**CRANFIELD UNIVERSITY**

**NEUS PLANAS PONT**

# VENTILATOR ASSOCIATED PNEUMONIA: ANALYSES OF VOLATILE FINGERPRINTS FOR IDENTIFICATION OF CAUSATIVE MICROORGANISMS, ASSESSMENT OF ANTI-FUNGALS AND USE OF *IN VITRO* MODELS FOR EARLY CLINICAL SAMPLE PREDICTION

**CRANFIELD HEALTH**

**PhD THESIS**
**Academic Years: 2007 – 2011**

**Supervisors:** **Prof. Naresh Magan and Dr. Catherine Kendall**
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**This thesis is submitted in partial fulfilment of the requirements for the degree of Philosophy**

# ABSTRACT

This study has involved the analysis of volatile fingerprints using a hybrid electronic nose (e-nose) to discriminate between and diagnose the microorganisms which cause ventilator–associated pneumonia (VAP), one of the most important infections in the hospital environment. This infection occurs in hospitalised patients with 48-72 hrs of mechanical ventilation. VAP diagnostics still remains a problem due to the lack of a precise diagnostic tool. The current tests are mostly based on quantitative cultures of samples from the lower lung airways with clinical findings, which do not often result in accurate diagnoses of the disease.

The objectives of this study were to examine the use of a hybrid e-nose system to investigate the discrimination of five different VAP microorganisms (*Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococ*cus strain, *Candida albicans* and *Aspergillus fumigatus*) using volatile fingerprints. Firstly, the medium to use was optimised, then the level of sensitivity of detection and the ability of discrimination in mixed cultures based on volatile fingerprints were examined; also, examination of the potential for discrimination when anti-fungal compounds are applied, and the use of complex and saline-based media to build *in vitro* models to distinguish between the VAP microorganisms was done to facilitate the diagnoses of a data set of clinical samples.

Initially, three different commercial media were tested: Nutrient broth (NB), Tryptone soy broth (TSB) and Brain heart infusion broth (BHIB). The analysis of volatiles with the e-nose using two microorganisms *(C.albicans* and *K.pneumoniae*) showed better discrimination after 24 hrs incubation with NB. This medium was used for the analysis of sensitivity of detection, mixed cultures and discrimination between the five VAP causing microorganisms.

The sensitivity threshold of detection using volatile fingerprints was found to be with $10^2$ cells $ml^{-1}$ for the bacteria after 24 hrs growth; 48 hrs for *C.albicans*; and 72 hrs for *A.fumigatus*. The potential discrimination of mixtures of two microorganisms, *C.albicans* + *E.cloacae* and *Staphylococcus* species + *E.cloacae* showed that in most cases one of the species (*E.cloacae*) was predominant. This suggests that the use of

volatile fingerprints for discriminating mixtures may depend on relative concentrations and growth rates and competitiveness of individual species.

The ability to discriminate between the five VAP microorganisms was compared in NB, and in saline solution which is used to obtain patient samples. This showed that it was possible to obtain discrimination between the five species within 24 hrs with an initial concentration of $10^4$ cells/spores $ml^{-1}$. Similar results were obtained with NB and saline solution. This suggests that it is possible to use the saline solutions from patients for analyses using the e-nose approach.

Complimentary studies were carried out to analyse the head space of VAP causing microorganisms by using GC-MS and SIFT-MS. Volatile profiles were investigated from all five VAP microorganisms on NA at 37°C for 24 hrs in nalophan bags. Key component for the microbial discrimination were: 1,4-pentadiene present only in *A.fumigatus* and *E.cloacae*; 4,4-dimethyl-1-pentene, 2-methyl-3-isopropylpyrazine and methyl mercaptan in *A.fumigatus*; mercaptoacetone in *C.albicans* and 2-methyl-1-propanol in *K.pneumoniae* and *E.cloacae* cultures. The ketone, 2-pentanone was found in all the species except in the *Staphylococcus* strain. 3-methyl-butanal was not seen in *A.fumigatus* and 3-methyl-1-butanol was remarkably found in *E.cloacae* but not in blank medium and *A.fumigatus*.

The efficacy of anti-fungal agents against *C.albicans* and *A.fumigatus* was evaluated to determine the potential of using volatile fingerprints to differentiate between $ED_{50}$ and $ED_{90}$ concentrations of benomyl, tebuconazole and fluconazole. Discrimination was obtained between $ED_{50}$, $ED_{90}$ and controls after 48 hrs for *A.fumigatus* with benomyl and tebuconazole at 37°C on solid medium; and after 48hrs slight discrimination was found for *C.albicans* in NB at 25°C. For the latter, no distinction between treatments was found at 37°C in saline solution. Interestingly *C.albicans* was resistant to fluconazole in saline solution at 37°C but not in NB at 25°C; *A.fumigatus* was resistant to fluconazole. There were only a few differences between volatile biomarkers in treatments. Some compounds were found to be more abundant in the presence of anti-fungal treatments. In $ED_{50}$ concentrations of benomyl, SIFT-MS showed that *A.fumigatus* produced more isoprene and methanol whereas more methyl pentadiene and ethanol were found when tebuconazole was present. GC-MS studies showed the

presence of some alcohols (3-methyl-butanol, 2-methyl-1-propanol) in *A.fumigatus* cultures and more presence of 2-methyl-1,3-pentadiene in tebuconazole $ED_{50}$ treatments. With *C.albicans*, regardless of fungicide treatment, no significant differences were found between treatments.

The data obtained in vitro was used to develop a model to try and discriminate between patient samples. This data set was obtained in a previous study. The clinical samples were divided into 4 groups: Gram-negative bacteria, Gram-positive, fungi and no growth. Two types of multivariate analyses were investigated, discriminant analysis (DA) and partial least square for DA (PLS-DA). The accuracy of prediction of clinical samples was 63.6% for both techniques in saline solution and 52.3% with PLS-DA and 53.4% with DA in NB.

The present study has demonstrated the ability of the e-nose to discriminate between five different VAP microbial species *in vitro*. The attempt of prediction of clinical samples was not successfully achieved but shows some promise. The provision of more clinical samples would be required to evaluate whether the *in vitro* models can be successfully used for determining which drug should be used for more effective treatment of VAP.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AMDIS | Automated Mass Spectral Deconvolution and Identification System software |
| ANN | Artificial Neural Networks |
| ANOVA | Analysis of variance |
| ARDS | Acute Respiratory Distress Syndrome |
| ART | Adaptive Resonance Theory |
| ATCC | American Type Culture Collection |
| ATD | Automated Thermal Desorption |
| BAL | Bronchoalveolar Lavage |
| BHIB | Brain Heart Infusion Broth |
| BP | Back-propagation |
| CA | Cluster Analysis |
| CDC | Centers for Disease Control |
| CFU | Colony Forming Units |
| C-NS | Coagulase-negative Staphylococci |
| CPIS | Clinical Pulmonary Infection Score |
| DG18 | Dichloran-Glycerol agar |
| DS | Direct standardisation |
| $ED_{50/90}$ | Effective Dose 50%/90% |
| e-nose | Electronic nose |

| | |
|---|---|
| GAs | Genetic Algorithms |
| GC | Gas Chromatography |
| HAP | Hospital-acquired Pneumonia |
| HPLC | High Performance Liquid Chromatography |
| ICU | Intensive Care Unit |
| IPA | Pronan-2-ol or Isopropanol |
| LDA | Linear Discriminant Analysis |
| LOO | Leave-one-out |
| LV | Latent variable |
| LVQ | Learning Vector Quantisation |
| MC | Mac Conkey |
| MDR | Multi-drug Resistant |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MS | Mass Spectrometry |
| MSSA | Methicillin-sensitive *Staphylococcus aureus* |
| MOS | Metal Oxide Semiconductor |
| MOSFET | Metal Oxide Semiconductor Field Effect Transistor |
| MSD | Mass Selective Detector |
| MVOC | Microbial Volatile Organic Compound |
| NA | Nutrient Agar |
| NB | Nutrient Broth |

| | |
|---|---|
| NIST | National Institute of Standards and Technology |
| NST | Nordic Sensor Technologies |
| OD | Optical Density |
| ORSA | Oxacillin-resistant *Staphylococcus aureus* |
| OSSA | Oxacillin-sensitive *Staphylococcus aureus* |
| PARC | Pattern Recognition |
| PCA | Principal Component Analysis |
| PCR | Principal Component Regression |
| PDS | Piecewise Direct Standardisation |
| PLS | Partial Least Square |
| PLS-R | Partial Least Square Regression |
| ppm | Parts per million |
| ppb | Parts per billion |
| QA | Quality Assurance |
| QC | Quality control |
| QCM | Quartz Crystal Microbalance |
| RT | Retention Time |
| SAW | Surface Acoustic Wave |
| SBHIA | Sabouraud Brain Heart Infusion Agar |
| SDA | Sabouraud Dextrose Agar |
| SIFT-MS | Selected Ion Flow Tube-Mass Spectrometry |

| | |
|---|---|
| SOM | Self-organising Map (Kohonen) |
| SPME | Solid-phase Microextraction |
| TB | Tuberculosis |
| TBA | Tracheobronchoalveolar aspirate |
| TD-GC/MS | Thermal Desorption GC-MS |
| TIC | Total Ion Current |
| TSB | Tryptone Soy Broth |
| UTI | Urinary Tract Infection |
| VAP | Ventilator Associated Pneumonia |
| VOC | Volatile Organic Compound |
| YES | Yeast Extract Sucrose (agar medium) |

# LIST OF EQUATIONS

# CHAPTER 1: LITERATURE REVIEW

## 1.1 INTRODUCTION

Ventilator associated pneumonia (VAP) is one of the significant infections in hospitals and has become of great medical importance because it is associated with antibiotic-resistant pathogens and might result in high rates of morbidity, mortality and increased medical care costs. The diagnosis of VAP is still difficult since there is no accepted clinical criterion to definitely establish VAP and the specificity and sensitivity of all existing diagnostics are of very poor accuracy. Clinical signs of VAP are not specific and the scoring systems are not absolutely reliable for VAP diagnosis.

Microorganisms are known to produce a wide range of different organic compounds which are released, with some of them being volatile. These microbial volatile organic compounds (MVOCs) may be used as fingerprints of microbial growth and perhaps to discriminate between different species. Sensor arrays have thus been developed trying to mimic the olfactory sense of mammals. Their ability to detect volatile fingerprints combined with pattern recognition systems would permit the early detection of specific microorganisms or groups of microorganisms.

These sensor array systems, so called electronic nose (e-nose), have been examined for potential applications in many fields such as food quality and spoilage, cosmetics and beverages industries, environmental monitoring and more recently in medical diagnostics (Gardner *et al*., 2000a). The use of e-nose systems in medicine for the early diagnosis of non-infectious diseases such as breast and lung cancers (D'Amico *et al*., 2010; Peng *et al*., 2010), kidney disorders and infectious diseases has been recognised (Pavlou *et al*., 2002a; Boilot *et al*., 2002; Pavlou *et al*., 2004; Humphreys *et al*., 2011).

Analytical approaches involving more complex devices such as gas chromatography associated with mass spectrometry (GC-MS) and selected ion flow tube mass spectrometry (SIFT-MS) have also been employed to detect volatile biomarkers in head space samples for similar applications. However, such approaches are more expensive and require a high level of expertise for analyses and interpretation of data sets.

## 1.2 VENTILATOR-ASSOCIATED PNEUMONIA

VAP is the acquired pneumonia that occurs in patients who require mechanical ventilator support by endotracheal tube or tracheostomy for ≥ 48hours (Hunter, 2006). Therefore, VAP is considered a nosocomial disease because it is acquired in the health care environment with no evidence that it is present or incubated at the time of hospital admission.

VAP can be caused by a range of individual or mixtures of microorganisms. Gram-negative enteric rods (*Enterobacteriaceae* such as *Klebsiella pneumoniae*, *Enterobacter* species, *Escherichia coli*), *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the predominant organisms responsible for this infection. However, many other microorganisms can cause VAP including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Acinetobacter baumanii* depending on the onset of the illness (Bouza *et al*., 2001). There are other opportunistic microorganisms such as *Candida albicans* and *Aspergillus fumigatus* which can play a role in immune-compromised patients. Polymicrobial infections appear to be especially high in patients with acute respiratory distress syndrome (ARDS) (Am. Thoracic Society, 2005).

Five microorganisms have been chosen from the available clinical isolations in this current study as it was found in a previous study (Sahgal, 2008) that a four group system gave sufficient discrimination between microorganisms. One or two from each main group of microorganisms were chosen: one Gram-positive (*S.aureus*), two Gram-negative (*Enterobacter cloacae* and *K. pneumoniae*) and two fungi (*A.fumigatus* and *C.albicans*).

A list of microorganisms isolated in different VAP studies is shown in Table 1.1. Please note that in some cases, the sum of percentages is above 100% due to more than one microorganism being present in the same patient sample.

**Table 1.1**: Aetiology of VAP in % obtained from diverse studies. (Key: MRSA: Methicillin-resistant *Staphylococcus aureus*; MSSA: Methicillin-sensitive *S.aureus*; ORSA: Oxacillin-resistant *S.aureus*; OSSA: Oxacillin-sensitive *S.aureus*; NS: Not stated).

| (a) No specification about initial treatment | *Streptococcus* spp | *S.aureus* | *P.aeruginosa* | *Acinetobacter* spp | *Enterobacteriaceae* | Anaerobic microbes | *Haemophilus* spp | *S.maltophilia* | Fungi | Others |
|---|---|---|---|---|---|---|---|---|---|---|
| Rello *et al.* (2005) (1) | 11.6 | 15.9* | 33.9 | 12.6 | 14.6 | NS | 8.6 | 2.6 | NS | 7.8 |
| Fagon *et al.* (2005) (2) | 12.1 | 20.4** | 24.4 | 7.9 | 14.1 | 0.9 | 9.8 | 1.7 | 0.9 | 7.8 |
| Papazian *et al.* (1996) (3) | NS | 21 | 27 | 5 | 36 | NS | 6 | NS | NS | 3 |
| Timsit *et al.* (1996) (4) | 14.5 | 26.1 | 15.9 | 11.6 | 10.1 | NS | 13 | NS | NS | 8.7 |
| Ibrahim *et al.* (2000) (5) | 0.7 | 19.7* | 23.7 | 3.3 | 16.3 | NS | 1.7 | 7 | 3.3 | 24.3§ |
| Trouillet *et al.* (1998) (6) | 14.7 | 21.3* | 15.9 | 9 | 17.9 | 2.4 | 6.1 | 2.4 | NS | 10.1 |
| Hunter,(2006) (7) | 2 | 35 | 31 | 4 | 11 | NS | 5 | NS | 7 | 10 |
| (b) With initial treatment | *Streptococcus* spp | *S.aureus* | *P.aeruginosa* | *Acinetobacter* spp | *Enterobacteriaceae* | Anaerobic microbes | *Haemophilus* spp | *S.maltophilia* | Fungi | Others |
| Kollef *et al.* (1998) (8) | NS | 23.1*** | 36.5 | 5.8 | 15.4 | NS | NS | 9.6 | 3.8 | 5.8 |
| Álvarez-Lerma *et al.* (1996 ) (9) | 2 | 20.1 | 43 | 18.8 | 15.4 | NS | 0.7 | NS | NS | NS |
| Luna *et al.* (1997) (10) | 3.1 | 23.5 | 9.2 | 28.6 | 24.5 | NS | 1 | NS | 4.1 | 6.1 |

* ORSA/OSSA; ** MRSA/MSSA; *** MRSA

1. Microorganisms isolated from 301 VAP episodes.
2. Microorganisms isolated by bronchoscopic techniques in 1689 episodes.
3. Microorganisms isolated from cultures of PSB $\geq 10^3$ CFU/ml.
4. Microorganisms isolated from $\geq 10^3$ CFU/ml for PSB and/or $\geq 10^4$ CFU/ml for BAL in 56 episodes.
5. Microorganisms isolated in 185 episodes of late-onset of VAP.
   § Others: 17% (multiple pathogens), 5% (other bacteria and viruses), and 2.3% (no growth).
6. Microorganisms isolated from 135 episodes of VAP.
7. Microorganisms responsible for VAP in 420 episodes.
8. Microorganisms isolated from mini-BAL cultures in 60 episodes.
9. Microorganisms isolated in 565 episodes receiving inappropriate antibiotic.
10. Microorganisms isolated in BAL samples from 65 episodes of VAP patients.

Identification of the microorganisms which may have the most adverse clinical impact on a patient, depends on the individual patient, the type of intensive care unit (ICU), duration of stay and on any previous antibiotic treatment (Chastre and Fagon, 2002). Several studies suggest that the time of onset determines which pathogens will infect and the prognosis of the disease (Kollef, 2005; Chastre and Fagon, 2002). For the latter authors *H.influenzae*, *S.pneumoniae*, methicillin sensitive *S.aureus* (MSSA) and some *Enterobacteriaceae* were common in early-onset VAP, while *P.aeruginosa*, *Acinetobacter* spp and methicillin resistant *S.aureus* (MRSA) were typically more common in late-onset infections. The latter species belong to the group of microorganisms which potentially can be multi-drug resistant (MDR). These pathogens have a high and very variable range of mortality, between 0-50%, due to their specific risk factors, patterns of clinical resolution and resistance (Rello *et al*., 2005).

