis of lactose malabsorption. Breath is collected and stored in a syringe for GC-MS analysis. This is again a research tool only.

Operation

Seven tungsten hot-wires are employed to measure the exhaled breath. These form part of a Wheatstone bridge circuit which is cooled by breath. The change in voltage can be translated into a flow rate. The breath is sampled via a set of nasal prongs. A 1ml sample is required and this can be collected in 0.5-4 minutes. The breath collected is stored in a glass syringe.

A schematic of the device is shown below:

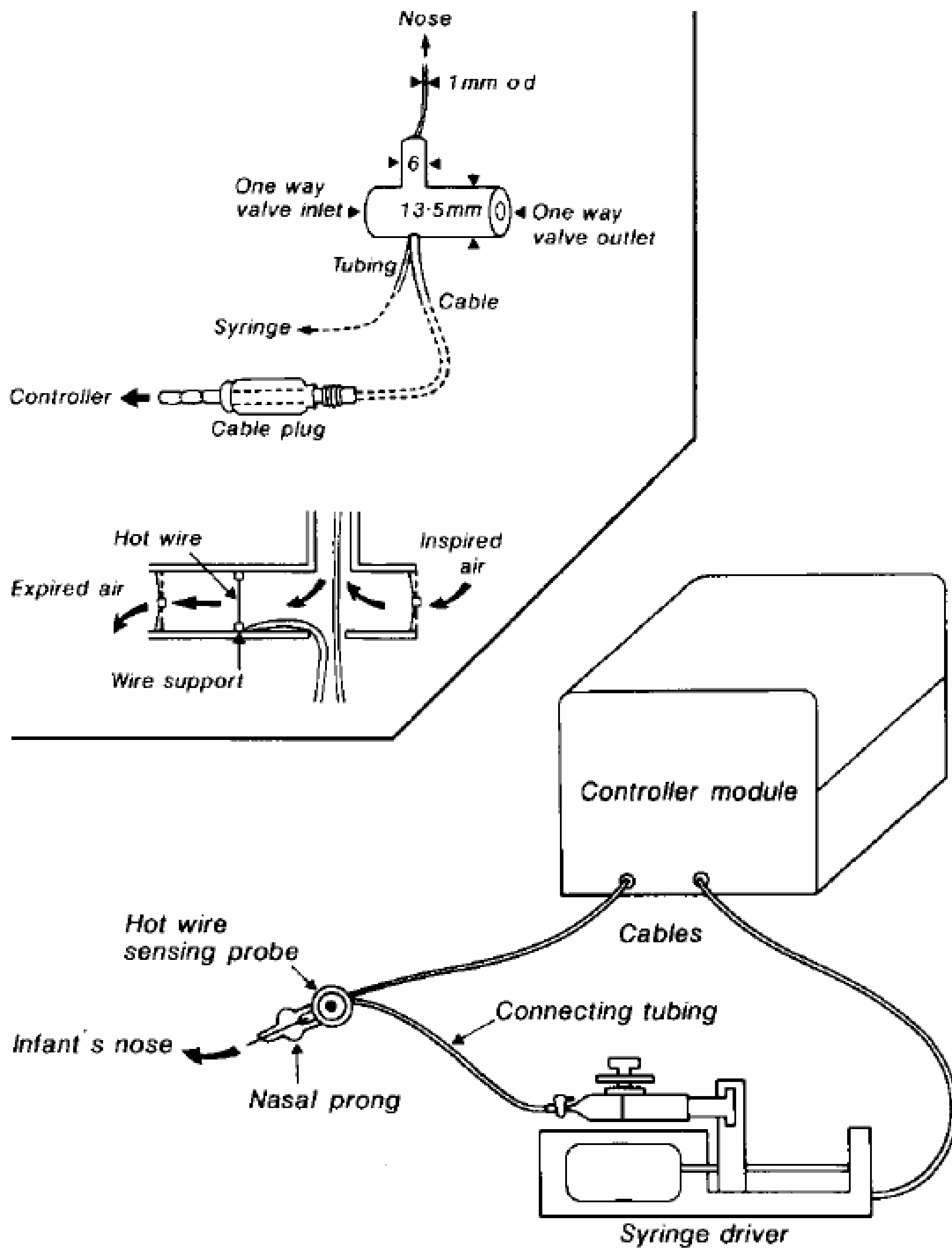


Fig 7.8 Schematic of End-Expiratory Air Sampling Device (Yeung et al., 1991)

7.4.4 Breath Sampler for Solvent Analysis (Dyne et al., 1997)[171]

This device is commercially available as the “Bio-VOC” sampler from Markes International Ltd. It is designed to collect alveolar breath samples and store them on a sorbent tube for GC-MS analysis.

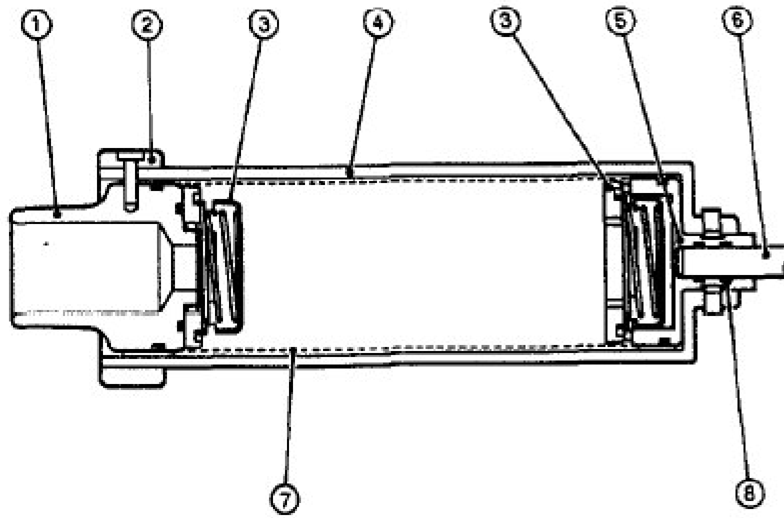
Advantages include:

1. Alveolar breath sampling
2. Easily portable
3. Simple to use
4. Designed to work with standard laboratory equipment

Operation

Again based on the Haldane-Priestly tube; one way valves trap the exhaled gas in the reservoir for collection. An 85ml Tedlar bag is housed inside a rigid case and the device is attached to a sorbent tube. The sample is forced into the sorbent tube by collapsing the Tedlar bag inside the case. The device utilises disposable mouthpieces and an aluminium case.