The incidence of VAP is estimated at between 8-28% (Chastre and Fagon, 2002; Kollef, 2005) and in all cases there has been a close relationship between VAP and the length of mechanical ventilation. Mortality rates due to VAP may reach values of up to 50%, especially in elderly populations, in severely ill patients, and those who may have received inappropriate previous drug therapy. However, some studies have not found any relationship between the high rates of mortality and VAP, but are mostly due to the underlying diseases (Am. Thoracic Society, 2005).

Pneumonia is the inflammation of the lower parts of the lung tissue due to the invasion by pathogenic microorganisms. Its severity and outcome depends on the competence of the hosts' immune system, on the virulence of the microorganisms, on the number of microorganisms present, and on the previous antibiotic therapy. There are a variety of mechanisms in healthy people to avoid lung infections such as the cough reflex, agents in saliva, mucus discharges and humoral and cellular immune responses. In hospitalised and mechanically ventilated patients these reactions are deeply compromised due to the illness itself and by the presence of the endotracheal tube in the upper airways. If the hosts' immune system and the initial therapy fail, VAP can prolong the patients stay and influence the relative outcome of the disease.

The way in which pathogenic bacteria reach the lung parenchyma (alveolar tissue in *sensu stricto*) in patients mechanically ventilated might be explained as follows:

bacteria residing in the oropharynx can settle between the endotracheal tube and the larynx walls creating a contaminated secretion pool that may be aspirated into the trachea and disseminated into the distal airways of lungs (Morehead and Pinto, 2000). Figure 1.1 shows this mechanism. VAP pathogens may also enter via other routes including the blood stream, by inhalation of infected aerosols, direct inoculation from a contaminated endotracheal tube or aspiration of gastric contents, although this is less common than oropharyngeal aspiration.

The diagnosis of VAP is difficult because there is no specific and single clinical criterion. A large number of diseases, even non-infectious, may present similar clinical signs such as ARDS, nosocomial tracheobronchitis, congestive heart failure, atelactasis, pulmonary thromboembolism and pulmonary haemorrhage. Likewise, the lack of an accurate diagnostic test may result in a non-specific and sometimes unsuccessful treatment, which exposes the patients to high levels of toxicity, longer stays in hospital, and elevates the costs and risks of having resistant microorganisms (Solomkin, 2005).

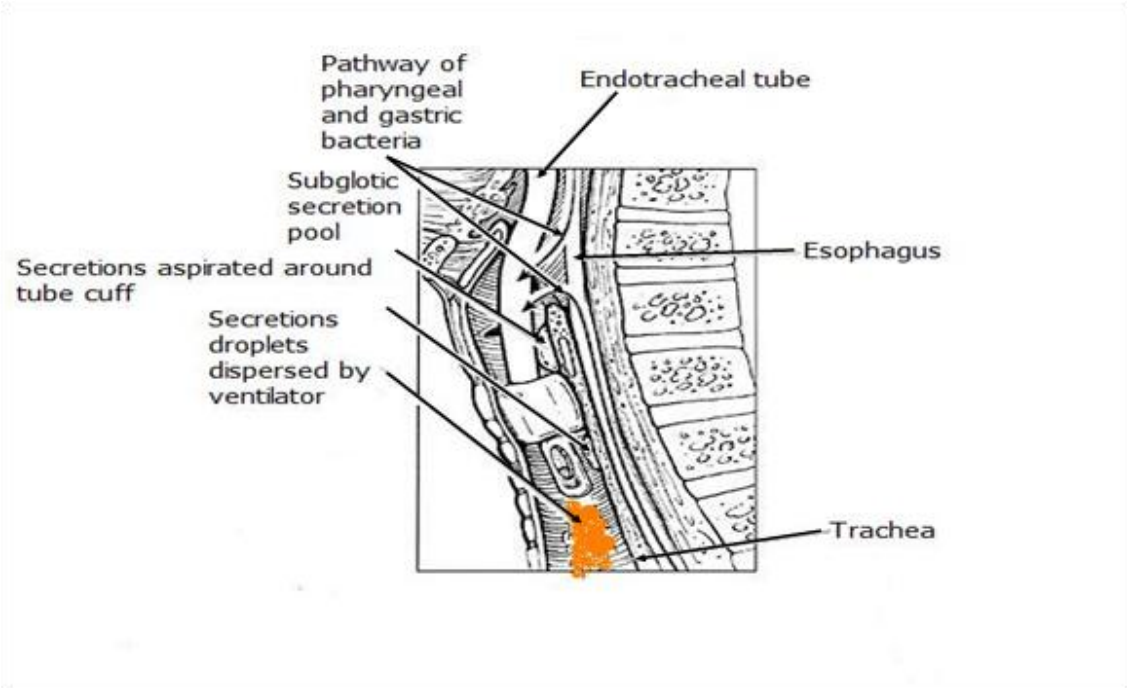



**Figure 1.1:** Potential pathways of contaminated secretions to the distal airways from oropharyngeal and gastric bacteria. Scheme modified from Morehead and Pinto (2000).

There have been various clinical strategies for VAP diagnosis such as CPIS (Clinical pulmonary infection score) developed in 1991 and modified by Pugin *et al*. (1991); Johanson criterion (Johanson *et al*., 1972) and Centers for Disease Control (CDC; Garner *et al*., 1988) usually based on clinical criteria and results of laboratory and other diagnostic tests. Table 1.2 summarises the criteria of those three systems. CPIS is the most widely used criterion based on 6 parameters: fever, tracheal aspirates, blood leukocytosis, oxygenation, radiographic infiltrates and semi-quantitative cultures of tracheal aspirates (Pugin, 2002). If a score of <6 an alternative diagnosis must be considered. However, some authors (*e.g*. Baughman, 2005) consider CPIS of little usefulness for daily evaluation due to the need of a waiting period for culture results, about 72 hrs until the identification of the pathogen and its antibiotic susceptibility testing, and for its low sensitivity and specificity. Also, CPIS has not been validated in immunosuppressed patients.

Many studies have investigated the accuracy of the clinical diagnosis of VAP by comparing with quantitative culture isolation of either protected specimen brush (PSB) or bronchoalveolar lavage (BAL) samples. These approaches may vary depending on whether one, or more than one, clinical finding is chosen for the diagnosis. Also, depending on how many clinical features are selected the values of specificity and sensitivity may vary in different ways. For instance, one of the reported studies (Am. Thoracic Society, 2005) found that using the presence of chest infiltrates with all of the three parameters (clinical signs such as fever, leucocytosis or purulent tracheal secretions) increases the specificity but reduces the sensitivity. Figure 1.2 compares sensitivity and specificity of some methods used for VAP diagnosis.

**Table 1.2:** Summary of features for three different criteria to diagnose VAP.

| **Johanson *et al*. (1972)** | **CPIS (modified by Pugin *et al*., 1991)** | **CDC[$] (Garner *et al*., 1988)** |
|---|---|---|
| * Chest radiograph infiltrates and one of the following parameters:<br><br>a) Leucocytosis (›12x$10^9$ $ml^{-1}$);<br><br>b) Temperature (›38.3°C);<br><br>c) Presence of tracheobronchial secretions. | * Suspicious of VAP when clinical score is ≥6:<br><br>a) Temperature (°C);<br><br>b) White blood cell count and number of band forms;<br><br>c) Type of tracheobronchial secretions (purulent or not);<br><br>d) Gram stain and semi-quantitative culture of tracheal aspirates;<br><br>e) Oxygenation: $PaO_2/FiO_2$, mmHg);<br><br>f) Chest radiograph (If infiltrate, diffused or localised and its progression) | * Nosocomial pneumonia when:<br><br>a) Chest percussion: if dullness and any of:<br><br>1. New purulent sputum or change character,<br><br>2. Isolation of pathogen from blood culture or lower respiratory specimen<br><br>b) Chest x-ray: if new or progressive infiltrate, consolidation, cavitation or pleural effusion and any of 1), 2) or<br><br>3) Detection of IgM or increase of IgG for pathogen |

[$]CDC developed a set of definitions for nosocomial infections which includes specific criteria for neonates and infants.

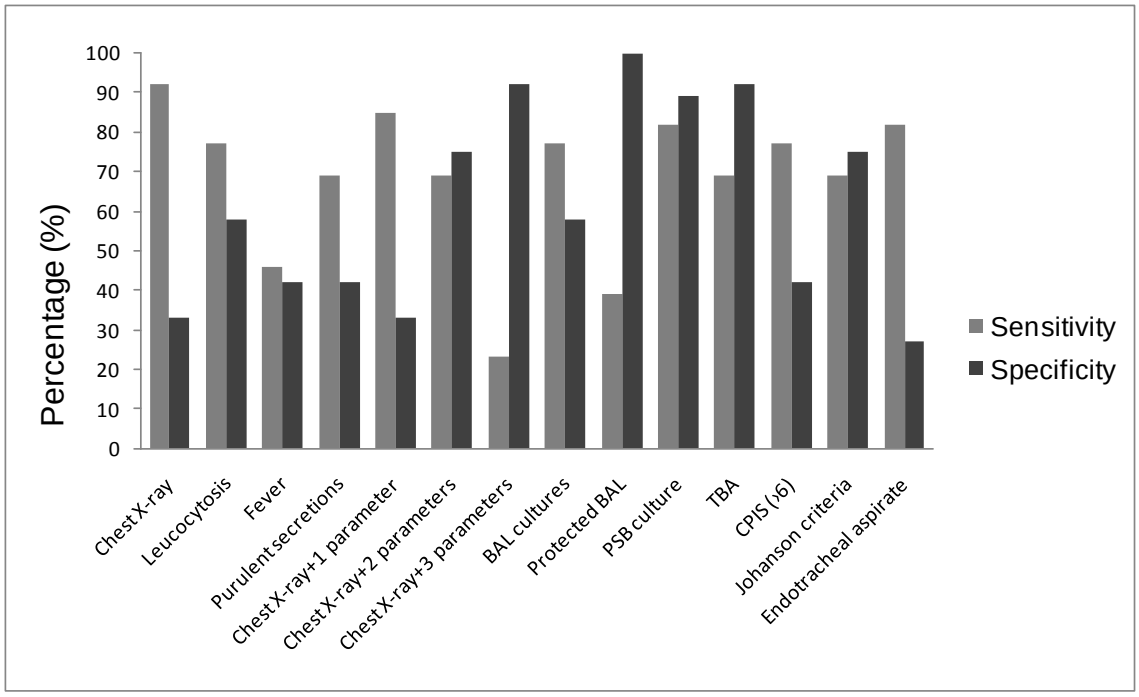



**Figure 1.2:** Relative sensitivity and specificity values for different criteria to diagnose VAP. Data obtained from Fábregas (1999) and Koenig (2006). (Key: BAL: Bronchoalveolar lavage; PSB: Protected specimen brush; TBA: Tracheobronchoalveolar aspirate; CPIS: Clinical pulmonary infection score).

Regarding VAP treatment, the decisive points to be considered once the therapy for VAP is chosen are the antibiotic selection and its duration. The empiric antibiotic therapy has to be based on knowledge of the most common microorganisms and its local resistance pattern within the ICU (Koenig, 2006), on the duration of mechanical ventilation and on the previous treatment if there was one. The duration of the therapy has been lately reduced due to the observations that prolonged therapies lead to colonization with antibiotic resistant bacteria such as *P.aeruginosa*, *S.aureus* or *Enterobacteriaceae*. This can result in a relapse of VAP, mainly in the second week of treatment (Dennesen *et al*., 2001). An early effective diagnosis and therapy are crucial for good outcomes with less antibiotic use and therefore for reducing mortality.

## 1.3 VOLATILE FINGERPRINTS-I: ELECTRONIC NOSES

### 1.3.1 WHAT IS AN ELECTRONIC NOSE?

The human nose is used as a qualitative analytical tool for discrimination/detection of odours from food (fish, meat, cheese, etc), perfumes (cosmetics, soaps) and flavours. Human personnel can be highly qualified and trained to detect odours, but there may be problems because of a lack of sensitivity over time and the variation between individuals. It can also sometimes be unsafe or unhealthy and variable depending on the individual (Nagle *et al*., 1998). To avoid these difficulties, attempts have been made to build an artificial olfactory model which can mimic the olfactory sense of mammals and which can detect and discriminate the production of volatile compounds from its sources. It was in the 1960s when the development of instruments to detect odours started using mechanical noses based on redox reactions. It was not until the 1980s when integrated e-nose systems began appearing as research tools and commercial devices. The currently accepted definition of an e-nose, regarding the chemical sensor arrays used to detect odorant molecules, was proposed by Gardner and Bartlett in 1994:

"An electronic nose is an instrument which comprises an array of electronic chemical sensors with partial sensitivity and an appropriate pattern recognition system, capable of recognising simple or complex odours".

The odorant molecules which interact with the surface of the sensors in an e-nose (equivalent to the stimuli in the olfactory system) are typically hydrophobic and volatile due to their molecular weight (30-300 Da), strength of the interactions between molecules and molecular shape (Gardner and Bartlett, 1994, 1999).

Many of the odorant molecules are produced by microorganisms. A wide range of MVOCs are released from both primary and secondary metabolites and obtained from the breakdown of protein and carbohydrates during the metabolic pathways. These compounds can be alcohols, ketones, esters, lactones, aldehydes, sulphur and nitrogen-based products. These MVOCs can be characteristic and different for each species (Larsen, 1997; Magan and Evans, 2000). Table 1.3 summarises some MVOCs produced by different species of microorganisms. They also can vary depending on temperature,

the age of cultures, the nutritional status (Fiedler *et al*, 2001) and the different water availability of the substrates (Magan and Evans, 2000).

**Table 1.3:** Some microorganisms and the volatile organic compounds which they are known to produce reported by different research studies using GC-MS and headspace concentration techniques in different substrates. (Key: DG18: Dichloran-Glycerol agar; MEA: Malt Extract agar; TSB: Tryptone Soy broth).

| Organism | Volatile organic compound | Authors<br>Substrate |
|---|---|---|
| *Campylobacter jejuni*<br>*Clostridium difficile* | Ethanoic, butanoic, pentanoic acid, benzaldehyde, ethanal, carbon disulphide, dimethyl disulphide, acetone, 2-butanone, 2,3-butanedione, 6-methyl-5-hepten-2-one, indole, 4-methylphenol. | Garner *et al*., 2007<br>Faeces |
| *Stachybotrys chartarum*<br>*Aspergillus penicillioides*,<br>*Chaetomium globosum* | 2-pentanol, 2-heptanone, 3-octanone, 1-octen-3-ol, 2-methyl-1-butanol. | Wady *et al*., 2003<br>MEA/DG18 |
| *Trichoderma harzianum* | Isoprene, 1,3,5-heptatriene, 2,6-dimethyl-2,4,6-octatriene, sesquiterpenes. | Fiedler *et al*., 2001<br>Beech wood |
| *Aspergillus fumigatus* | Heptane, undecane, isoprene, 2,4-hexadiene, 1,3-octadiene, 2-methyl-1,3-pentadiene, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-ethyl-1-hexanol, 1-octen-3-ol. | Fiedler *et al*., 2001<br>MEA |
| *Proteus mirabilis*<br>*Klebsiella pneumoniae*<br>*Pseudomonas aeruginosa*<br>*Staphylococcus aureus* | Dimethyl disulphide, isobutanol, isopentanol, methyl ketones, 2-heptanone, methylbutanal, 2-butanol. | Zechman, *et al*., 1986<br>TSB |

Typically, an e-nose consists of three parts: a sensor array, where the sensors measure electrical changes generated by adsorption of volatiles to the odour-sensitive chemical surface; a transducer which converts signals into readable values and the software for the analysis of the data. This structure has been copied from the olfactory organ of mammals in the sense that the active material of the sensors in an e-nose device (olfactory receptors) interacts with the volatiles creating a sensor response (electric signal or pattern) which is transmitted to the pre-processor (olfactory bulb) where the output is amplified and the noise is reduced. In the final stage the simplified signals (nerve impulses), as patterns of responses, are processed into the data-analysis system (hypothalamus and olfactory cortex in the brain). Figure 1.3 shows the diagrammatic analogy between the human olfactory sensing and e-nose system.

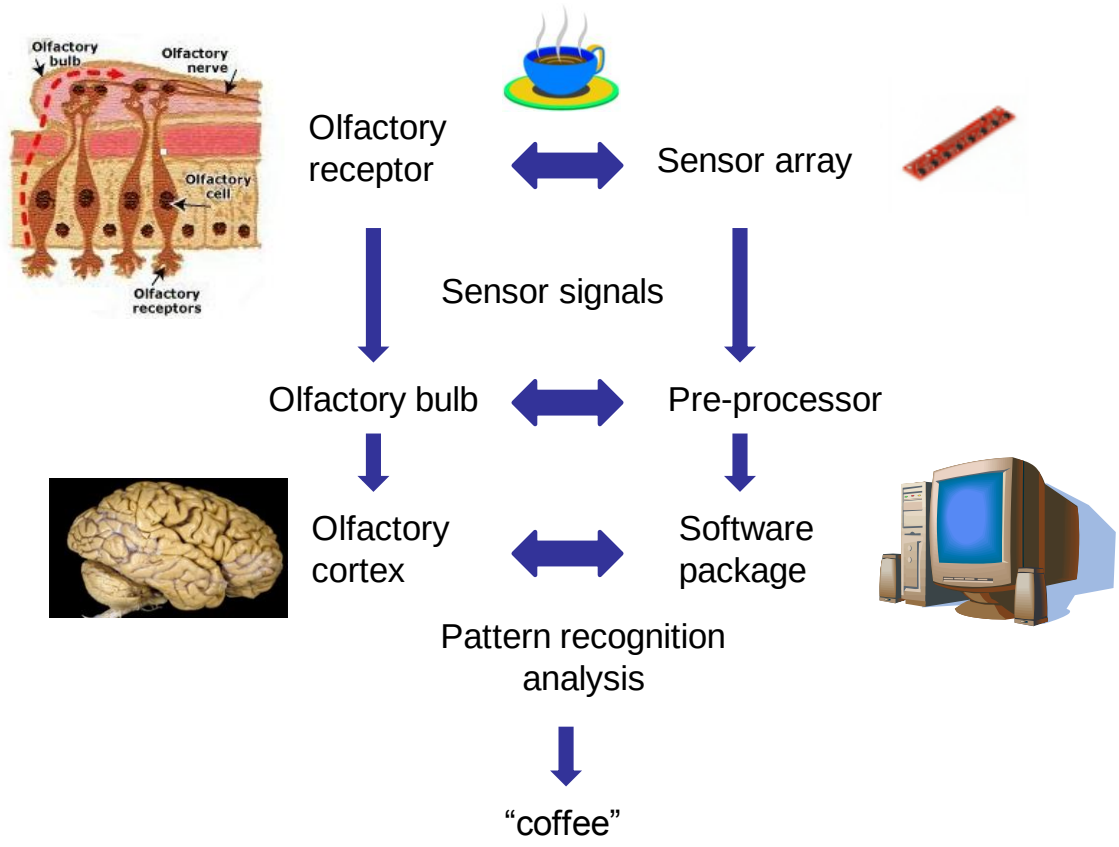



**Figure 1.3:** Scheme of the olfactory human sense and its analogy with the e-nose. Adapted from Pearce *et al*. (2003).

### 1.3.2 TYPES OF ODOUR SENSORS

Since the first model of an e-nose reported by Persaud and Dodd (1982), many advances have been made in the development of sensor technology. Some of the most important aspects of sensors are that they must be sensitive enough to the chemicals even at low concentrations in the ppm range and each sensor in the array must have partial sensitivity. Thus, response to a range of different volatiles rather than to a specific one is an advantage. This variable sensitivity leads to the production of distinct qualitative volatile fingerprints, enabling the identification of different patterns.

The e-nose sensor technologies can be classified according to the sensor material and its transduction or conversion principle. Table 1.4 provides a summary of the main sensor transducer systems and their characteristics.

**(i) ELECTROCHEMICAL SENSORS**

a) **Conductivity sensors:** these exhibit a change in resistance when they are exposed to volatile organic compounds. There are two types of conductivity sensors:

   1. **Conducting polymer sensors (CP sensors)**: This kind of sensors have reversible physiochemical properties in terms of change in conductivity under ambient temperature conditions when the active material (such as polypyrroles, triophenes) is exposed to the chemicals, which bond with the polymer backbone. They are extremely sensitive and the standardisation of sample presentation is crucial for avoiding problems with humidity and drift over time.

   2. **Metal oxide semiconductor sensors (MOS sensors)**: In this case, the doped materials with which the volatile organic compound (VOC) interacts are oxides that can be made of tin, zinc, tungsten. Those materials are deposited between two metal contacts over a heating element which operates at high temperatures, 200-650˚C. When the sample interacts with the oxide material, oxidation occurs. The output signal is produced by a change in resistance caused by the reaction

between the adsorbed oxygen and the gas molecules. They are quite sensitive (5-500ppm) and this can be improved by using noble metals or changing the operating temperature. They are also resistant to humidity but they may have drift problems over time and they may be poisoned by irreversible binding by different compounds such as sulphur or weak acids.

b) **Metal oxide silicon field-effect sensors (MOSFET sensors)**: They consist of three layers: a semiconductor which can be made of silicon (Si) or silicon carbide (SiC); a thick oxide layer ($SiO_2$) as insulator; and on top a thin catalytic metal layer made of platinum (Pt), iridium (Ir) or palladium (Pd). In these sensors, the contact between the gas molecules and the catalytic gate metal affects the voltage and thus change the capacitance through the transistor. The gas detection is recorded as a voltage change in the sensor signal. The sensitivity and selectivity can be determined by the choice of the operation temperature, gate metal and structure of the gate metal. The sensitivity of the sensors is normally high for low concentrations of the gases, while it becomes saturated for high concentrations of gases. MOSFET and MOS sensors operate at high temperatures and are considered to be less sensitive to humidity with less carry-over from one measurement to another (Schaller *et al*., 1998).

**(ii) PIEZOELECTRIC SENSORS**

They can measure temperature, mass changes, pressure, force and acceleration. The e-nose with piezoelectric sensors is designed to detect mass changes. There are two types of piezoelectric sensors:

**a) Quartz crystal microbalance (QCM sensors)**: They consist of a resonating quartz disc which vibrates at a characteristic frequency (10-30 MHz). When the volatiles are absorbed there is an increase in the mass of the disc reducing the

resonance frequency. This reduction is inversely proportional to the mass of the odorant absorbed.

**b) Surface acoustic wave devices (SAW sensors)**: These sensors measure mass changes too but differ from the above ones in that they operate at much higher frequencies and require waves to travel over the surface of the device. They can be less sensitive than other piezoelectric sensors.

**(iii) OPTICAL SENSORS**

They are carbon nanotubes or glass fibres coated with a thin chemically active material which contains a fluorescent dye. When the volatiles interact with the dye under a light source of a determinate frequency, or narrow range of frequencies, the polarity light of the fluorescent dye is altered and then a shift is produced in the emission spectrum. This change of colour (or of fluorescence, chemo luminescence, absorbance or reflectance) can be measured. Optical sensors have wide biological applications though they present bleaching problems with sunlight.

In the last few decades new sensor technologies have been developed in the field of biosensors which are devices that consist of a biological probe element (*e.g.* an antibody, a protein, an enzyme, or a whole organism) and a signal transducer. Based on any of the signal transduction methods mentioned earlier, the reaction between the analyte and the bioreceptor is converted into a measureable effect by the transducer. With the emergence of nanotechnology, biosensors have become micro-sized devices, namely nano-biosensors. Other categories of sensors are the calorimetric sensors based in the thermal signal transducer system where changes in temperature are detected or holograms used combined with biological materials in the so-called holographic biosensors which may detect metabolites such as glucose, urea or penicillin G (Marshall *et al*., 2004) due to the changes in the holographic reflection.

In this study a hybrid sensor array formed by MOS and MOSFET sensors was used due to their sensitivity and higher resistance to humidity which has been shown to be a drawback with CP sensors. Furthermore, Sahgal (2008) found, when comparing both

systems, the hybrid sensor array gave better results in terms of earlier discrimination between different microbial species.

**Table 1.4:** Summary of the sensors technologies for e-noses. Adapted from Pearce *et al*. (2003) and Mahmoudi (2009).

| Category | Measures | Material | Sensitivity | Principle |
|---|---|---|---|---|
| Electrochemical | | | | |
| MOS<br>MOSFET<br>CP | Resistance<br>Capacitance<br>Resistance | Oxides<br>Catalytic metals<br>Polymers | 5-500ppm<br>ppm<br>0.1-100ppm | Changes in current or in potential |
| Piezoelectric | | | | |
| SAW<br>QCM | Piezoelectricity | Polymer-coated resonating quartz disc | 1 pg mass change<br>1ng mass change | Changes in mass, density, viscosity and acoustic phenomena |
| Optical | | | | |
| Optical fibres<br>Fluorescence<br>Chemoluminescence | Intensity/Spectrum | Fluorescent dyes | Low ppb | Changes in light intensity |

### 1.3.3 APPLICATIONS

The e-nose has been used to examine applications in a wide range of areas including food quality control, medical diagnoses, environmental monitoring, safety and security.

**a. Food:** E-nose technology has been applied in many fields, but the most frequently considered has been the analysis of raw or manufactured food products; freshness and maturity monitoring; shelf-life investigations; microbial detection and control of food packaging material. In this field, many studies have been performed in order to find the quality of raw materials like grains (Jonsson *et al*., 1997; Evans *et al*., 2000); to evaluate the olive oil oxidation during storage (Buratti *et al*., 2005); for freshness evaluation of meats or fish; to classify a wide range of beverages such as coffee, beer, wine (Di Natale *et al*., 1997); to recognise spoilage bacteria and yeasts in milk (Magan *et al*., 2001); to detect spoilage fungi in bread (Keshri *et al*., 2002) and to monitor the ripening of Danish blue cheese (Trihaas *et al*., 2005).

Today, there is significant interest in detecting the levels of mycotoxins produced by fungi in many food products. Thus, diverse studies using the e-nose technology have been carried out to differentiate between mycotoxigenic and non-mycotoxigenic strains of *Fusarium*, *Aspergillus*, *Penicillium* in food raw materials (Keshri and Magan, 2000; Sahgal *et al*., 2007) and in ham-based medium (Camardo *et al*., 2011). Discrimination between mycotoxin producers and non-producers strains is based, as some studies have reported, on the different volatile fingerprints produced because of the different biosynthetic pathways for mycotoxin production used.

Food application has received significant attention because of the ease of consistent sample presentation, sample size that may be required, non-invasive technique and the lack of sample preparation. Thus potential exists in using this approach as part of a QA system (Magan and Sahgal, 2007).

**b. Medical:** If the e-nose is able to recognise volatiles from bacterial and fungal growth on food substrates, it may also be able to detect and diagnose certain illnesses. There is a need for rapid, inexpensive and intelligent systems for clinical diagnosis. E-nose technology could provide a tool for early detection and reduce the high costs and the time consuming nature of using molecular, microbiological cultures and serological tests in the detection of diseases like *Helicobacter pylori* (Pavlou *et al*., 2000) and tuberculosis (Pavlou *et al*., 2004). Furthermore, there has been an increasing need for early detection of microbial infections in the hospital environment. Dutta *et al*. (2005) distinguished between different types of *S.aureus* species: MRSA, MSSA and

coagulase-negative staphylococci (C-NS) from swab samples of infected patients using a sensor array. Other acquired diseases of great importance in hospitals are those related to prolonged mechanical ventilation like sinusitis, bronchitis and pneumonia (Rumbak, 2005; Humphreys *et al*., 2011).

Previous *in vitro* studies suggest it could be possible to diagnose microbial pathogens of medical importance such as anaerobic bacteria *Clostridium* spp and *Bacteroides fragilis*, and even to discriminate between different *Clostridia* based on volatiles produced (Pavlou *et al.,* 2002b) and for diagnoses of bacterial contaminants in urinary tract infections (UTI) (Pavlou *et al*., 2002a). Also, diseases such as tuberculosis (Turner and Magan, 2004) and cancer pathologies have been monitored. For example, Machado *et al*. (2005) studied the exhaled breath from individuals with lung cancer and compared this with subjects with non-cancer diseases and a healthy group as non-cancer control volunteers. They demonstrated that the e-nose could identify the different characteristics of exhaled breath in patients with lung cancer in a non-invasive way. More recently, Peng *et al*. (2010) were able to differentiate between distinct cancer types by means of patient breath analysis using a nanosensor array.

Some recent studies have demonstrated the capacity of a hybrid metal oxide/metal ion based sensor systems to discriminate between different species of *Trichophyton* which cause dermatophytosis (Sahgal *et al*., 2006). At present the diagnosis of these skin diseases takes up to 1-2 weeks. They were able to differentiate between species and distinct concentrations within 4 days of growth. This would permit a rapid diagnosis, the appropriate use of anti-fungal drugs and also monitoring their activity during the treatment. Naraghi *et al*. (2010) studied the potential of using an e-nose for discriminating between five anti-fungal treatments against two dermatophytes species within 96-120h. This approach is important due to the lack of accurate susceptibility testing, especially for dermatophytic fungi.

**c. Environmental:** Pollution has become one of the main concerns among public opinion and for local governments. The water supply can be contaminated with faecal microorganisms and with chemical products discharged from industrial, agricultural and

animal farm activities. The traditional methods for detecting indicator microorganisms of pollution, such as microbiological cultures, can underestimate their presence in water. Sensor arrays have been used to monitor wastewater in treatment plants, sewage treatment works and to discriminate between different products from sources as diverse as leather and automotive industries.

A wide spectrum of organisms, both bacteria and fungi, and chemical components such as pesticides and insecticides can contaminate water supplies (Canhoto and Magan, 2005). E-nose systems have also been used to monitor the quality of potable water for testing cyanobacterial species (Gardner *et al*., 2000b) or detecting and differentiating between *Streptomyces* spp spores in reverse osmosis and tap water (Bastos and Magan, 2006). Recently, Bastos and Magan (2007) showed that soil quality and microbial status could be differentiated using volatile production patterns under different environmental regimes. Other interesting applications such as control of health and safety air quality in the aircraft cabin environment have gained special interest (Spengler and Wilson, 2003).

## 1.4 VOLATILE FINGERPRINTS-II: MICROBIAL DETECTION AND DISCRIMINATION STUDIES

This section summarises previous research which has addressed microbial discrimination and sensitivity of detection issues, mixed cultures and anti-microbial screening using volatile fingerprints detected by e-nose systems. Table 1.5 provides an overview of some of the studies.

Microbial detection has been investigated in detail in a range of food matrices relevant to the food industry, especially for QC systems. Needham *et al*. (2005) used a CP sensor array to examine microbial (one bacterium, *Bacillus subtilis*; one yeast, *Pichia anomala*; and one filamentous fungus, *Penicillium verrucossum*) and non-microbial spoilage (enzymatic) on bread analogue substrates. They were able to differentiate between microbial and non-microbial spoilage. However, poor discrimination was obtained

between the bacterium and the yeast. Magan *et al*. (2001) investigated the discrimination between five milk-spoilage microbial species three bacteria (two *Pseudomonas* spp and *Bacillus cereus*) and two yeasts (*Candida pseudotropicalis* and *Kluyveromyces lactis*) after 5 hrs incubation at 25°C with skimmed milk-based media. They found discrimination between control samples, unspoiled and that containing bacteria or yeasts.

With the high demand for QC in the food industry, the e-nose technology has been successfully used to monitor the ripening of Danish blue cheese. Trihaas and Nielsen (2005) successfully monitored the maturation stage of Danish blue cheese which is a complicated process that depends on *Penicillium roqueforti* growth. They were able to classify the ripening age of the cheese from three different batches of two manufacturing units and from two different types of cheese using a CP e-nose sensor and multivariate methods for the data analysis. In another study, Trihaas *et al*. (2005) demonstrated that the e-nose technology with partial least square regression (PLS-R) and a correction of the e-nose features showed similar success as GC-MS in predicting 25 aroma compounds from different manufacturing plants of Danish blue cheeses. Also e-nose systems have been able to identify closely related fungi to a species level as Karlshøj *et al*. (2007b) achieved in a study with 20 isolates of several cheese-associated species of genera *Penicillium* and *Geotrichum* and *P.expansum* a non-cheese-associated fungus. The isolates were inoculated on yeast extract sucrose agar (YES) and analysed daily by the e-nose for 7 days. They not only found correct identification of the different species but the e-nose showed a potential for predicting mycotoxin production on a synthetic substrate. The same group of researchers in another study (Karlshøj *et al*., 2007a) built classification models for patulin prediction in *Penicillium expansum* spoilage of apples used for juice industry with the e-nose system and PLS regression analysis.

Regarding quantitative analyses, Magan *et al*. (2001) found slight segregation when three concentrations of *Pseudomonas aureofaciens* were tested ($10^6$, $3.5x10^8$ and $8x10^8$ CFU $ml^{-1}$). Also Boilot *et al*. (2002) found that their CP sensor array was able to separate all six bacteria tested suspended in saline solution for direct e-nose analysis for a given concentration, but not between different concentration values. Gardner *et al*.

(1998) investigated the ability of a tailor-made array of six metal oxide, one temperature and one capacitive humidity sensors to predict the type and growth phase of two bacteria (*Escherichia coli* and *S.aureus*) grown in a standard nutrient medium using ANN. All the unknown *S.aureus* samples were correctly classified (100% accuracy) and 92.2% of the *E.coli*. Lag phase samples were correctly predicted in only 14.3% of the cases, whereas log and stationary phases were discriminated in 95.1% and 73.1% respectively.