A cross section of the device is shown below:



1. Mouth piece
2. Outer ring
3. Non return valve
4. Outer tube
5. Absorbtion tube adaptor
6. Tenax absorbtion tube
7. Tedlar bag
8. O-rings

Fig 7.9 Breath sampler in cross section (Dyne et al. 1997)

7.4.5 CO_2 Alveolar Gas Sampler (Schubert et al., 2001)[111]

This device was used to collect alveolar gas samples from mechanically ventilated patients. The alveolar phase of expiration was detected by measuring exhaled CO_2 levels. Sampled breath is stored on sorbent tubes for GC-MS analysis. This is a device for research applications only. It was designed to evaluate the use of expired CO_2 concentration as a trigger for alveolar breath sampling.

Operation:

A 930 Siemens-Elema CO_2 analyser is used to detect the alveolar phase of respiration. The analyser works on the principle of infra red adsorption to detect CO_2. When the CO_2 concentration reaches 5-6% a valve is activated to direct the breath onto the sorbent traps. Medical grade plastics are used in the construction of this device as are glass and stainless steel components. A schematic is shown below:

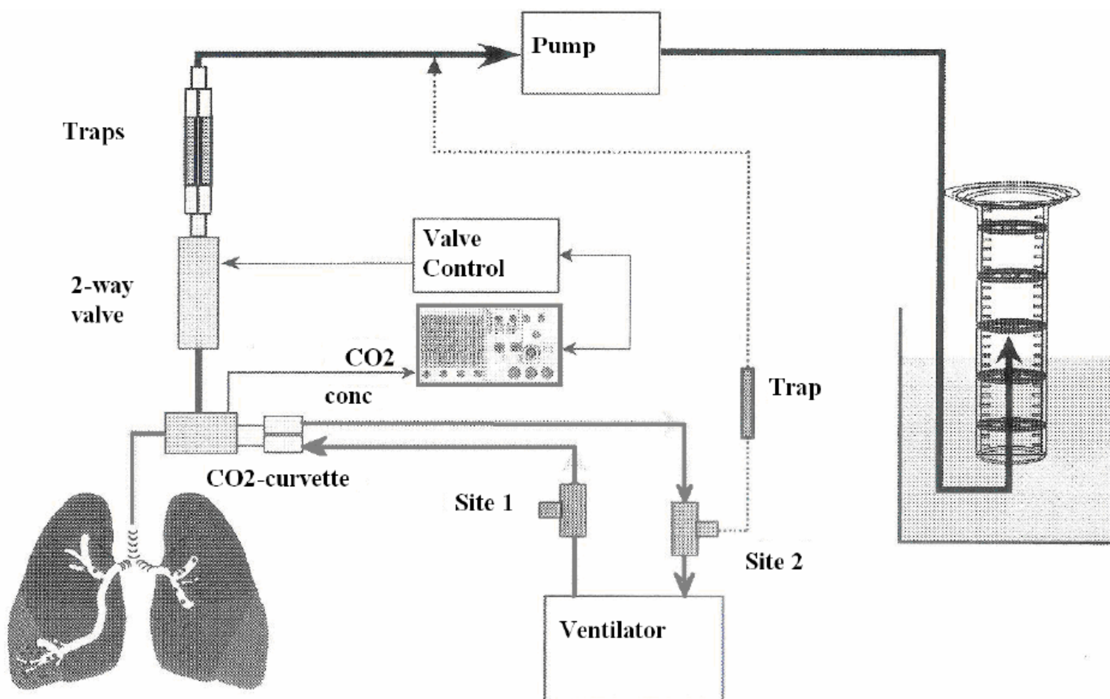


Fig 7.10 Schematic of the CO_2 Controlled Alveolar Sampler

7.4.6 Alveosampler (Quinitron)

This is a commercially available, disposable device produced by Quinitron. It allows collection of alveolar breath samples which are then stored in an ordinary syringe.

Operation

Again this device is based on the Haldane-Priestly tube, however this device employs a bag as the reservoir as opposed to a rigid tube. The majority of the device is made from medical grade plastic but the bag material is not disclosed. Mouthpieces are disposable and cardboard. A picture of the device is shown below:

Fig 7.11 The Quinitron Alveosampler (www.quinitron-usa.com)

7.5 Research Aims

The aim of this study is to attempt to differentiate between the breath of patients who have microbiological growth on BAL samples and the breath of those with no microbiological growth. We are attempting to identify those mechanically ventilated patients who have or who are at risk of developing VAP. We are also evaluating the Breathotron as a means of sampling alveolar breath.

7.6 Materials and Methods

The same set of patients that have been described in the previous chapters are studied in this chapter. A total of 102 samples were collected. 101 from patients and 1 ambient air sample. 2 data files were lost by the software and 6 data files were unusable due to problems with the sensor equilibrating. This left a total of 94 samples from the MMOS sensor for analysis.

Samples were taken at the same patient encounter as the BAL samples described earlier in this thesis. The BAL sample was taken first and then the Breathotron was connected into the ventilator circuit, this was to eliminate any effect the normal saline lavage may have on the respiratory tract.

The Breathotron was set up and allowed to equilibrate for 15 minutes prior to each sampling episode as detailed in the operating instructions appended. Sample collection was performed according to the operating instructions. The sensor response is stored on

the PDA as a text file. The text files were then imported into Microsoft Excel to allow further analysis in Matlab 7.2.

7.7 Results

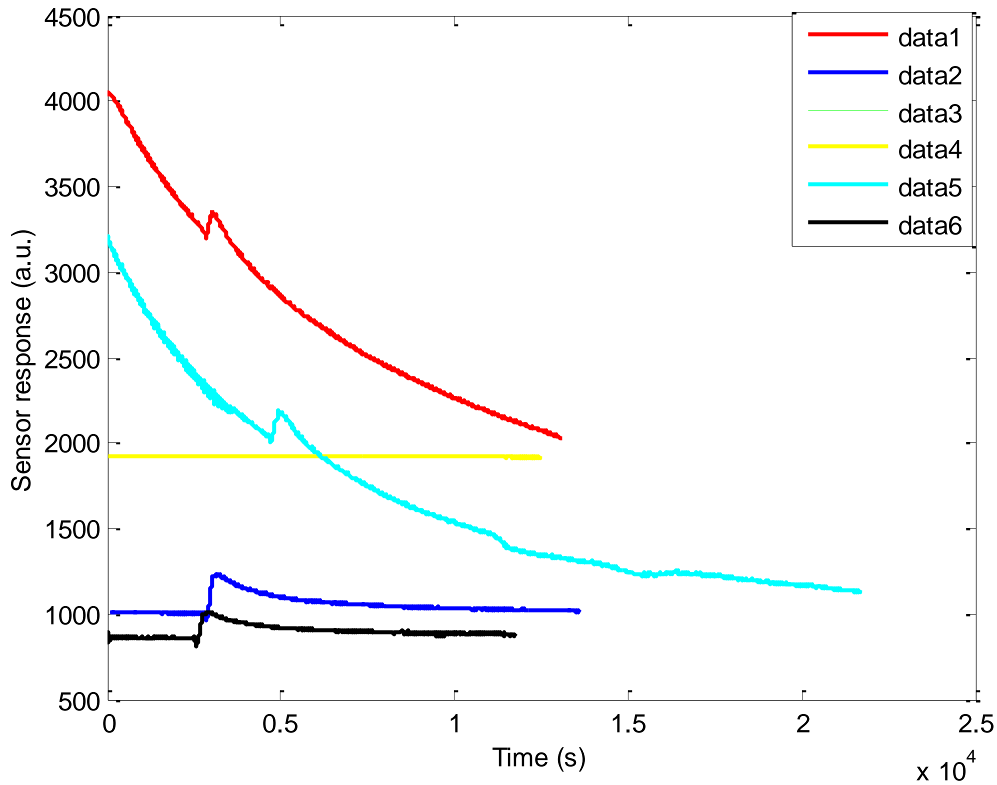


Fig 7.12 Examples of traces from Breathotron MMOS sensor from multiple sampling episodes.