The minimum number of microbial cells that may be detected in food substrates has been suggested to be in the range $10^3$-$10^4$ CFUs for grain and milk and $10^3$ CFUs for bread and cheese (Magan and Sahgal, 2007). Regarding bacterial infections, Boilot *et al*. (2002) found $10^4$ CFU $ml^{-1}$ was the threshold of detection when they checked for bacteria causing eye infections. Similar concentration ($10^3$-$10^4$) was found in skimmed milk experiments (Magan *et al*., 2001).

Few studies have been undertaken using mixtures of microorganisms. Pavlou *et al*. (2000) studied the head space of pure cultures of *H.pylori* and a mixed infection of *Proteus mirabilis*, *E.coli* and *Enterococcus faecalis* by using a CP sensor array. Later, Pavlou *et al*. (2004) tested sputum samples from patients with the same CP sensor array. Samples consisted of three bacteria (*Mycobacterium tuberculosis*, *M.avium*, and *P.aeruginosa*), a mixture of these microorganisms and control sputum, all treated with lipase enzymes. The addition of specific enzymes may also enhance volatile head space generation when non-nutritional medium is employed or real samples are analysed (sputum, BAL, urine).

The use of volatile fingerprints to investigate the efficacy of anti-microbials has not been examined extensively previously. Besides Naraghi *et al*. (2010) who were able to discriminate between the effects of $ED_{50}$ and $ED_{90}$ concentrations of itraconazole on *Trichophyton* spp within 96-120 hrs of growth based on volatile fingerprints, practically no other studies have been carried out to address anti-microbial efficacy of compounds against medically important microorganisms.

This thesis has concentrated on VAP because it is one of the gravest respiratory diseases in which diagnosis represents one of the main concerns and problems. Practically no

detailed studies have been carried out to examine the potential use of e-nose systems for this purpose. It may well be possible to use the qualitative volatile fingerprints for early detection and discrimination of the key microbial species which may be involved in VAP. Humphreys *et al*. (2011) investigated the diagnosis of VAP by detecting microorganisms in bronchoalveolar lavage (BAL) fluid in a prospective comparative study of e-nose and microbiology using linear discriminant analysis (LDA) for the data analysis. 77% of the samples were correctly classified and 68% when patients on antibiotics were included. Sahgal (2008) performed an *in vitro* study with 13 bacterial and yeast species using a hybrid electrochemical sensor array. She found that the microorganisms were discriminated from control samples but not between species. When all the samples, including controls, were split into 4 groups *i.e*. Gram-positive, Gram-negative, fungi and controls better segregation was found and 83% of *in vitro* samples were accurately predicted. However, no detailed studies have been carried out to examine whether it is possible to use volatile fingerprints of the key species either in rich liquid culture media or in saline solution which is used for obtaining BAL samples and try to build a lab-based model to classify unknown clinical samples. Furthermore, as the early detection of which microorganisms may be involved is critical to drug treatment, it is also important to have knowledge of the potential efficacy of anti-fungal compounds on the fungal species which may be involved in VAP.

**Table 1.5:** Overview of studies of volatile fingerprints using e-nose systems for discriminating between different microorganisms. (Key: YES: Yeast extract sucrose; HPLC: High Performance Liquid Chromatography).

| References | Results |
|---|---|
| Needham *et al*., 2005 | - Differentiation between microbial (fungal and bacterial) and non-microbial spoilage (enzymatic) of bakery products in bread analogue substrates.<br>- No discrimination between the bacterium (*B.subtilis*) and the yeast (*P.anomala*). |
| Magan *et al*., 2001 | - Discrimination of unspoiled milk from that containing spoilage bacteria or yeasts in skimmed milk-based medium.<br>- Threshold of detection: $10^3$-$10^4$ CFU $ml^{-1}$ .<br>- Relatively good quantitative differentiation between 3 concentrations of *P.aureofaciens* ($10^6$, $3.5x10^8$ and $8x10^8$ CFU $ml^{-1}$). |
| Trihaas and Nielsen, 2005 | - Accurate classification of the maturation stage of three batches of two manufacturing Danish blue cheese units and two different types of other Danish blue cheeses. |
| Trihaas *et al*., 2005 | - Prediction of 25 identified compounds by e-nose in Danish blue cheeses from different units. |
| Karlshøj *et al*., 2007b | - Correctly identification of closely cheese-associated fungi grown on YES medium.<br>- Potential for mycotoxin production prediction. |
| Karlshøj *et al*., 2007a | - Prediction models for patulin concentration in apples on the basis of e-nose analysis and correlated to HPLC patulin quantification. |
| Boilot *et al*., 2002 | - Good discrimination between bacteria suspended in saline solution for one given concentration but not for separating different concentrations of the same bacterium.<br>- Threshold of detection: $10^4$ CFU $ml^{-1}$. |
| Gardner *et al*., 1998 | - Two bacteria (*E.coli* and *S.aureus*) were successfully discriminated by type and growth phase (lag, log and stationary).<br>- Low prediction of the lag phase samples (14.3%). |
| Pavlou *et al*., 2004 | - Effective discrimination between infected human sputum samples with 3 microorganisms and a mixed infection. |

| References | Results |
|---|---|
| Pavlou *et al*., 2000 | - Good separation of *in vitro* samples with *Helicobacter pylori* and gastroesophageal isolates. |
| Naraghi *et al*., 2010 | - Clear separation between inoculated and un-inoculated itraconazole treatments against two dermatophytes (*Trichophyton rubrum* and *T.mentagrophytes*), and the negative controls grown at 25°C on SDA after 96h incubation. |
| Humphreys *et al*., 2011 | - 68% correct classification of BAL samples from patients diagnosed with VAP on antibiotics. |
| Sahgal, 2008 | - 83% accuracy prediction of 16 microbial species involved in VAP based in a 4-group model. |

## 1.5 VOLATILE FINGERPRINTS-III: GC-MS AND SIFT-MS TECHNIQUES

Techniques such as gas chromatography (GC), gas chromatography linked with mass spectrometry (GC-MS) and selected ion flow tube-mass spectrometry (SIFT-MS) have been used to analyse volatile compounds. SIFT-MS was developed in 1976 for real-time quantification of gas analysis of ambient air, exhaled breath and the head space above liquids (Španěl and Smith, 2007a, 2007b). Head space analysis of liquid samples such as urine to detect nephrotoxicity (Boudonck *et al*., 2009), blood to detect bacterial growth (Scotter *et al*., 2006) and human sweat to investigate stimulant compounds for mosquitoes causing malaria (Meijerink *et al*., 2000) have also been examined.

A large number of studies have been carried out to identify MVOCs growing on a diversity of matrices using chromatographic techniques but only a few have investigated these compounds from microorganisms involved in VAP. Humphreys (2010) used GC-MS for patient's breath analysis in order to identify patients with VAP. Scotter *et al*. (2005) used SIFT-MS to find the fingerprints of volatiles from medically important fungi (*Aspergillus flavus, A.fumigatus, C.albicans, Mucor racemosus, Fusarium solani* and *Cryptococcus neoformans*) grown on a variety of laboratory media (SDA, BHIB, Columbia agar, blood agar). Others (Wady *et al*., 2003) identified MVOCs present in diverse building materials and from isolated and sub-cultured pure moulds cultures (*Stachybotrys chartarum, Aspergillus penicillioides* and *Chaetomium globosum*) related to allergic status using GC-MS and solid-phase microextraction (SPME). Finally, there has been interest in monitoring mycotoxigenic fungal strains of *Penicillium roqueforti* using SPME with GC-MS by the detection of some metabolites formed in the biosynthesis of the mycotoxins (Demyttenaere *et al*., 2003).

In this study, both methods, GC-MS and SIFT-MS, have been used to analyse and identify specific biomarkers patterns from *in vitro* bacterial and fungal species which may be involved in VAP in order to assess which volatile compounds the e-nose potentially responded to and therefore to explain the e-nose results.

## 1.6 AIMS AND OBJECTIVES

The overall aim of the present study was to examine the potential for using qualitative volatile fingerprints of specific microorganisms involved in VAP infections using the hybrid sensor e-nose to discriminate between different species; to build an *in vitro* model based on these data sets to interpret samples from VAP patients to enable better and more accurate treatments to be applied.

The detailed programme of work involved in this project was:

- To evaluate the potential of a hybrid e-nose array based on MOS and MOSFET sensors for detecting and discriminating volatile production patterns between different microorganisms involved in VAP infections (*e.g.*, *K.pneumoniae*, *Staphylococcus* species, *E.cloacae*, *C.albicans* and *A.fumigatus*).
- To investigate and compare nutritional media which can be used to optimise discrimination between VAP microorganisms *in vitro.*
- To determine the relative sensitivity for the detection of different concentrations of these VAP causing microorganisms over 24-96 hr periods
- To analyse the ability of the e-nose to distinguish between mixtures of different microorganisms.
- To determine the volatiles produced by these microorganisms using GC-MS and SIFT-MS techniques and correlate these with the results with the e-nose
- To evaluate the potential of using the e-nose to discriminate efficacy of different anti-fungal agents using volatile fingerprints.
- To compare the use of a simulated lung fluid medium (saline solution amended with 0.2 % Glucose-0.2% Peptone) to discriminate between VAP causing microorganisms.

The thesis layout consists of Chapter 2, which presents all the relevant Materials and Methods including the e-nose operation and statistics tools for data analysis, and GC-MS and GC-MS linked with SIFT device used; Chapter 3 describes the series of results

obtained in relation to (a) analyses of volatiles produced by VAP microorganisms in different media, (b) sensitivity of detection, (c) mixed cultures, (d) effect of anti-fungals, (e) GC-MS and SIFT-MS analyses, (f) use of saline solution as a lab-based medium and (g) development of statistical models for prediction of clinical samples; Chapter 4 contains the Discussion of the different key components of the research in the context of the available literature; and Chapter 5 summarises the overall Conclusions and possible avenues for future work. Figure 1.4 summarises the different phases of the work which is presented in this thesis and the links between them.

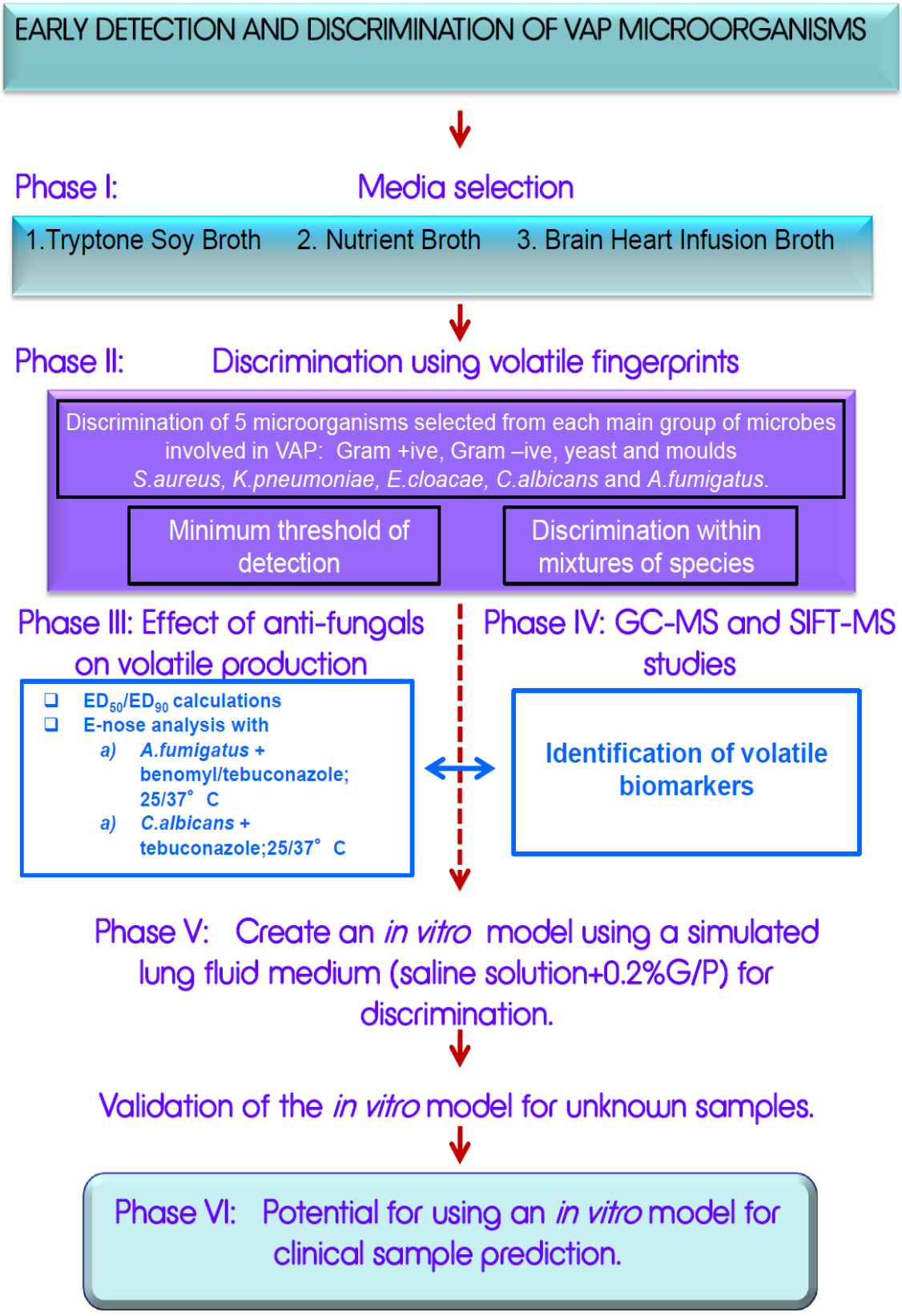



**Figure 1.4:** Flow diagram of the different phases of research and the links.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 BACTERIAL AND FUNGAL CULTURES

*In vitro* studies were carried out with five microorganisms, two Gram-negative bacteria (*K.pneumoniae, E.cloacae*), one Gram-positive bacterium, (a *Staphylococcus* species for the initial experiments, *S.aureus* for the later ones), one yeast (*C.albicans*) and one filamentous fungus (*A.fumigatus*). Appendix A explains the origin of the microorganisms used. The five microorganisms were chosen from clinical isolations at Gloucestershire Hospitals NHS Foundation Trust, with the exception of the *A.fumigatus* isolate, in order to have at least one representative specimen from each group: filamentous fungi, yeasts, and Gram-negative and Gram-positive bacteria. All of them may have a role in the cause of VAP.

The bacterial strains were cultured on Nutrient Agar plates (NA; Oxoid, UK). *C.albicans* was cultured on Sabouraud Agar (SA; Oxoid, UK) and *A.fumigatus* on Malt Extract Agar (MEA; Oxoid, UK). All of the cultures were stored at 4°C for at least 2 weeks until they were sub-cultured on the respective same solid medium and incubated at 37˚C to obtain fresh 3-7 day old cultures either for storage or for experiments. Shaker incubators were used for liquid culture studies. An orbital incubator DJB (Weiss Gallenkamp, UK) was used in the Phase I and Phase II experiments and this was replaced by an IKA® KS4000 (Germany) for the Phase III experiments with saline solutions.

API 20 E® (bioMérieux SA, France) strips were used to check the identification of the Gram-negative bacteria. Gram stains were prepared regularly for all bacteria and microscopic observations were made for *A.fumigatus* and *C.albicans.*

## 2.2 ANALYSES OF VOLATILES OF VAP MICROORGANISMS IN DIFFERENT MEDIA

Three different liquid media were tested: Brain Heart Infusion Broth (BHIB; Oxoid, UK), Nutrient Broth (NB; Oxoid, UK) and Tryptone Soy Broth (TSB; Oxoid, UK). Table B.1 (Appendix B) summarises the composition of these three media.

For liquid culture experiments, 250 ml flasks were used. For controls, 200 ml of medium and for microbial treatments (e.g. *K.pneumoniae* or *C.albicans*) 150 ml of medium was used. All the flasks, sealed with cotton wool and aluminium foil were autoclaved prior to inoculation. A cell suspension was prepared by taking loops of cells and placing them in 10 ml of saline solution in a Universal bottle. The concentration was determined using a haemocytometer (Helber chamber, Marienfeld GmbH & Co. KGM, Germany). The cell concentration was diluted as required by addition of sterile saline solution to obtain a target final concentration of $10^4$ cells $ml^{-1}$ of microorganism in each flask. The flasks were incubated at 37˚C in the orbital incubator at 150 rpm for three days. Daily, 5 ml sub-samples were taken from each replicate of each treatment and placed in sterile special 30 ml vials sealed with a screw cap containing a teflon septum before placing in the autosampler of the e-nose.

## 2.3 SENSITIVITY OF DETECTION OF VAP MICROORGANISMS

This study was performed exclusively in NB. Initial cell suspension of each microorganism (*E.cloacae, K.pneumoniae, C.albicans, Staph.* species and *A. fumigatus*) were prepared in saline solution and the concentration of cells calculated using the haemocytometer and serial dilutions were made to obtain: $10^2$ cells/spores $ml^{-1}$, $10^4$ cells/spores $ml^{-1}$ and $10^6$ cells/spores $ml^{-1}$ for each microorganism. For *A.fumigatus*, a spore suspension was prepared in sterile saline containing a drop of Tween-80 (Acros Chemicals, Belgium). The conical flasks were incubated in the orbital incubator at 37°C

for three days. Five replicates per treatment were used and uninoculated media served as controls. 5 ml of each sample were placed in the e-nose specific sterile 30 ml vials for volatile fingerprints analyses every 24 hours for three days.

## 2.4 DISCRIMINATION USING VOLATILES IN MIXED CULTURES

For these experiments the following microorganisms were used (a) a *Staphylococcus* strain and *E.cloacae* and (b) *C.albicans* and *E.cloacae*. Experiments were performed in NB. The cell suspensions were determined and diluted as required in saline solution to obtain $10^2$, $10^3$, $10^4$ and $10^6$ cells $ml^{-1}$ concentrations of each one. In the first set of experiments: $10^2 + 10^4$ cells $ml^{-1}$, $10^3 + 10^3$ cells $ml^{-1}$, $10^4 + 10^2$ cells $ml^{-1}$, and $10^6$ cells $ml^{-1}$ of *Staphylococcus* species and *E.cloacae* were used and the bacteria alone or NB medium serving as positive and negative controls. Table 2.1 summarises the mixtures and the proportions for each microorganism. This experiment was repeated once. A second experiment was carried out to examine mixtures of the yeast and a bacterium. Thus, *C.albicans* and *E. cloacae* were mixed in the same proportions as detailed above. All the treatment flasks were incubated at 37°C in an orbital incubator for three days and sub-samples removed and analysed every 24 hrs as described previously.

From the literature, positive cultures from suspicious VAP patient samples have more than $10^3$, $10^4$ or $10^5$ CFU $ml^{-1}$ depending on the type of sample: BAL, mini-BAL and tracheal aspirate. This experiment could have been planned mixing different initial amounts of each microorganism. However, as an initial approach, two mixtures with different concentrations and one mixture with equal amounts of each microorganism were prepared in the range of $10^2$ and $10^6$ CFU $ml^{-1}$ also single cultures with the highest concentration were analysed.

**Table 2.1:** Summary of mixtures for two microorganisms with their initial concentrations.

| Mixtures | *Staphylococcus* spp/*C.albicans* (CFU $ml^{-1}$) | *Enterobacter cloacae* (CFU $ml^{-1}$) |
|---|---|---|
| 1 | $10^2$ | $10^4$ |
| 2 | $10^4$ | $10^2$ |
| 3 | $10^3$ | $10^3$ |
| 4 | $10^6$ | - |
| 5 | - | $10^6$ |

Colony counting of the two species was done by spread plating 100 µl on agar media in order to check how many cells grew of each microorganism after 72 hrs incubation at 37°C. To do that it was necessary to use one selective medium for Gram-negative bacteria, such as Mac Conkey (MC; Oxoid, UK) and one non-selective one, NA. The total number of colonies on MC corresponded to the Gram-negative bacterium population whereas the colonies on NA minus the ones grown on MC corresponded to the other microorganism (*Staphylococcus* species or *C.albicans*).

## 2.5 DISCRIMINATION BETWEEN SPECIES IN NUTRIENT BROTH AND SALINE SOLUTION

For the experiments in NB it was used the same as it was described in previous sections (Oxoid, UK). 100 ml of medium was placed in 250 ml flasks for autoclaving. Afterwards, for the experiments with five microorganisms (*E.cloacae*, *K.pneumoniae*, *C.albicans*, *Staphylococcus* strain and *A.fumigatus*), cell/spore suspensions were made in sterile saline+Tween-80. The number of cell/spores was counted with the haemocytometer and diluting until achieving the final concentration of $10^4$ cell/spores

$ml^{-1}$. The inoculum was added to a four replicates of each microorganism and no inoculum was added to the blank samples (negative controls). All the samples were incubated in agitation at 37°C for 72 hrs and daily analysed by the e-nose.

Saline solution (0.85% NaCl) was obtained by diluting one tablet of saline solution (Oxoid, UK) in 500 ml of distilled water. This was combined with either 0.1% or 0.2% of D-(+)-Glucose (Sigma, Germany) and Bacteriological Peptone (Lab M, UK). These were compared to determine the best combination which would support growth such that volatile production patterns could be used for discrimination between the five test microorganisms: *E.cloacae, K.pneumoniae, C.albicans, S.aureus* and *A.fumigatus*. The pH of the media was 6.6. Three experiments using saline solution with 0.2% glucose and 0.2% peptone were performed on different days. Cell suspensions were made in sterile saline solution and spore suspensions in sterile saline solution+Tween-80. Cell concentrations were determined using the haemocytometer and diluted appropriately to obtain final concentration of $10^4$ cells/spores $ml^{-1}$ per flask and per microorganism. The inocula were added to five replicates of the medium before incubation in the orbital incubator at 37°C for 72 hrs. Also five replicates without inoculum were incubated as negative controls. Samples were taken every 24 hrs as described previously for e-nose analysis.

## 2.6 USE OF VOLATILE PRODUCTION PATTERNS TO DISCRIMINATE BETWEEN DIFFERENT ANTI-FUNGAL TREATMENTS AGAINST *A.FUMIGATUS* AND *C.ALBICANS*

### 2.6.1 EFFECT OF $ED_{50}$/$ED_{90}$ ANTI-FUNGALS ON GROWTH

The fungal strains used in this study were *A.fumigatus* and *C.albicans.*

Three anti-fungal agents were tested in this study: fluconazole (PLIVA Pharma Ltd, UK), tebuconazole (Folicur® SE, Bayer CropScience, Germany) and benomyl

(Benopron®, Probelte SA, Spain). Fluconazole solution was obtained ready for use in a concentration of 2 mg $ml^{-1}$. 1mg $ml^{-1}$ stock solutions were prepared for tebuconazole and benomyl in sterile reverse osmosis water containing 100% of the active ingredient.

For *A.fumigatus* different concentrations of the anti-fungals were incorporated into molten MEA in the range 0.1, 0.5, 1, 2.5, 5 and 10 mg $ml^{-1}$ for tebuconazole and benomyl. The concentrations of spores were determined as described previously in saline+Tween-80 sterile solution. With a sterile loop, a drop of approximately 5 mm wide from a suspension of $10^6$ spores $ml^{-1}$ was inoculated in the middle of five replicates in 9 cm Petri plates per treatment and incubated at 25°C and 37°C. Five replicate control plates were used for comparison. The diametric growth of the colonies in all treatments were measured daily in two directions at right angles to each other over a period of 9 days. Using the growth rate data at different concentrations and by making comparisons with the control growth data it was possible to calculate the $ED_{50}$ and $ED_{90}$ values of the anti-fungals treatments that inhibited 50% and 90% growth respectively. The $ED_{50}$/$ED_{90}$ were calculated by plotting in the relatives growth rates (mm $day^{-1}$) to compare to treatments using Microsoft® Excel.

For *C.albicans* studies were carried out in NB and saline-based solution liquid cultures. In NB, the anti-fungal concentrations of 0.1, 1 and 5 mg $ml^{-1}$ of fluconazole and tebuconazole were added to the broth media. Three replicates 250 ml flasks were used for each treatment and control. Each replicate was inoculated with 1.5 ml of an initial spore suspension of $10^6$ cells and incubated on an orbital incubator at 25°C as described previously. In saline solution experiments (with 0.2% glucose-0.2%peptone) the media were modified with tebuconazole to obtain concentrations of 0.02, 0.05, 0.15, 0.25, 0.5, 1, 1.5, 2 and 5 mg $ml^{-1}$. A cell suspension was prepared in saline solution as described previously to obtain an inoculum of $10^4$ cells $ml^{-1}$. This experiment was carried out for 6 days at 37°C.

Growth was monitored by measuring the optical density (OD, 640 nm wavelength) in a spectrophotometer (Camspec MS350, UK) for both NB and saline solution experiments. The optical densities were taken for more than 3-4 days or more depending on the concentration of anti-fungal.

Baranyi's sigmoid function (Baranyi, 1993; Baranyi, 1994) was used to find the maximum growth rate of each treatment for *C.albicans* with tebuconazole in saline solution. The raw data was obtained by measuring optical density per each treatment and control over time. These data sets were fitted to the Baranyi's sigmoid function (Baranyi, 1993; Baranyi, 1994) by using DMFit version 2.1 model (Institute of Food Research, UK) and the maximum growth rates ($\mu_{max}$) were obtained for each treatment. The different $\mu_{max}$ were plotted in an Excel chart to allow the calculation of the anti-fungal concentrations where the growth was inhibited by 50% and 90%. The Baranyi model was selected because this minimises the subjectivity in the calculation of the growth rate.

### 2.6.2 EFFECT OF $ED_{50}$/$ED_{90}$ OF ANTI-FUNGALS ON VOLATILE PRODUCTION PATTERNS AND DISCRIMINATION BETWEEN TREATMENTS

For *A.fumigatus*, the $ED_{90}$ and $ED_{50}$ concentrations for tebuconazole and benomyl were added to molten MEA and poured into 9 cm Petri plates. Five replicates of each treatment were prepared. All the plates except for blank samples were inoculated with 0.1 ml of a spore suspension of $10^6$ spores $ml^{-1}$ and spread plated on the agar surface with a sterile glass spreader. Five control MEA plates were also prepared as blanks. All the replicates were incubated at 25 and 37ºC. For the e-nose measurements of volatile fingerprints, four 2 cm diameter agar plugs were sampled using a sterile cork borer and placed in the 30 ml sterile vials, sealed with septa and screw caps. A destructive sampling regime was used such that every 24 hrs a different set of replicates were analysed over a 96 hrs period. Please note that no analysis of treatments was done after 24 hrs incubation because very little growth had occurred in the anti-fungal cultures (Figure C.1, Appendix C).

*C.albicans* was inoculated in: (1) NB with the $ED_{50}$ and $ED_{90}$ of tebuconazole and fluconazole; and (2) for saline solution amended with 0.2% of D-(+)-Glucose and 0.2%

Bacteriological Peptone with the $ED_{50}$ and $ED_{90}$ of tebuconazole in 250 ml conical flasks. Each flask or replicate contained 100 ml of medium. Cell suspensions were prepared in sterile saline solution and serial dilutions were made where necessary. Each replicate was inoculated with $10^6$ cells $ml^{-1}$ in NB or $10^4$ cells ml $^{-1}$ in saline solution. The treatments were sampled every 24 hrs for 72 hrs and samples placed in the 30 ml e-nose vials for analysed as described previously.

## 2.7 GC-MS AND SIFT-MS EXPERIMENTS

Several analyses were carried out using two different mass spectrometric techniques, GC-MS and SIFT-MS: (1) Volatiles from five microorganisms grown on NA (*A.fumigatus*, *C.albicans*, *K.pneumoniae*, *E.cloacae* and *Staphylococcus* species) were analysed using GC-MS and SIFT-MS techniques; (2) Volatiles from *A.fumigatus* growing on MEA treated with the $ED_{50}$ concentrations of the two fungicides, tebuconazole and benomyl, were analysed using GC-MS and SIFT-MS techniques; (3) Volatiles from *C.albicans* treated with tebuconazole in saline solution amended with 0.2% of glucose and 0.2% of peptone were analysed using a different GC-MS system. In all experiments, negative and positive control samples were also analysed.

**(a) Sampling procedure for GC-MS and SIFT-MS**

In the first set of experiments five microorganisms used previously were cultured on 9 cm Petri dishes with NA. Appropriate cell/spore suspensions were prepared per microorganism and 0.1 ml of each suspension was spread out on the plates in sterile conditions. Three plates per microorganism and negative controls were placed per bag and incubated at 37°C. Only one bag per microbial species was analysed once after 24 hrs incubation.

For the analysis of the head space above *A.fumigatus* cultures, 0.1 ml of the initial suspension was spread out with a sterile spreader on MEA plates (9 cm Petri dishes) to reach a final concentration of $10^6$ spores $ml^{-1}$. Three replicates were prepared of

*A.fumigatus* without anti-fungal compound; three for treated samples (*A.fumigatus* with $ED_{50}$ concentrations of two anti-fungals, tebuconazole and benomyl); and two replicates for blank samples (MEA untreated and MEA treated with $ED_{50}$ concentrations of both anti-fungals). A total of fifteen samples were analysed after 48 hrs and 72 hrs of incubation at 37°C respectively.

For the chromatographic analyses, plastic bags were constructed from Nalophan rolls, 210 mm width and long enough to accommodate three plates per bag (about 600 mm length). One side of each bag was attached to a ¼ inch Swagelok fitting and tightly closed with two tie-wraps. The other side was left open in order to place the plates when ready then sealed with one more tie-wrap. Afterwards, the bags were filled with air BOC zero grade and left them for incubation.

In both sets of experiments, the cultures were left with their lids on. After each incubation period, the bags containing the solid cultures were taken out from the incubator, removed the lids without opening the bags and placed in a preheated oven at 37°C for 5 minutes before connecting the bag fitting through the wall of the oven to the inlet capillary of the SIFT-MS. After these 5 minutes, each bag end was connected first to the SIFT-MS instrument for the analysis and later to a pump in order to capture the volatiles in the automated thermal desorption (ATD) tubes for the posterior analysis by GC-MS. The SIFT-MS used was a Model Profile 3, manufactured by Instrument Science Ltd. (UK). Then the head space samples passed through the heated capillary (at 60° C) and into the flow tube where the three precursor ions reacted with the sample. This process was recorded during 60 seconds to generate mass spectra at *m/z* values between 10 and 200. The count rates of the precursor ions ($H_3O^+$, $NO^+$ and $O_2^+$) and the resulting product ions of the reactions are determined by a mass spectrometer downstream. This can be operated in two modes: a) The full-scan mode (FSM) which provides the spectrum of all ions across a chosen mass range (*m/z*); b) The multi-ion monitoring mode (MIM) which determines the count rates for targeted compounds in the sample. It is possible to calculate the concentrations of trace gases by SIFT-MS at the parts per billion (ppb) and parts per million (ppm) levels knowing the reactions and the corresponding constants rates, the flow rates and the pressures in the instrument. Some of those parameters are constant such as carrier gas flow rate and others such as

the rate coefficients and ion products have been provided from SIFT-MS studies of diverse types of compounds with the three precursor ions (Turner *et al*., 2009). The present study was carried out at a helium carrier gas pressure of 0.345 Torr L $s^{-1}$ (equals roughly to 27 ml $min^{-1}$) at room temperature (23-27° C).

0.5 l of each sample was collected into the ATD tubes using a pump which was taking out the headspace from the bags during 5 min at a 100 ml $min^{-1}$ flow rate. The volatiles captured in the ATD tubes were analysed using a Perkin Elmer system which comprises a TurboMass mass spectrometer 4.1 (Software version) and an AutoSystem XL GC equipped with an Automatic Thermal Desorption system ATD 400 (Perkin Elmer, Wellesley, MA, USA). The carrier gas was CP grade helium (BOC gases, Guildford, UK) passed through a combined trap for removal of hydrocarbons, oxygen and water vapour. ATD tubes were desorbed by purging for 2 min at ambient temperature then for 5 min at 300°C. A wall-coated Zebron XB624 chromatographic column (30m x 0.4mm x 0.25mm) was used (Phenomenex, Torrance, CA, USA). The ATD tubes contained two parts: 50% Tenax TA and 50% of Carbotrap (Markes International Limited, UK) and were stored at 4°C once they were conditioned by purging with He gas for 2 min at 25°C followed by 1 hour at 320°C.

**(b) Sampling procedure for alternative GC-MS system**

Side-arm flasks were used for culturing *C.albicans* in 100 ml of saline solution (0.85 % NaCl) medium amended with 0.2% of D-(+)-Glucose and 0.2% of Bacteriological Peptone and $ED_{50}$ and $ED_{90}$ of tebuconazole. Saline solution was obtained by dissolving one tablet of saline solution in 500 ml of distilled water. An initial suspension of *C.albicans* was prepared in sterile saline solution and 0.1 ml was inoculated in each replicate to reach $10^4$ cells $ml^{-1}$. Three replicates per treatment and positive controls and two replicates for negative controls were incubated at 37°C for 24 hrs. Only $ED_{90}$ treatments were incubated one day more and analysed.

At the moment of the sample collection, the side-arm of each flask was connected through transparent Silicone tubing to the steel sorption tubes containing Tenax TA/Carbopack in series. With the help of a pump every sorption tube was sampled with

the head space from each flask at a flow rate of 100 ml $min^{-1}$ during 10 minutes. A cork was perforated and a Pasteur pipette was placed to allow air replacement in all the flasks.