Fig 7.12 above shows the features of the response curve from the Breathotron MMOS sensor. Data 2 and 6 show typical examples of the response curve with the initial baseline, peak and then return to baseline. The other traces are examples of bad samples where the sensor did not have enough time to equilibrate before sampling began.

Fig 7.13 below shows an example of how a best fit curve has to be constructed to compensate for the noisy data.

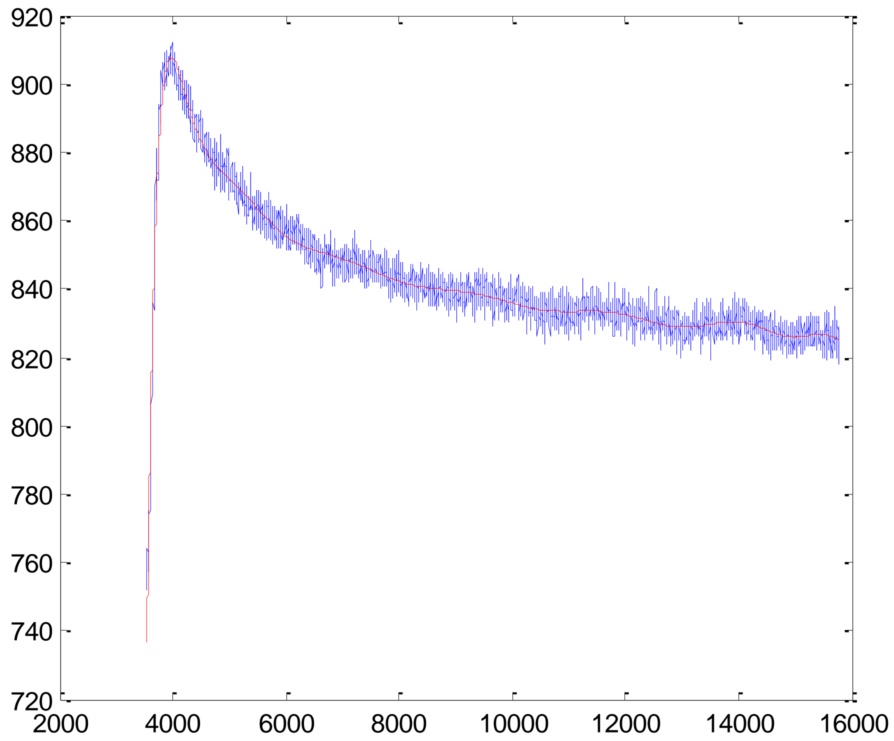


Fig 7.13 Best fit curve applied to MMOS sensor data.

7.8 Discussion

The drive towards non-invasive near patient diagnostic techniques is growing in the medical community. The Breathotron represents an attempt to combine two technologies currently in use for breath analysis. The MMOS sensor analysis represents a true rapid, near patient diagnostic tool; whilst the collection of samples for GC-MS analysis allows for quantitative analysis of the compounds involved. The use of a single sensor as opposed to a multi sensor array approach is attractive but one sensor may not have the ability to discriminate in the same way as a multi sensor array. Following the GC-MS analysis it may be possible to change the single senor once the target compounds of choice have been identified, this approach may allow the use of a single sensor to be effective.

The Breathotron mark IIa as used in this study is portable although not lightweight. It does make a loud buzzing noise whilst in use. This is not a significant problem for unconscious patients but can become wearing for the operator performing multiple back to back sampling. After initial instruction and demonstration in its use, it was on the whole easy to operate. The battery life of the PDA became a problem when the machine was not in use as the battery goes flat in about 5 days. This causes loss of the enostril software, but not data as this is stored on the SD card as opposed to the internal memory. This is a minor annoyance but is a problem with PDA's in general and not the actual Breathotron itself. The software is easy to reinstall once the PDA has been recharged.

The Breathotron is easy to connect into the ventilator breathing circuit with only a short disconnection time for the patient. This caused no more patient discomfort than undergoing routine suctioning of secretions. Towards the end of the study, the MOS sensor began to take longer to equilibrate than it had in the early stages. This may be a result of sensor drift after repeated use. It could also be a result of the high water content of breath. This did not seem to affect results as long as sampling was delayed until the sensor appeared stable on the PDA display.

Analysis of the raw data was performed according to the feature extraction techniques described by Jon Lee-Davey in 2004.[164] The initial upstroke following the plateau phase of the curve represents the point at which the Breathotron switches the flow of the breath sample from the sorbent trap for GC-MS analysis to the MMOS sensor. This point has to be manually marked on each sample curve via a Matlab 7.2 plug in. This allows data to be normalised form this point. The time at which the switching occurs is different because it is determined by the volume of breath sampled rather than a set time.

PCA analysis of this data did not show any separation when compared with the growth or no growth BAL results. This is most likely a reflection of the use of a single sensor as opposed to a sensor array. Another issue is the choice of sensor employed in the Breathotron. As already discussed this is designed for general air quality measurements and not for breath sampling. The single sensor approach may be valid in this setting if more information on the VOC's of interest can be established through GC-MS or SIFT-

MS analysis. This may allow the choice of sensor used to be "tuned" to these VOC's allowing for more accurate discrimination.

7.9 Conclusions

The Breathotron represents an ambitious attempt to create a portable, near patient device for breath analysis combining two distinct methods of analysis. Its usefulness as a collection device for breath samples onto sorbent traps to allow GC-MS analysis has been demonstrated. The use of the single sensor approach as opposed to a sensor array has not yielded any useful results in this work. Future generations of the Breathotron need to be lighter, quieter and have a modified single sensor. It may be that a mini sensor array of 4 or 5 sensors could be created which would retain the portability but improve accuracy.