The samples collected from every flask were analysed by thermal desoprtion (TD) and GC-MS system. The chromatographic device was formed by a Markes TD-100 two-stage thermal desorber linked to an Agilent 6890 GC (Agilent Technologies UK Limited, UK), fitted with a capillary column, Agilent DB-5 (60mx0.50mmx0.25mm) and coupled to a 5973 mass selective detector (MSD). The sorption tubes were conditioned in the TD-100 by heating to 320°C in a flow of 50ml/min for 15 minutes. For the analysis, all the samples were run with 0.5 µl of the internal standard (IS, D8-toluene) followed by an empty sorption tube and purged each with dry gas at 80 ml/min for approximately three minutes. A maximum of 20 samples are run before running an unused tube. After checking by visual inspection the chromatograms and named the compounds using the internal library or the NIST library the concentrations of the most abundant compounds were calculated. The concentrations in ng $l^{-1}$ were calculated either using the calibration curve for the specific compounds or using toluene calibration factor.

## 2.8 ELECTRONIC NOSE SYSTEM USED

Two e-noses manufactured by AppliedSensor AB (Sweden), NST 3320 system, were available for the sample analyses. Both consist of a non-specific sensor array of 12 MOS sensors, 10 Field Effect sensors (FE sensors) and a capacitance-based relative humidity sensor. The AppliedSensor FE sensor technology is based on the field effect generated by gases in metal oxide semiconductor field-effect transistor (MOSFET) devices with catalytic metals. The unique difference between both e-noses is the absence of a $CO_2$ sensor in one of them. Figure 2.1 shows the e-nose used in this study, the one not provided with the $CO_2$ sensor. Table B.2 (Appendix B) shows the sensors and the wide range of volatile compounds detected. The e-nose also has a control software package for analysing the collected data.

**Figure 2.1:** NST 3320 system by AppliedSensor AB.

### 2.8.1 OPERATING PRINCIPLE OF THE E-NOSE SYSTEM

The NST 3320 e-nose system comprises a 12 position carousel autosampler unit and a sampling system. The samples consisted of 30 ml glass vials sealed with screw open-top caps with septa. The sampling system consists of two needles, one for collecting the sample gas and other for replacing the head space with a clean air (reference gas) that has been filtered through two gas filters: a drying column of silica gel which acts as a main moisture trap and a hydrocarbon filter which is divided in two parts, one is a highly efficient moisture filter and the other is an active carbon adsorbent. The air enters the system at a flow rate of 60 ml $min^{-1}$. The sample phase comprises three more phases in which the temperatures can be set. These phases are: the idle, the standby and the incubation phase. All the samples are maintained at 37°C during standby and incubation phases and at room temperature during the idle phase.

All the measurements realised by the e-nose are performed in cycles during which the sensors are exposed to the sample head space. One measurement cycle lasts 4 min and 20 seconds and comprises 30 seconds of baseline, 30 seconds for the sample phase and 200 seconds for the recovery phase including 60 seconds for flushing. Before and after the sampling phase a reference gas is passed over the sensor surface in order to prepare it for measurement. Figure 2.2 shows the measurement cycle in the NST 3320 e-nose system. For each cycle, for each sample, several characteristic parameters are calculated and represented as a curve (Figure 2.3). Some of the parameters are: divergence (the maximum response from the baseline), adsorption (rate of increase in response), desorption (rate of decrease in response) and area (area under the curve).

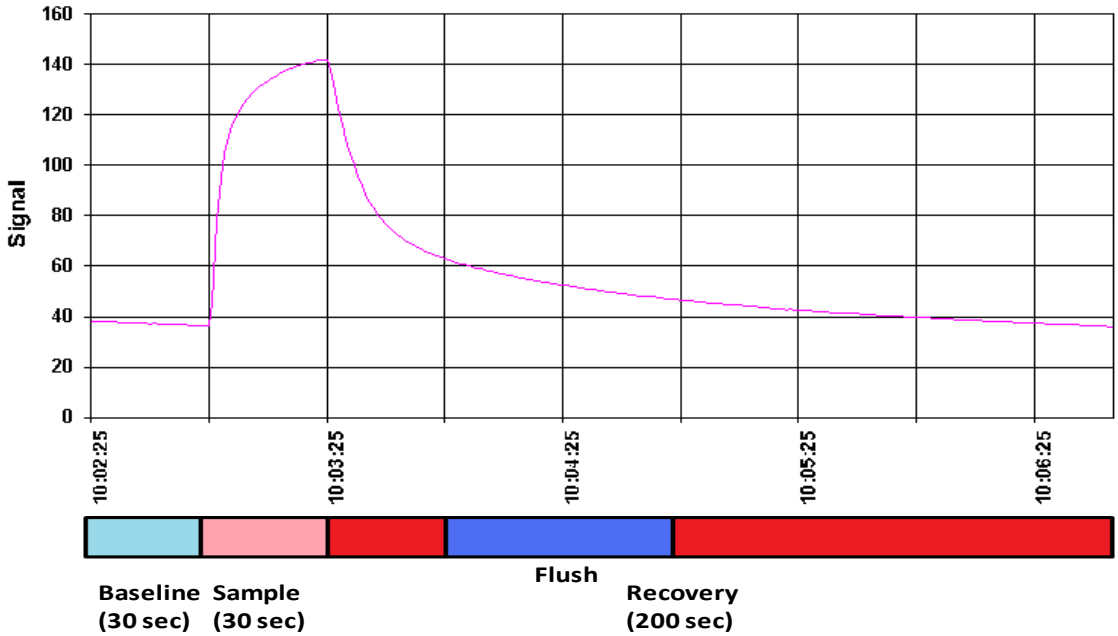



**Figure 2.2**: Measurement cycle in NST 3320 e-nose for one sensor (FE102A).

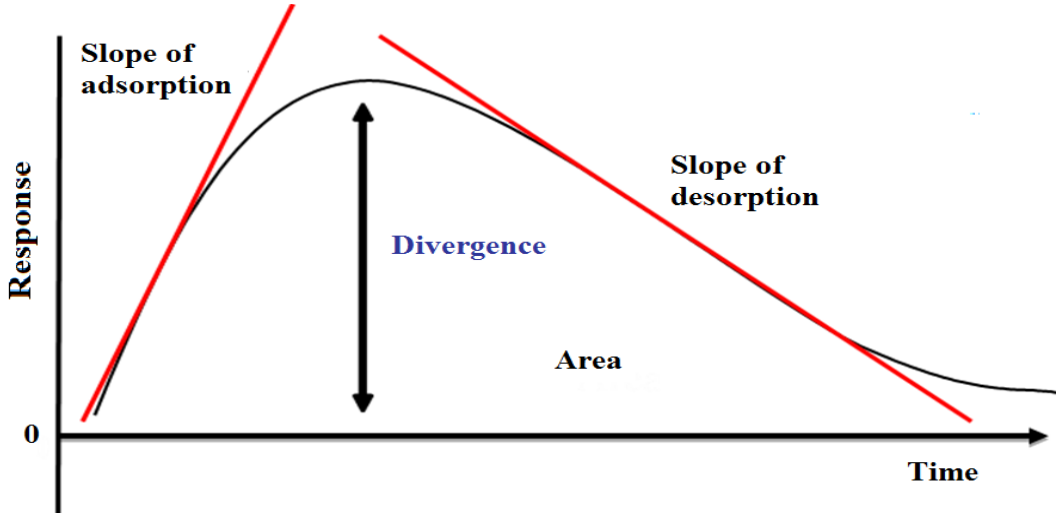



**Figure 2.3**: E-nose sensor response curve showing some measurable parameters.

Many others parameters can be calculated such as baseline, response, absolute response, on derivative, on integral, off derivative, off integral, difference and as many parameters as slopes are on the curve. In this current study the measurement mostly used has been the response which is calculated as the subtraction of the absolute response minus the baseline values which are calculated as an average over a certain time interval in order to minimise the signal-to-noise ratio.

### 2.8.2 SAMPLE PREPARATION FOR E-NOSE ANALYSES

The 30 ml glass vials were cleaned and sealed with aluminium foil, autoclaved and heated overnight in a dry oven. Septa were autoclaved separately in aluminium foil sealed beakers. The open top caps were cleaned and dried but not autoclaved. There was no contact between the caps and the samples.

Each sample was placed in the 30 ml glass vials and sealed with screw caps containing teflon septa (teflon layer towards the sample) (Figure 2.4). The samples were left for one hour at 37°C in the incubator in order to generate the head space with the volatiles

from the samples. Afterwards, the head space generated was analysed using the e-nose. The same media without inoculum were used as controls. Four or five replicates per treatment were tested.

In all cases the 30 ml vials were kept for 1 hr at the same temperature as the samples were incubated in order to increase head space volatile organic compound prior to sampling. 5 ml of sample were always analysed using the e-nose.

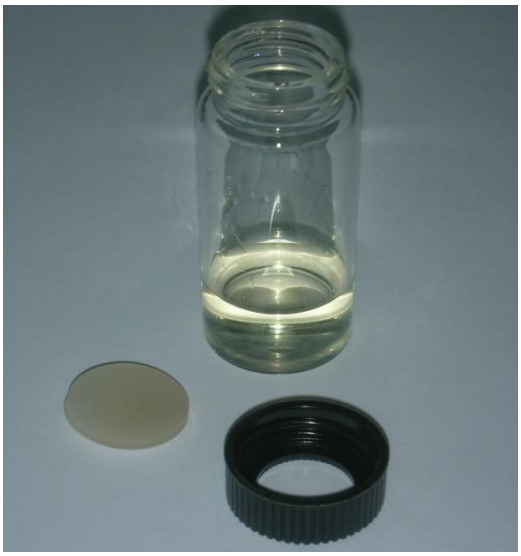


**Figure 2.4:** Special vials with septa and open-top caps for the e-nose.

### 2.8.3 E-NOSE DATA ANALYSIS

In order to understand the signal processing in the e-nose instrumentation it is necessary to consider how the data were obtained. The input signal is represented as a concentration vector, a matrix with one row and as many columns as components in the odorant. After contact with the sensor material, the input signal is converted in an electrical signal, for example, a difference in voltage. Then, the output signal from the sensor array is transformed in a new vector called array response vector.

The data collected from an e-nose based on more than one (response) variable are analysed using multivariate data analysis. These techniques permit the classification of data to determine the relationships between the sensor responses (independent variables)

and odour classes (dependent variables) (Pearce *et al*., 2003). Several methods can be used which may be either unsupervised or supervised. With unsupervised methods the unknown samples are discriminated but not classified by the differences between their measurements. The supervised methods permit classification of the unknown samples, also called the validation set, using a set of previously classified samples, the so called training set. Multivariate data methods can be subdivided into two approaches: statistical, also known as parametric, and neural networks, referred as non-parametric approach. For each two groups there is a possible quantitative data analysis and a qualitative technique commonly known as pattern recognition (PARC). The statistical multivariate analysis looks for the relationships between data in a probabilistic way. Artificial neural networks (ANN) solve multivariate problems using the human reasoning with an artificial neural network. Figure 2.5 shows some multivariate techniques broadly used in multidimensional data analysis and mainly in the e-nose field.

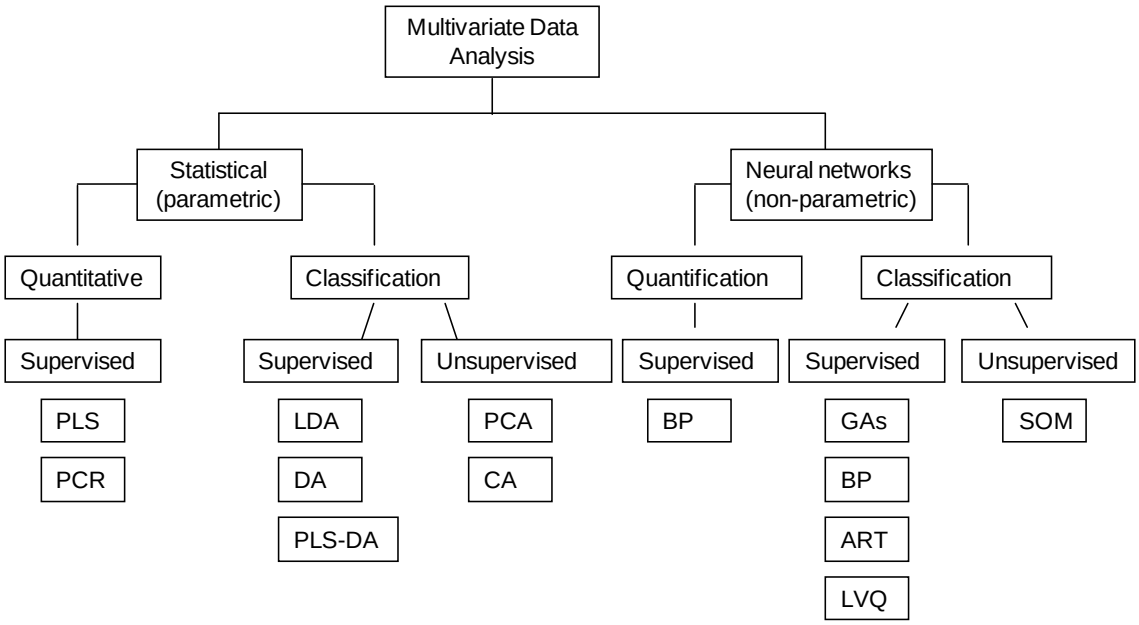



**Figure 2.5:** Brief scheme of some multivariate data analysis. Modified from Gardner and Bartlett (1999) and Jurs *et al*. (2000). (Key: ART: Adaptive resonance theory; BP: Back propagation; CA: Cluster analysis; DA: Discriminant analysis; GAs: Genetic algorithms; LDA: Linear discriminant analysis; LVQ: Learning vector quantisation; PCA: Principal component analysis; PCR: Principal component regression; PLS: Partial least square; SOM: Self-organising map).

In the present study, the data obtained from the e-nose were analysed using Principal Component Analysis (PCA) and Cluster Analysis (CA) as exploratory techniques to investigate the trend of the data. Discriminant analysis (DA) was utilised to build a model for validation samples also partial least square for DA (PLS-DA). These multivariate methods, also the standardisation, scale and cross-validation methods are explained below.

**Principal Component Analysis (PCA)**: This is a statistical multivariate method, unsupervised or untrained that allows reducing the dimensionality of data trying to capture maximum variance with a fewer number of variables. These new variables are called principal components (PCs). Then, after the analysis, each PC is sorted by its amount of variance in decreasing order. The simplification of the data permits the display of multivariate data sets in two dimensions (2 PCs) or three dimensions (3 PCs) in space instead of n-dimension space, making it easier to analyse the data without losing too much information. Other advantage of PCA is also classification and outlier detection (Wold *et al*., 1987). PCA is also a linear technique since there is a linear relationship between the independent and the dependent variables or in other cases there has been a linearisation of the data. The e-nose software package provides the PC analysis and performs a pre-processing of the array signals in default as the data obtained from the e-nose is previously mean-centred. Other scale alternatives are available from the NST Senstool software such as normalisation, auto-scale, unit variance and the combination of unit variance and normalisation. Standardisation and diverse types of range-scale are others scale methods to use. Range-scale between <+1 and >-1:

$$\boldsymbol{yij} = \frac{(\boldsymbol{xij} - \bar{\boldsymbol{xj}})}{(\mathbf{max}(\boldsymbol{xj}) - \mathbf{min}(\boldsymbol{xj}))} \qquad \textbf{Equation 1}$$

And range-scale between 0 and +1:

$$\boldsymbol{yij} = \frac{(\boldsymbol{xij} - \mathbf{min}(\mathbf{xj}))}{(\mathbf{max}(\boldsymbol{xj}) - \mathbf{min}(\boldsymbol{xj}))} \qquad \textbf{Equation 2}$$

The PC analyses are based on covariance matrixes instead of correlation matrices because they are more appropriate when the differences are sought in the amount of variability between the variables.

In order to improve the PCA results, sometimes it was necessary to remove replicates considered outliers if they were located far away from the mean group. Most of the time, outliers corresponded to the first measured sample. The first measurement of each cycle often gives outliers when the instrument has been switched off for some time. The criteria for considering an outlier was: first, the outlier was identified visually when it was located outside the expected group and secondly, an outlier is a data point well outside the range of others in the box and whisker plots for most of the variables as it is shown in Figure 2.6.