Chapter 8

Conclusions

The diagnosis of VAP remains a difficult and contentious issue. We have investigated several novel techniques that could be employed in this area. Of all these; the Enose analysis of BAL fluid looks to be the most promising. The accuracy achieved in the lab based model is comparable to the microbiological techniques which are used currently in the diagnosis of VAP. The accuracy did decrease when clinical samples were introduced and the possible reasons for this have been discussed in Chapter 5. Further studies are needed with much larger sample numbers in order to gain a true picture of the role Enose technology has to play in this field. In particular, the use of "surveillance BAL sampling" in patients not on antimicrobial therapy, would yield large numbers of BAL samples with no growth which should allow us to establish more clearly what non-colonised or infected samples "smell" like.

The GC-MS analysis of breath samples did not yield any biomarkers as we had hoped. What did become clear is that the science of breath analysis is very complex with huge amounts of data generated from each individual sample. Reducing this data and comparing it with a database of potential compounds requires large amounts of skill, experience and indeed time. The wide variation of compounds found in the breath of normal healthy volunteers, let alone the critically ill patient is one of the most difficult aspects of this diagnostic approach to deal with. Breath analysis is quite a way from becoming an accepted mainstream diagnostic technique for these reasons and also due to the lack of agreement amongst those that work in this field on how to standardise the data and how to deal with the problems of establishing which compounds are exogenous.

Despite these shortcomings, breath analysis still remains a tantalising and exciting area of research with the potential to detect disease painlessly and non-invasively.

Finally, we evaluated the Breathotron with its single MMOS sensor. As discussed the single sensor approach did not yield any useful results in this study. However replacing the sensor with a different model which is more sensitive to the compounds we have found during the GC-MS analysis may help to improve the unit's performance. It may also be that a sensor array will be necessary as no single sensor has the ability to distinguish the compounds of interest with any accuracy. The Breathotron did prove an accurate and reliable method of breath collection for GC-MS analysis. This could be improved if the unit could be made smaller, lighter and quiter.

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Appendix 1

Ethical Approval

Gloucestershire Research Ethics Committee

Gloucestershire Royal Hospital

Great Western Road

Gloucester

GL1 3NN

Telephone: 01452 395726

Facsimile: 01452 395720

02 November 2005

Mr Martyn LEE Humphreys

Clinical Research Fellow

Cranfield Postgraduate Medical School

Gloucestershire Royal Hospital

Great Western Road

Gloucester

GL1 3NN

Dear Lee

Full title of study: **Diagnosis of Ventilator Associated Pneumonia: Tandem Breath Analyser versus Bronchoalveolar Lavage**

REC reference number: 05/Q2005/119

The Research Ethics Committee reviewed the above application at the meeting held on 26 October 2005. Thank you for attending to discuss the study.

Documents reviewed

The documents reviewed at the meeting were:

Document	*Version*	*Date*
Application		10 October 2005
Investigator CV		
Protocol	1	10 October 2005
Peer Review		03 October 2005
Participant Information Sheet	1	10 October 2005
Participant Consent Form	2	10 October 2005
Assent Form	2	10 October 2005
CV for Catherine Kendall		
Relative Information Sheet	1	10 October 2005
Declaration of Conformity		10 October 2001

Provisional opinion

The Committee would be content to give a favourable ethical opinion of the research, subject to receiving a complete response to the request for further information set out below.

Authority to consider your response and to confirm the Committee's final opinion has been delegated to the Chair.

Further information or clarification required

- Please produce a simplified version of Patient Information Sheet for patients who later have to give retrospective consent. Data from patients who do not give retrospective consent must be destroyed.
- Assent Form
    - Second statement change to '*I agree to samples from my relative being used in the research project as described in the information sheet*.'
    - Add a space for the name of the patient and the relationship the patient has to the person who has given assent.
- Application Form
    - A36, NHS indemnity does not cover non-negligent harm. Normal trust complaints procedures apply and the trust may make an ex-gratia payment.
    - A51, the committee require more detail on the sample size calculation.
    - A53, the committee would like to know why you are using 'Principal Component Analysis'.
- You need to investigate whether the filters on the ventilators remove the gases being studied. It is thought that only bacteria organisms are removed but clarification is needed.
- Relative Information Sheet
    - It is not clear on the information sheet for relatives that should they not give assent then their relative will not be included in the study.
    - The applicant was asked to personalise the information sheet for relatives e.g. '*Will my details be confidential'* should be changed to '*Will my relative's details be confidential?*' This is suggestive, not exhaustive. All elements need personalising.

- The tandem breath analysis should be carried out before the BAL. This will exclude any potential confounding.
- The management and security of electronic patient data is adequate. However the application lacked detail on the storage of paper documentation e.g. consent forms. It is not clear as to where, by whom and for how long these will be retained.

When submitting your response to the Committee, please send revised documentation where appropriate underlining or otherwise highlighting the changes you have made and giving revised version numbers and dates.

The Committee will confirm the final ethical opinion within a maximum of 60 days from the date of initial receipt of the application, excluding the time taken by you to respond fully to the above points. A response should be submitted by no later than 02 March 2006.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q2005/119	**Please quote this number on all correspondence**

Yours sincerely

Miss Alix Stevenson

Chair

Email: hazel.gage@glos.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments.*

Copy to: Dr Birgit Whitman, GHNHSFT

Appendix 2

Breathotron Mk IIa Operating Instructions

Operating Instructions for the Cranfield University Tandem Breath Analyser (Breathotron Mk IIa)

Version 0.0

February 2006

Outline of operation

The Breathotron is an instrument which samples expired human breath for chemical analysis. It is a prototype unit which was developed in the Department of Analytical Science and Informatics at Cranfield University. It is a stand-alone, mains-powered, portable unit, which is software controlled using the incorporated hand-held computer (personal digital assistant or PDA).

To use the instrument, the volunteer wears a facemask and continues to breathe normally (i.e. no specialised manoeuvres need to be learned). Non-return valves in the mask ensure that the volunteer inhales ambient air and exhales though a wide-bore tube containing a flow rate sensor. The sensor is used to monitor the flow rate during exhalation and this is used to control the sampling process. A small proportion of each expired breath is drawn off via a narrow-bore sampling line and passed into the instrument.

The current version of the eNostril software is designed to enable the Breathotron to sample alveolar breath. The signal from the flow rate sensor is integrated so that the cumulative exhaled volume for any given breath is known, and sampling is initiated when this exceeds the estimated volume of the upper respiratory tract and the dead space of the face mask assembly.

The sampler incorporates two distinct methods for measuring compounds contained in the breath. In the first method, breath is passed through a cartridge containing granules of special polymers and/or activated carbon which trap the compounds of interest for later analysis in the laboratory. The second method uses a semiconductor sensor (mixed metal oxide sensor, MMOS) which produces an electrical signal in response to a broad range of compounds typically found in breath. This signal is digitised and stored on the

PDA for subsequent numerical analysis. After sampling, breath is vented to the atmosphere and no gaseous samples are retained.