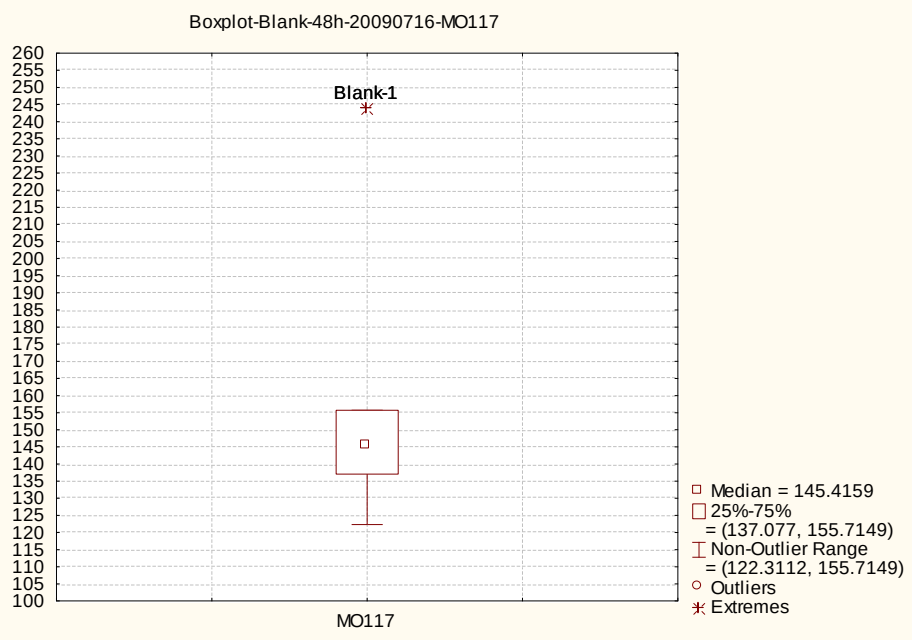



**Figure 2.6:** Box and whisker plot of 5 replicates of blank samples for one sensor (MO117).

**Cluster Analysis (CA)**: This is another unsupervised multivariate technique that classifies the data into clusters (groups). In every cluster the samples share some common trait according to some defined distance measure. In this way, data considered similar are clustered or partitioned into the same cluster, and data considered different are sorted in different clusters. The result of this analysis is a graphic called a dendrogram or tree-diagram. There are various ways of computing distances and several types of clustering methods. Statistica version 7/8 (StatSoft, USA) was used to calculate the dendrograms choosing the Euclidean distances with the Ward's procedure in all cases.

**Discriminant Analysis (DA):** The general purpose of this multivariate technique is to find which variables discriminate better between different groups and then to use those variables to predict new cases (unknown samples). Computationally, DA is very similar to analysis of variance (ANOVA) and regression analysis, with the difference that the DA dependent variable is a categorical variable rather than numerical. JMP® 8.0.2 (SAS Institute Inc., USA) was used for DA.

**Partial Least Square for Discriminant Analysis (PLS-DA)**: Is a multivariate discrimination method used to classify samples. It combines PLS regression and PCA. From PCA, the dimensionality of the data is reduced but keeping the characteristics of the data that contributes mostly to its variance; and from PLS the most discriminant variables are sought attempting to maximise the separation between groups of observations. Therefore, PLS-DA tends to increase the covariance between the input data and the output class. PLS Toolbox 3.5, Eigenvector Research Inc., USA) was employed in Matlab 7.3 (Mathworks Inc., USA) was used for PLS-DA analyses.

**Monte Carlo simulation**: This type of model assesses for the accuracy of a model by simulating the prediction several times (replications) showing whether the model predicts well by chance or not. The data generated from the simulation can be seen as probability distributions (or histograms).

**Cross validation methods**: Are used to estimate how well a model can predict a new data set. In these methods the data set is partitioned into subsets. The analysis is performed on one subset (training set) and validated on the other subset (validation set).

This has to be done several times, or rounds, with different partitions. Leave-one-out (LOO) is one of the methods to validate models where only one sample is removed every time.

**Multiplicative Drift Correction (MDC):** This method implemented for changes over time in NST Senstool was performed based in the measurements of the blanks samples related with the more frequently analysed samples (*i.e.*, saline solution with no inoculum). The ratio between the first and the second sets of measurements (the average of the response signals) is calculated for each sensor. Then the calibration is made by multiplying all the signals parameters with the appropriate ratio.

**Piecewise direct standardisation (PDS):** Is an adjustment method for enabling the comparison of measurements that may differ. This approach develops a model for predicting a new set of measurements from previous ones made in a standard instrument. This is normally motivated when different instruments have been used, also when it may want to use a historical database.

### 2.8.4 OPTIMISING SENSOR COMBINATIONS

In order to optimise the discrimination observed in the PCA score plots, it was sometimes necessary to follow some steps. Firstly, all the e-nose measurements are plotted in a PCA plot with all the sensors to obtain an initial impression of the sensor responses. This facilitated the identification and removal of outliers from the data set. This also enabled the selection of the most discriminating sensors either by examining the loading plots or the correlation table selecting the sensors with highest correlation values (positive or negative) in relation to each class or group of samples.

All the PCA score plots shown in this thesis have been built using all the sensors, MOS, MOSFET and humidity sensors. In some cases, specific sensors or groups of sensors were removed to obtain better discrimination. For instance, the following example shows how the discrimination between groups can be improved by removing sensors. In the experiment with different initial concentrations of *K.pneumoniae* ($10^2$, $10^4$, $10^6$ CFU $ml^{-1}$) grown in NB, after 72 hrs incubation the PCA with all the sensors shows (Figure

2.7) little discrimination among inoculated samples. However, by removing MOSFET and humidity sensors, and an outlier which corresponded to the first measurement of one of the cycles, the PCA showed (Figure 2.8) better discrimination with three clusters: the same clusters for blanks and two additional groups, one for the lowest initial concentration and a second for the rest of inoculated samples.

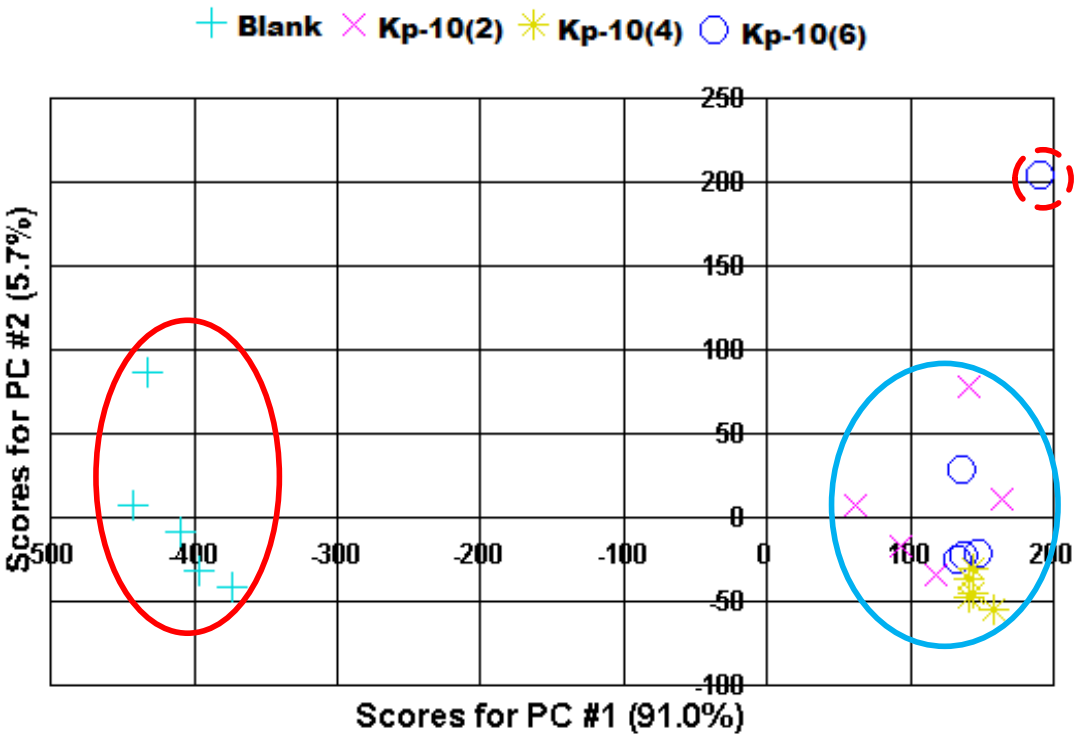



**Figure 2.7:** PCA score plot of *K.pneumoniae* after 72 hrs incubation at 37°C in NB. Red dotted line shows an outlier. (Key: Kp: *K.pneumoniae*: 10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ cells $ml^{-1}$).

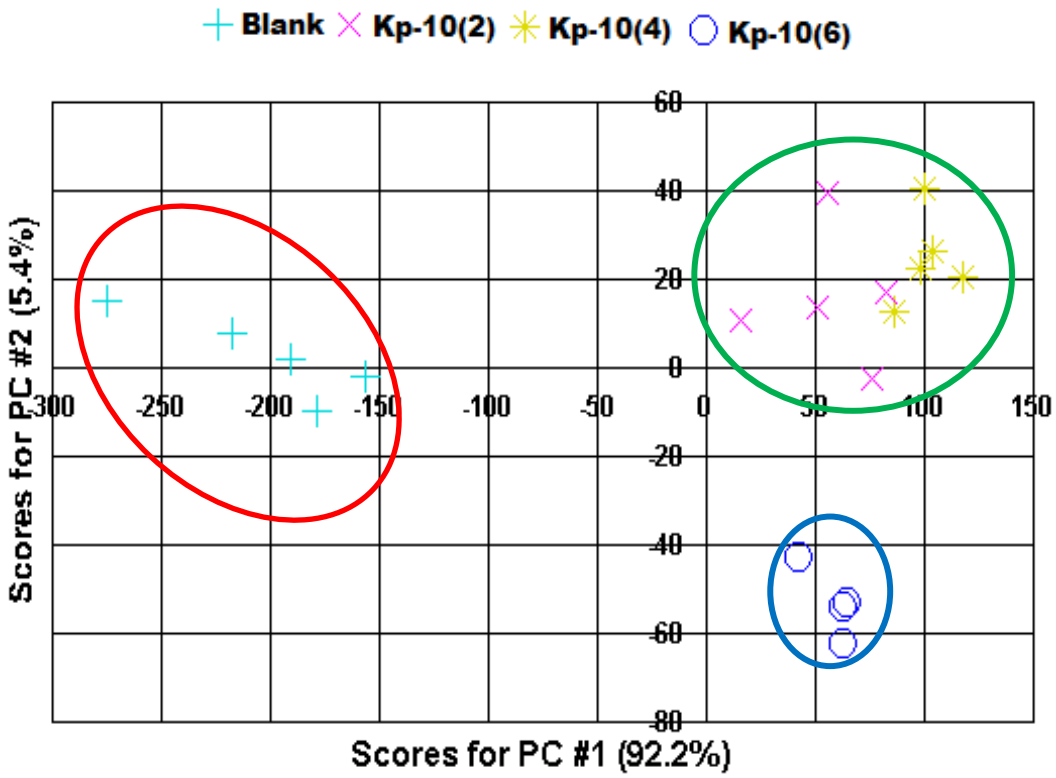



**Figure 2.8:** PCA score plot of *K.pneumoniae* after 72 hrs incubation at 37°C in NB. MOSFET, humidity sensors and one outlier have been removed. (Key: Kp: *K.pneumoniae*: 10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ cells $ml^{-1}$).

### 2.8.5 CONTROL OF SENSOR RESPONSES

One of the most common problems of gas-sensor technology is known as sensor drift. This means these sensors may not always give us exactly the same measurement over time (Pearce *et al*., 2003). Sensor drift can be due to ageing of the sensors, or by contact with sulphur compounds which bind to the sensor surfaces, hampering their working order. If the drift is caused by condensation of vapours which do not poison the sensors' surfaces, it can be reversible.

By using always the same reference substances over time it is possible to check the stability of response for each sensor therefore, in order to ensure that the hybrid sensor array did not drift significantly over time, two diluted substances (isopropanol and acetone, 0.1% concentration) and distilled water were analysed on a regular basis. Figure 2.7 shows the PCA score plot of 24 measurements over a period of 24 months. Figure 2.8 a, b shows an example of the mean of the sensor responses of 0.1% acetone in 24 different days over that period for four different sensors: MOSFET 101A, 102A, MOS 101 and 102. The mean percentage of the standard error was 7.04, 8.22, 1.86 and 3.18% for those sensors respectively. Table B.3 (Appendix B) resumes the mean of responses, the standard deviations and the percentages of the deviation for all the sensors and for all three compounds analysed over time.

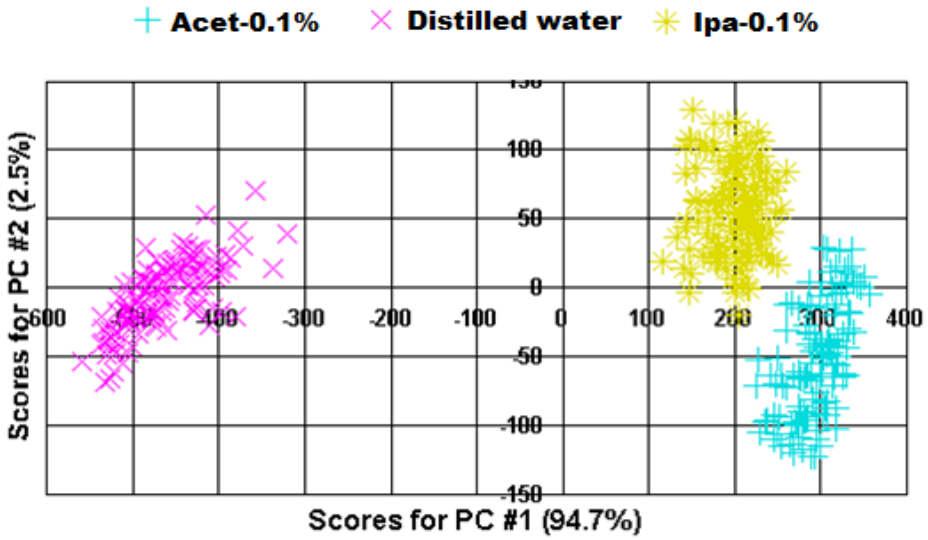
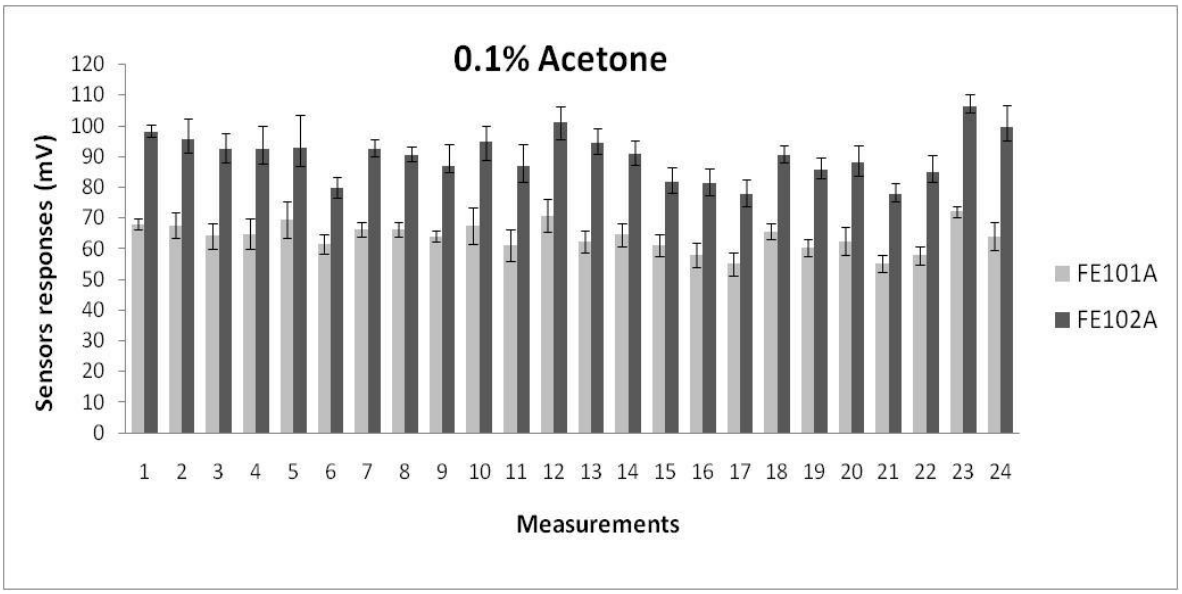



**Figure 2.9:** PCA score plot for all the replicates for acetone (Acet.), isopropanol (Ipa) 0.1% and distilled water measured in 24 occasions over a period of 2 years.