In order to present a defined sample to the MMOS sensor, the Breathotron incorporates a sampling loop of fixed known volume. To pass a sample across the sensor, the loop is first switched into the sampling position so that it becomes charged with air or breath drawn in via the sampling port. When fully charged the loop is switched into the flush position, in which its contents are driven across the sensor using cleaned air at a fixed flow rate. Appropriate pumping and switching arrangements are provided so that a constant flow rate (required for correct operation) is maintained across the sensor while the instrument is in operation.

Setting up

Connections

The instrument case houses the power supply, mains cable and USB lead for the PDA. The PDA itself, facemask, valve and sampling line are separate. Make the following connections:

1. Lead from the power supply to the three-pin power connector at the centre rear of the top panel.

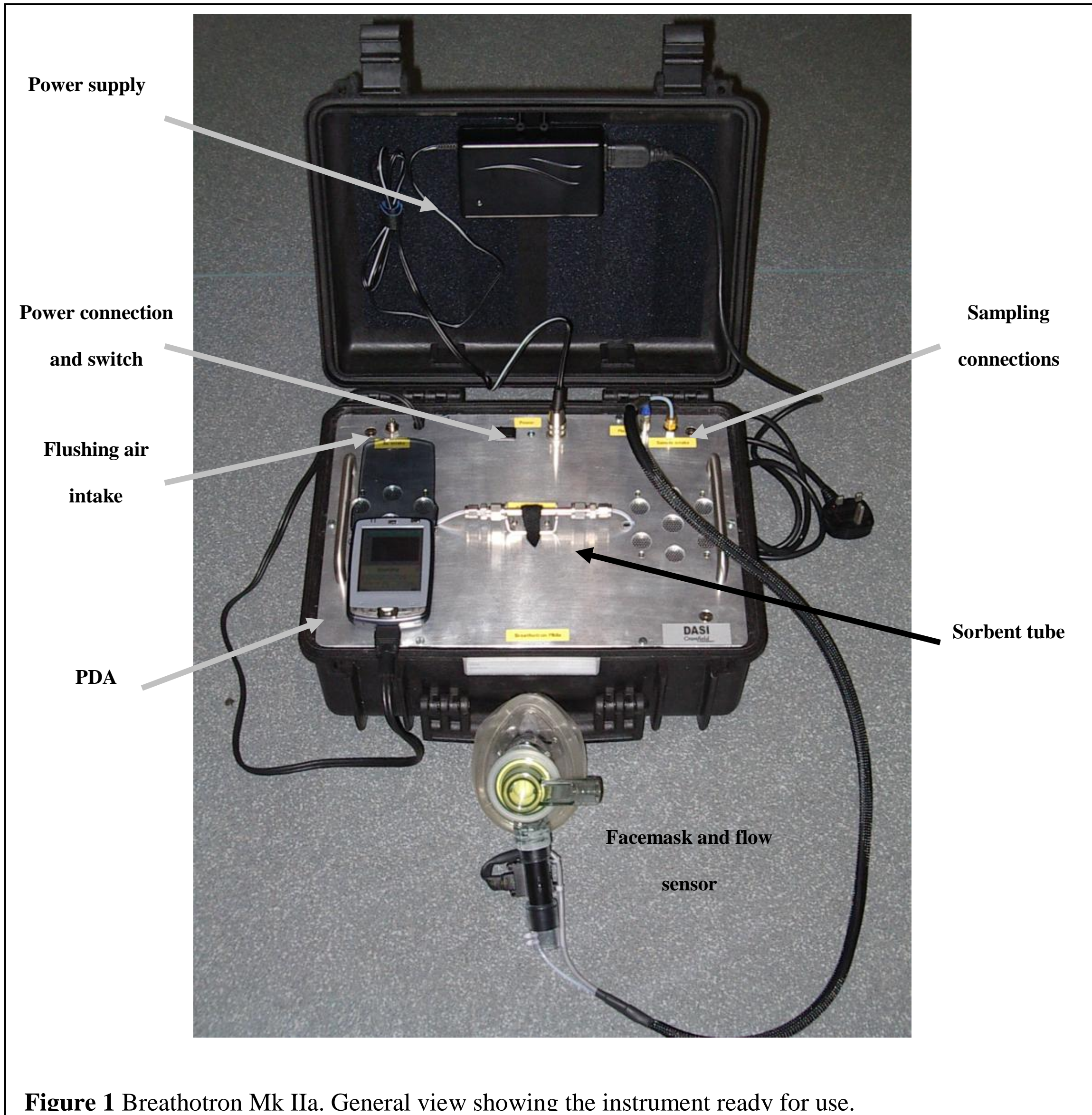

Figure 1 Breathotron Mk IIa. General view showing the instrument ready for use.

2. The sampling line to the screw thread connector at the right rear of the top panel. The nut should be done up finger tight then secured with approximately ¼ clockwise turn using an 11mm spanner, to ensure an airtight seal.
3. The lead from the flow rate sensor to the 4-pin socket adjacent to the sampling line connector.
4. The USB connector to the socket in the base of the PDA.
5. Mains connector to a convenient outlet. Note that the power supply can be detached from the lid of the case if greater reach is needed.

Figure 1 shows the Breathotron and its connections.

Startup

On switching on (the on/off switch is the only hardware control) the blue power indicator will light and then, after a five-second pause, the sampling pumps will start to run. The Breathotron uses two pumps, for the sorbent and MMOS channels respectively. MMOS sensors require continuous airflow to operate effectively; therefore this pump operates whenever the instrument is switched on. The sorbent channel pump is operated as needed for breath sampling under software control.

As soon as the instrument is running, the PDA may be switched on. It is important to check that the PDA date and time are set correctly since this information is used to timestamp the data. The eNostril programme may now be started (select **Start ► eNostril**). If the software cannot be found at this location, select **Start ► Programs**, then tap on the eNostril icon on the page of displayed applications. Check section 5.5 for information on re-installing the application.

After switching on, the Breathotron requires a minimum of 10 minutes warm-up before it can be used. Once this has elapsed, change to the Configure screen (**Menu ► Configure**) and ensure that the MMOS output signal lies between 0.5V and 1.5V. The controls on this screen are preset to optimal values and should not usually be changed by the operator. However, if necessary, the baseline can be adjusted using the Offset slider control. See below for more information on the features provided in eNostril.

Sorbent cartridge

The instrument is supplied with a dummy cartridge which should be replaced when it is not in use to avoid contamination. To remove the dummy cartridge, release the Velcro tie which holds it in place and lift it away from its mounting bracket. Sufficient play for this is allowed in the connecting tubes. Unscrew the unions at each end using a Caplok

tool and spanner and remove the nuts. It is good practice at this point to seal the dummy cartridge using either permanent or temporary end caps.

Remove the end caps from the live cartridge and fit the nuts removed from the dummy. Fitting the cartridge is the reversal of removal. Note that the engraved ring denotes the upstream end of the cartridge.

Facemask and valve assembly

A standard single-use anaesthetic mask is used in conjunction with a Laerdal non-rebreathing valve (part # 560200). The flow rate sensor is connected to the output port of the valve. These components are all push-fit so that the mask and valve may be replaced as necessary.

The mask selected should provide a snug fit around the volunteer's nose and mouth. For most applications, the mask will be held in place by a headstall. Mask adjustment should be checked by carrying out a positive pressure test. This is achieved by placing the palm of a hand over the end of the flow rate sensor and asking the volunteer to exhale slightly. The face seal should expand slightly against the volunteer's face, and the volunteer should not be conscious of any escaping air.

Collecting samples

Breath

Go to the Setup screen in eNostril **(Menu ►Setup)** and complete the information fields (free-text) as required.

To collect a sample, ensure that eNostril is set to the main screen (**Menu ►Main;** shown in Figure 2) and set the sample volume. The Volume control is a drop-down list

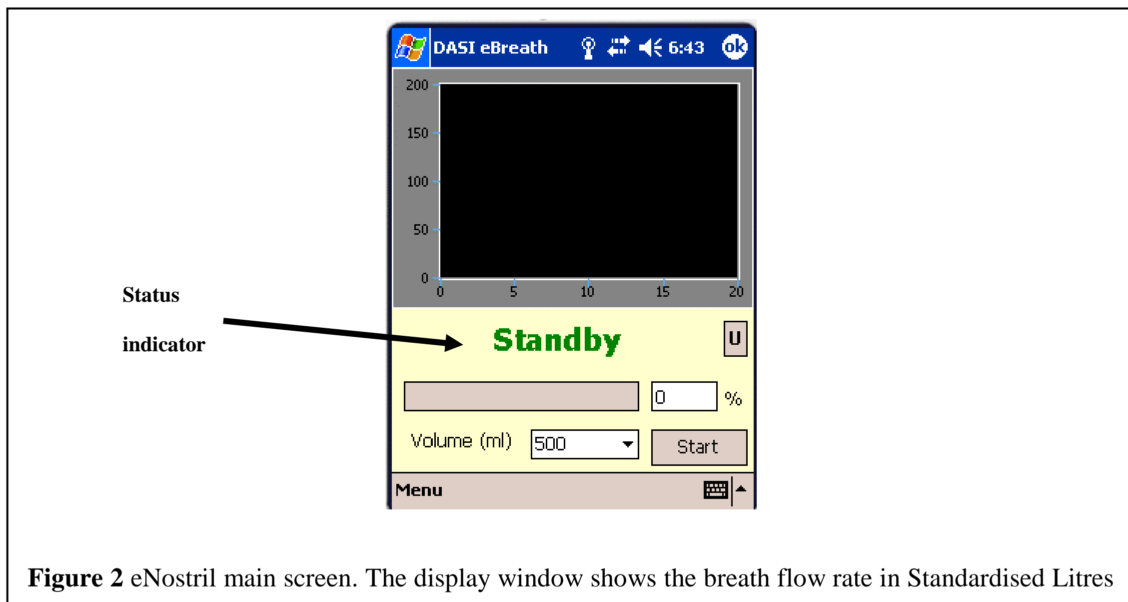

Figure 2 eNostril main screen. The display window shows the breath flow rate in Standardised Litres

which allows the total volume of breath to be collected to be set in increments of 500 ml. It is recommended that a particular sample volume is specified for any given experimental protocol and then used exclusively.

Tap on the **Start** button. The status indicator will change from **Standby** to **MMOS tests: 0** and the instrument will then automatically sample alveolar breath up to the set volume. Progress is indicated on the thermometer bar and as the percentage of the total sample collected. The expiration flow rate is displayed graphically in the window at the top of the screen; the vertical axis is calibrated in standardised litres per minute (SLPM).

Because of the long recovery time of MMOS sensors, only one breath is analysed for each 500ml of breath collected, the remainder passing through the sorbent tube. The eNostril software controls the sampling sequence such that the first sample for MMOS analysis is drawn after 100 ml has been collected, with subsequent samples being drawn

at 500 ml intervals. The number of MMOS samples is shown on the status indicator. The run can be terminated at any time by tapping on the Start button again.

Note that, since sampling is controlled on the basis of volume collected, runs do not take a specific length of time. As a rough guide, however, allow 1 minute per 100 ml sample for healthy individuals sitting quietly.

As soon as the run is complete, the volunteer may remove the mask. The sorbent tube should be removed from the instrument immediately and capped. Permanent caps are recommended. The tube should then be replaced by another live tube or the dummy; the tube connections should not be left open to the air for any length of time.

Background

Since the concentration of volatiles in breath is very low, it is essential to obtain samples of ambient air for comparison. An absolute minimum would be one sample for

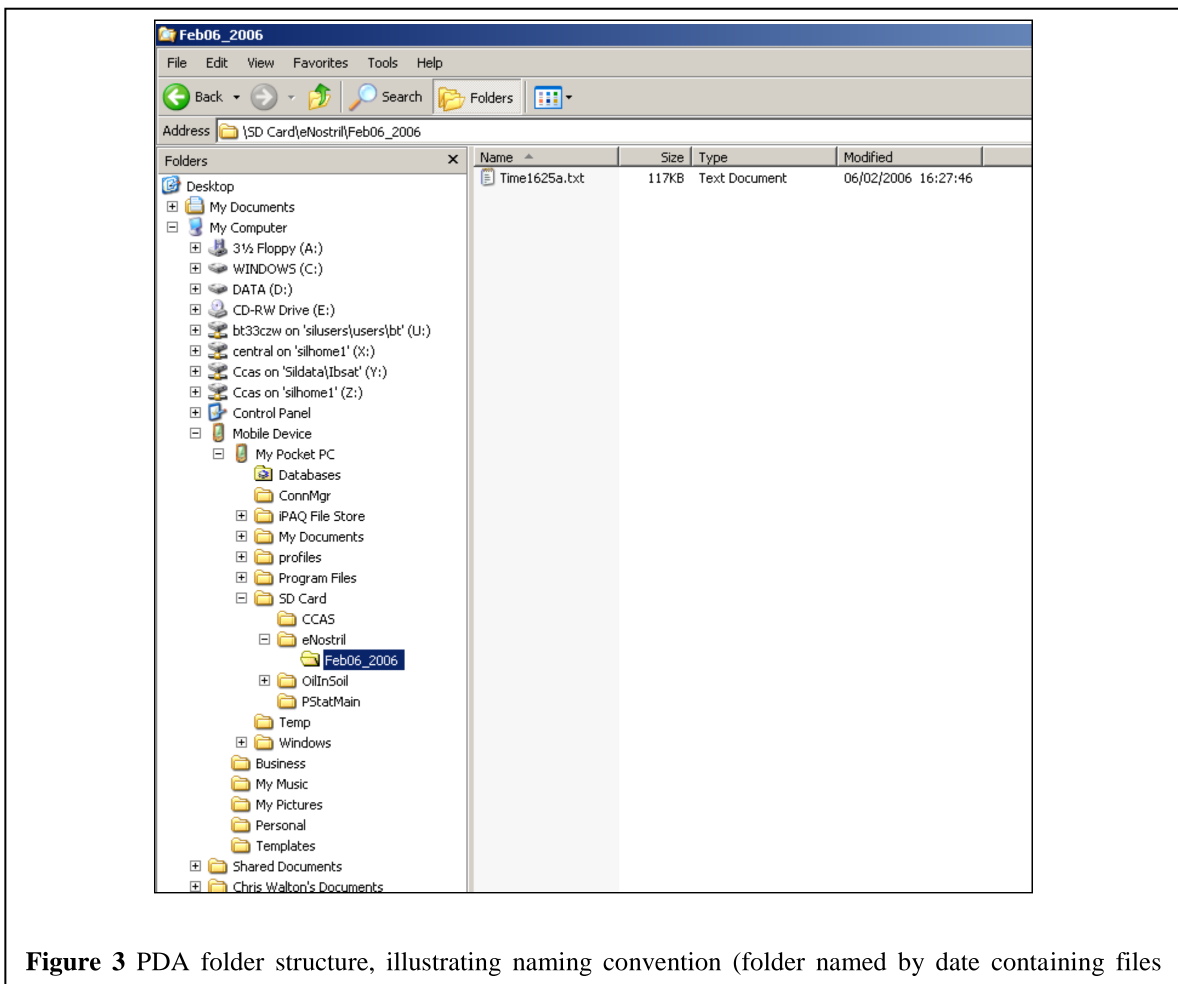

Figure 3 PDA folder structure, illustrating naming convention (folder named by date containing files

each day of operation. However, we recommend taking at least two, one at the beginning and one at the end of each day.

Fresh background samples are also required if the instrument is moved (e.g. into another room) or if there is any other marked change in ambient conditions.

Background samples are obtained by disconnecting the sampling line from the sampling port and then proceeding exactly as for breath sampling. Set the same volume for breath and background samples.

MMOS data

The Breathotron stores its MMOS data as text files on the PDA which are suitable for emailing as attachments or for import into spreadsheet programmes such as Excel. These files also hold other information including the breath flow rate, sample volume and software settings. They are held on a removable memory card if one is fitted, or in the PDA's main memory if not. In the later case, however, data will be lost if the PDA battery becomes discharged.

A sample volume of 500ml will produce a file of around 150kB. Although not large, they cannot reliably be opened by the Gatesware lurking on the PDA. Files should therefore be copied to a PC or network disk drive as soon as practicable.

Connect the PDA to a PC running ActiveSync and drag them across using Windows Explorer. Figure 3 shows the PDA folder structure. Files are named by their time of collection, and are arranged in folders which are named by date. We recommend that this structure is also used on the target drive.

Using eNostril

Graphical displays

The Main, Configure and Monitor screens all allow graphical display of data. The Main and Configure screens show breath flow rate and MMOS output respectively, whilst the Monitor screen allows either of these to be selected. The vertical axes of these displays may be zoomed by tapping on the display at the lower and upper values to be expanded. The display will then be rescaled to cover this range. Successive expansions can be obtained by repeating this process.

The display can be reset by tapping the “U” (Unzoom) button, which steps the display back through the previous zoom settings.

Replay

The data stored in the output text file may be replayed by eNostril in real time. The file to be replayed must first be copied from the secure data card to the My Documents folder using File Explorer: this requirement is a limitation of the PDA software.

1. Start ► File Explorer.
2. From the dropdown folder list Select My Device ►SD card ►eNostril.
3. Then select folder from the relevant date and then the file to be copied.
4. Press (tap & hold) on the file and select Copy from the pop-up menu.
5. Navigate to My Documents, select Edit ► Paste.
6. Close File Explorer.
7. Start eNostril and select Replay, then select file to be replayed.

Data are displayed as for actual sampling. Additionally, it is possible to switch freely between the Main, Monitor and Configure screens and between sensor and breath display on the Monitor screen. This feature is especially useful for examination of the MMOS sensor output.

Configure screen

This screen (Figure 4) includes a number of controls which allow the instrument to be

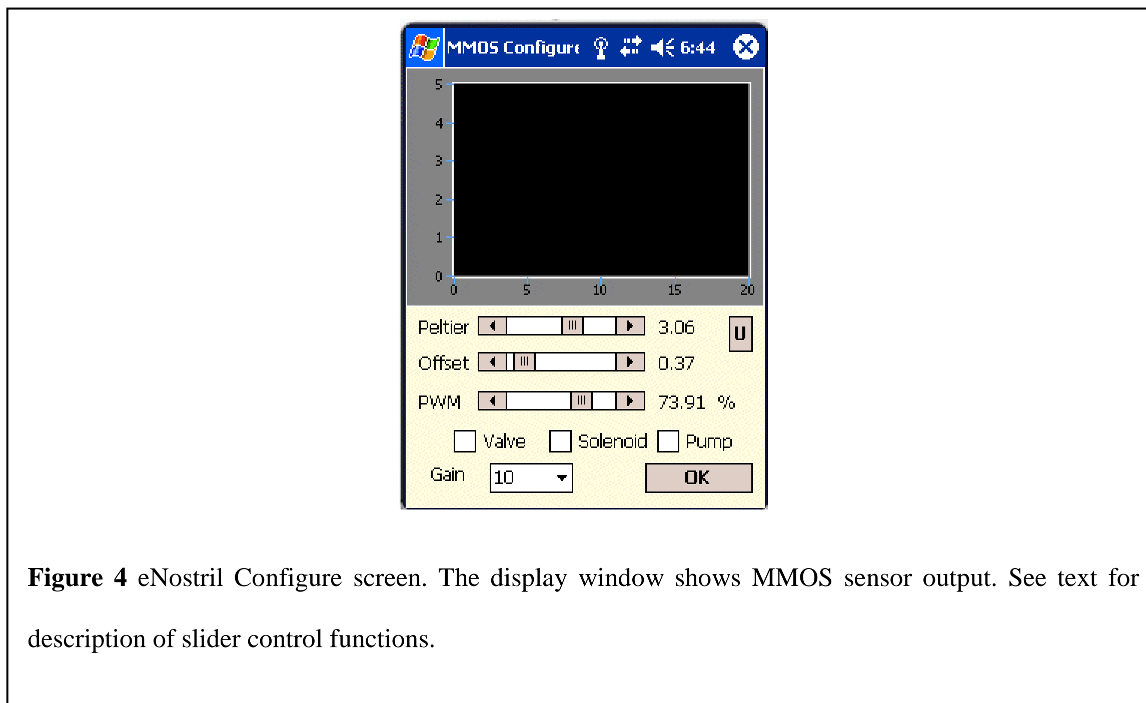

Figure 4 eNostril Configure screen. The display window shows MMOS sensor output. See text for description of slider control functions.

manually controlled.

1. Peltier. This controls the temperature of the air or breath which is passing across the MMOS sensor. The chamber in which the sensor is mounted is heated or cooled as required using a Peltier device.
2. Offset. Determines the offset of the MMOS signal amplifier. At the concentrations of volatiles found in breath, the sensor signal takes the form of a few millivolts superimposed upon a much larger baseline. Subtracting the baseline is the only practical way in which a usefully large amplifier gain may be realised.
3. PWM. This controls the temperature of the MMOS sensor itself (which typically operates around 400 °C). The value actually set on this control is the duty cycle of the Pulse-Width Modulated signal which excites the sensor heater.
4. Gain. This is a drop-down list which allows the gain of the MMOS signal amplifier to be set. The actual gain is found by multiplying the value of this control by 50.
5. Pump. Checking this box turns on the pump for sorbent tube sampling.
6. Solenoid. Checking this box opens the sampling valve, causing air or breath to be drawn in at the sampling port and passed through the sorbent tube. The state of the valve checkbox determines whether or not the sample loop becomes charged.

7. Valve. With this box unchecked, the sample loop is placed in the sampling circuit so that it becomes charged with air or breath when the sampling valve is opened. When checked, the sample loop is placed in the MMOS circuit, so that its contents are flushed across the sensor.

The Pump, Solenoid and Valve controls are also available on the Monitor screen.

Monitor screen

The Monitor screen (Figure 5) incorporates a graphical display which can be set to show either breath flow rate or the MMOS sensor signal. It also includes digital displays

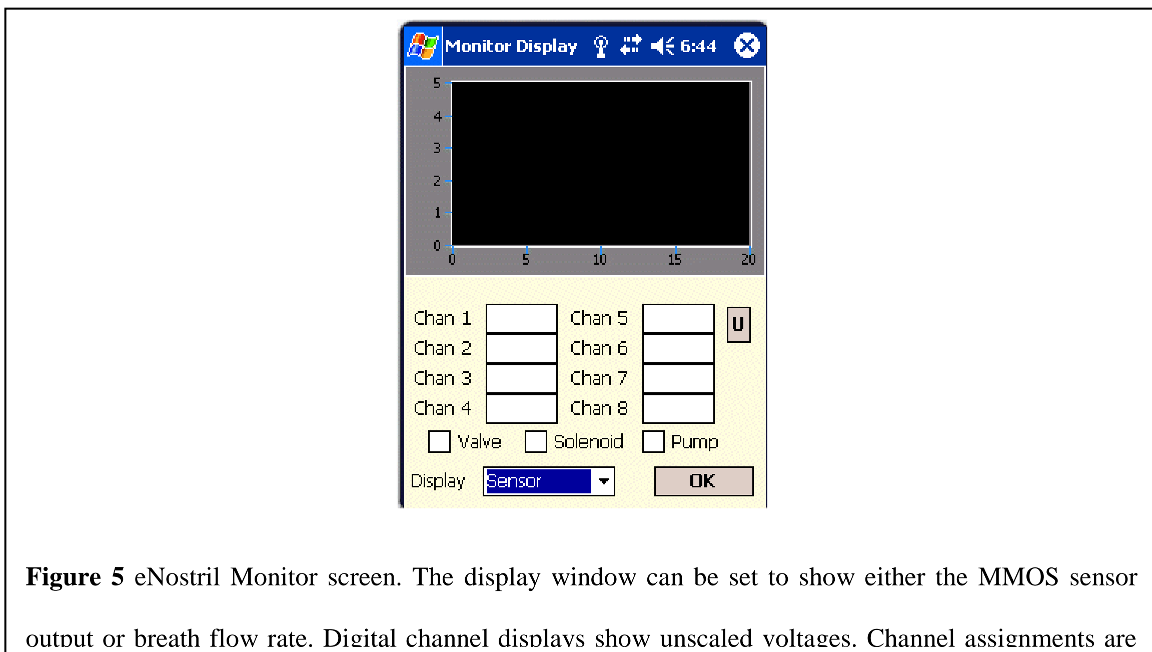

Figure 5 eNostril Monitor screen. The display window can be set to show either the MMOS sensor output or breath flow rate. Digital channel displays show unscaled voltages. Channel assignments are

of a number of parameters such as the temperature and relative humidity inside the MMOS sensor chamber. These displays are all unscaled voltage readings and are not useful in routine operation. They are listed here for completeness (see Table 1).

1. The Pump, Solenoid and Valve checkboxes may be used to take a sample manually, while monitoring the MMOS sensor signal in real time:
2. Select **Sensor** on the Display drop-down. (If necessary the sensor offset may be adjusted on the Configure screen.)
3. Ensure that the Solenoid and Valve checkboxes are unchecked.
4. Check the Pump checkbox to start the sampling pump.
5. Check the Solenoid checkbox. This causes the sample to be drawn into the sampling loop.

6. Wait at least 2 seconds for the sampling loop to fill, then uncheck the Solenoid and check the Valve checkbox. This switches the sampling loop into the flush position so that the sample is passed across the sensor.
7. Uncheck all three boxes.

Channel number		**Channel number**	
1	MMOS signal	5	Chamber temperature
2	Breath flow rate	6	Not used
3	Sampling flow rate	7	Chamber relative humidity
4	MMOS chamber flow rate (MFC)	8	MMOS chamber flow rate

Table 1 Breathotron analogue channel assignments.

Reloading the software

The iPAQ Pocket PC device stores its application programs and data in volatile memory. As long as there is some charge in the battery, memory contents are retained. However, if the device is left unattended for several weeks, the contents of memory are lost as the battery becomes fully discharged.

To overcome this problem, the software looks for a non-volatile SD Flash Memory Card in the iPAQ when the application is started. All Breathotron data is stored on the iPAQs memory card if one is detected, which retains its contents even when battery becomes fully discharged. If no SD Card is found, volatile iPAQ memory is used (the 'My Documents' folder), and data saved here should be backed up to a PC using ActiveSync as soon as possible.

When no battery power is present, the application program may also be lost, and will then need re-installing. The complete package of Breathotron application software is

distributed in one file called “BreathlMain_PPC.ARM.CAB”, which can be found on the supplied CD. This file should be transferred to a folder on the iPAQ file using ActiveSync – any folder will do. Using the iPAQ stylus and screen search for the file just downloaded and tap on it. This will cause the application to be automatically installed, then delete the original .CAB file from the local iPAQ folder. A warning about version compatibility may appear, but this can be safely ignored as the software has been configured to run of all versions of the Pocket PC operating system.