**a)**




**b)**

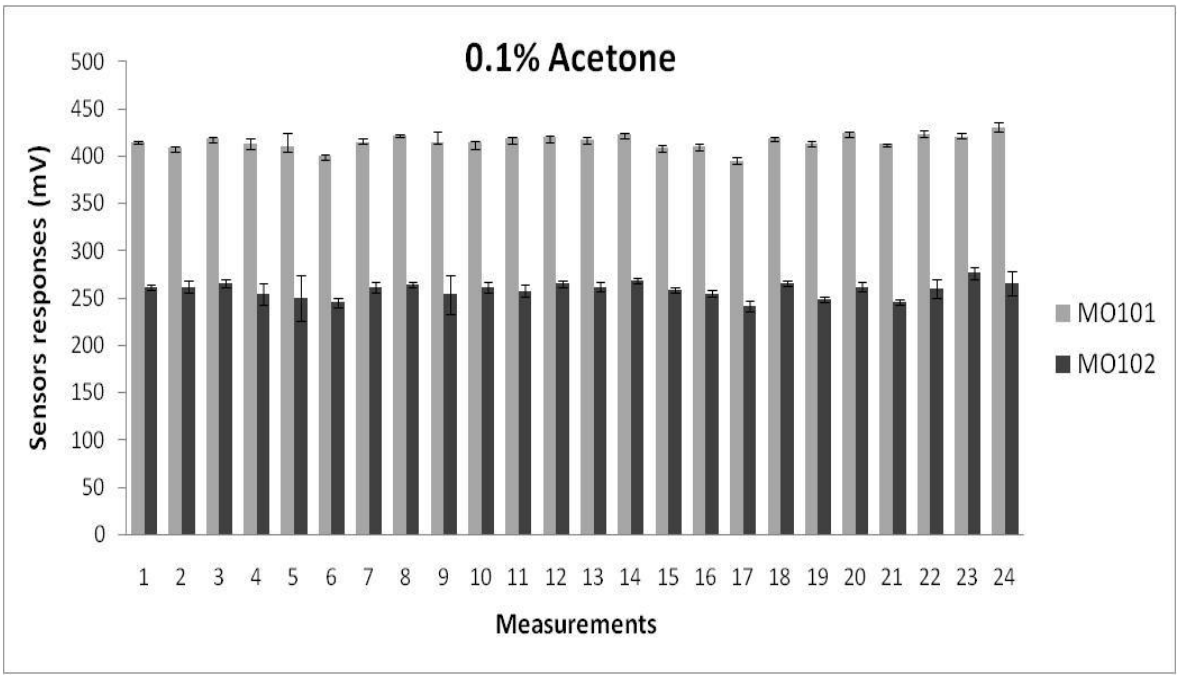



**Figure 2.10:** Bar graphs showing the mean responses of the replicates of 0.1% acetone over 24 different measurements for (a) 101A and 102A sensors and (b) 101 and 102 MOS sensors. I: depicts the standard error bars.

# CHAPTER 3: RESULTS

## 3.1 ANALYSIS OF VOLATILES OF VAP MICROORGANISMS IN DIFFERENT MEDIA

A comparison was made of the various test media for the generation of the volatile fingerprints which would be best for discriminating between the various VAP microorganisms using the e-nose. For this study two of the species were examined (*C.albicans* and *K.pneumoniae*). It was possible to obtain discrimination after 24 hrs on NB, as shown in the PCA plot in Figure 3.1. Figure 3.2 shows the discrimination between the different media types tested after 48 hrs incubation. TSB and BHIB did not show any discrimination between species until after 48 hrs incubation. The CA confirms the relative clustering of the different treatments after 24 and 48 hrs (Figures 3.3 and 3.4). This shows that after 48 hrs there were more distinct clusters, with differences between the blanks being detected.

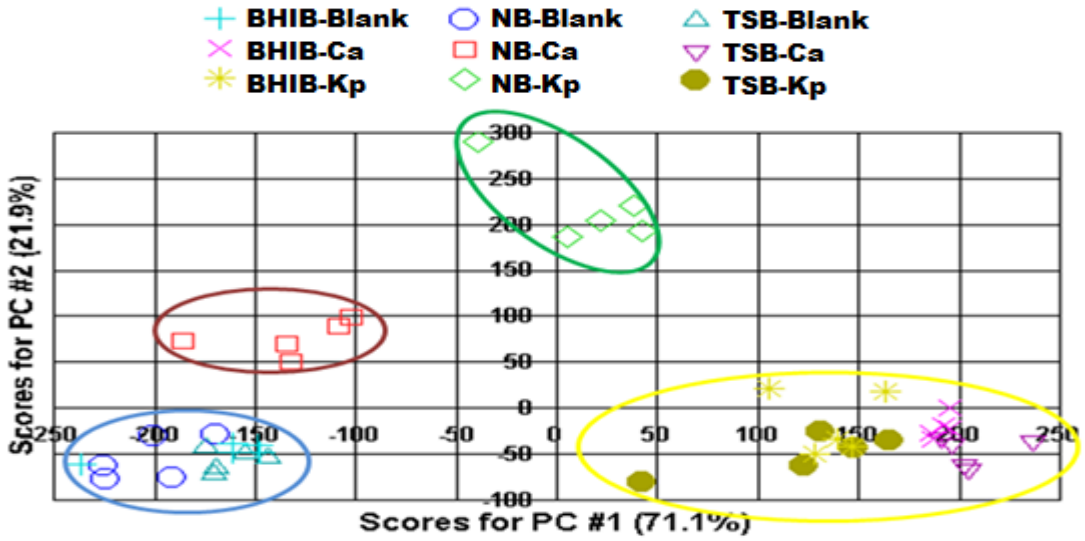



**Figure 3.1:** PCA score plot of different media after 24 hrs incubation at 37°C. (Key: Ca: *C.albicans*; Kp: *K.pneumoniae*; BHIB: Brain Heart Infusion Broth; TSB: Tryptone Soy Broth; NB: Nutrient Broth).

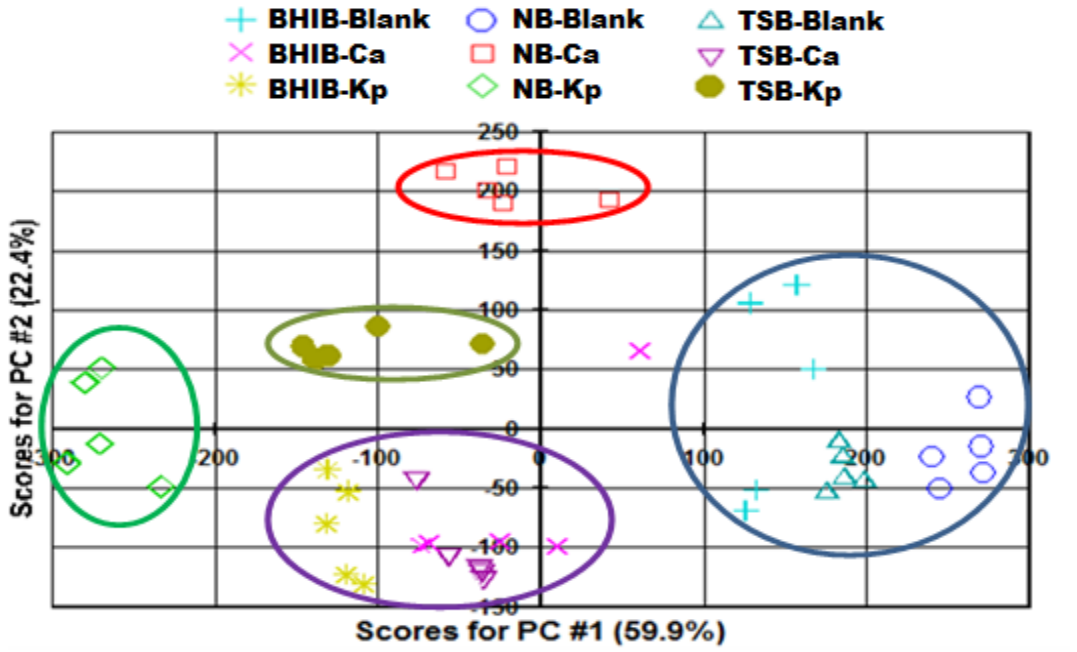



**Figure 3.2:** PCA score plot of different media after 48 hrs incubation at 37°C. (Key: Ca: *C.albicans*; Kp: *K.pneumoniae*; BHIB: Brain Heart Infusion broth; TSB: Tryptone Soy broth; NB: Nutrient broth).

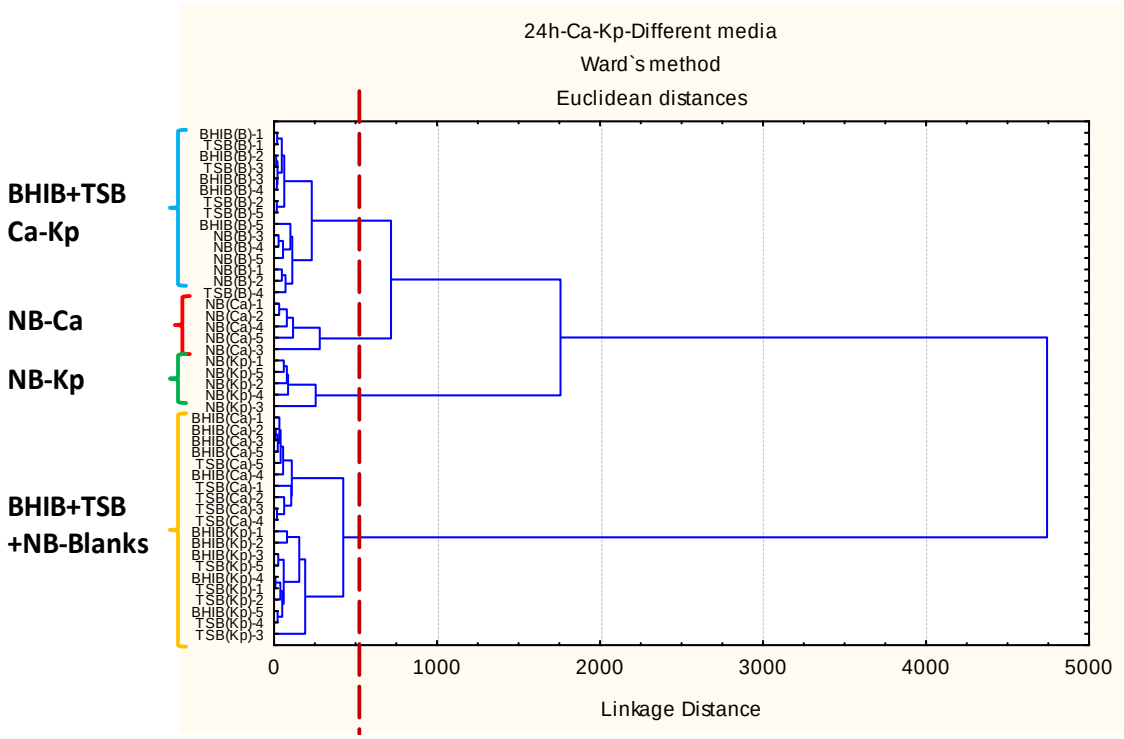



**Figure 3.3:** Dendrogram after 24 hrs incubation at 37°C. (Key: same as Figure 3.2).

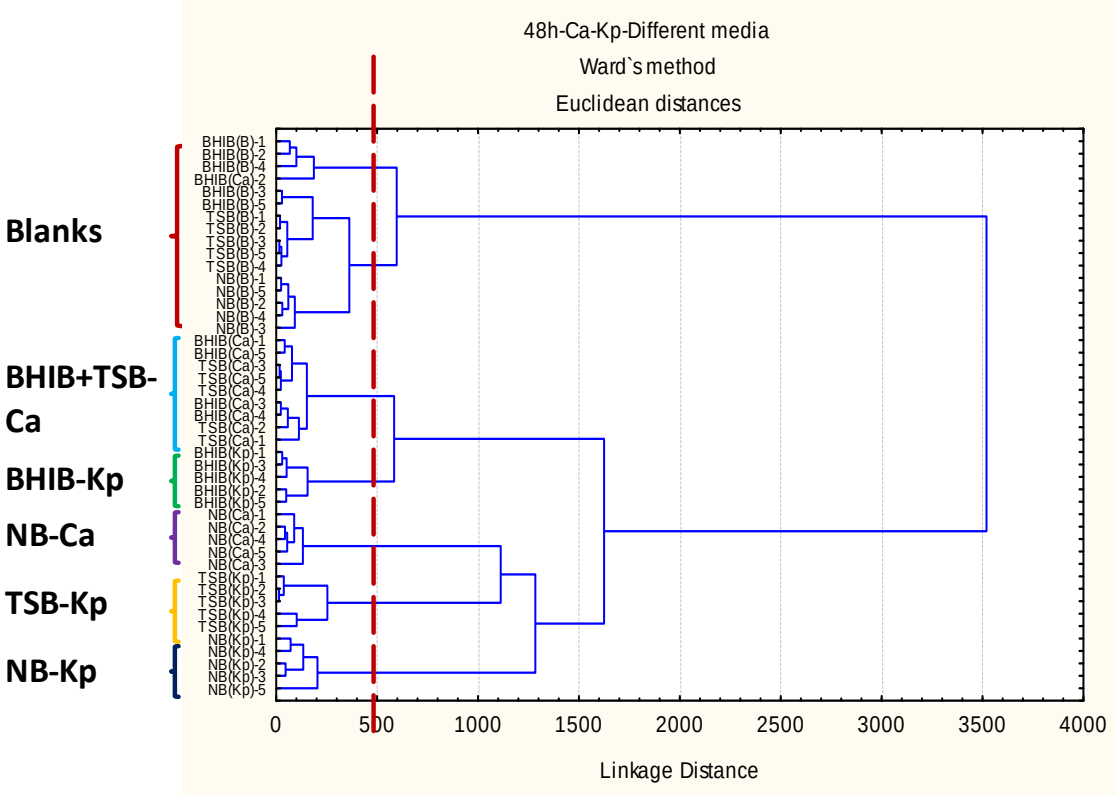



**Figure 3.4:** Dendrogram after 48 hrs incubation at 37°C. (Key: Ca: *C.albicans*; Kp: *K.pneumoniae*; BHIB: Brain Heart Infusion broth; TSB: Tryptone Soy broth; NB: Nutrient broth).

## 3.2 SENSITIVITY OF DETECTION OF VAP MICROORGANISMS

A series of experiments were carried out to evaluate the sensitivity for detection of the various VAP microorganisms, including the bacteria, yeast (*C.albicans*) and the filamentous fungus *A.fumigatus*. Overall, the sensitivity of detection was different depending in which microorganism was tested. After 24 hours of incubation of $10^2$, $10^4$ and $10^6$ spores $ml^{-1}$ as initial concentrations of *A.fumigatus* it was observed with the PC analysis (Figure 3.5) that the lowest concentration tested ($10^2$ spores $ml^{-1}$) was not differentiated and it was clustered together with the negative control samples. Also samples with the initial concentration of $10^4$ spores $ml^{-1}$ were classified close to them. Only $10^6$ cells $ml^{-1}$ samples were identified from the beginning as a clear separate

cluster. Discrimination between negative controls and the rest of the samples was achieved after 72 hrs incubation. Over time, a separation between concentrations, with respect the negative controls was observed, until all the *A.fumigatus* treatments were clustered together (72h). Therefore, the lowest concentration $10^2$ spores $ml^{-1}$ was discriminated from blank after 72 hrs incubation. Figure 3.6 shows the dendrogram after 72 hrs incubation obtained for *A.fumigatus*.

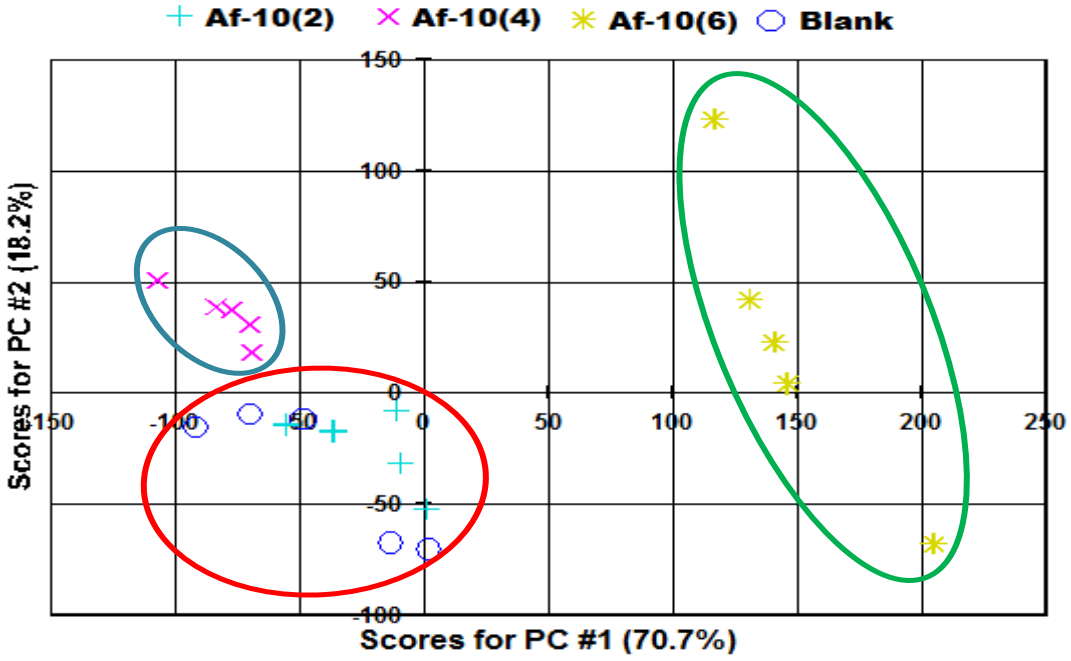



**Figure 3.5:** PCA score plot of *Aspergillus fumigatus* after 24 hrs incubation at 37°C in NB. (Key: Af: *Aspergillus fumigatus*: 10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ spores $ml^{-1}$).

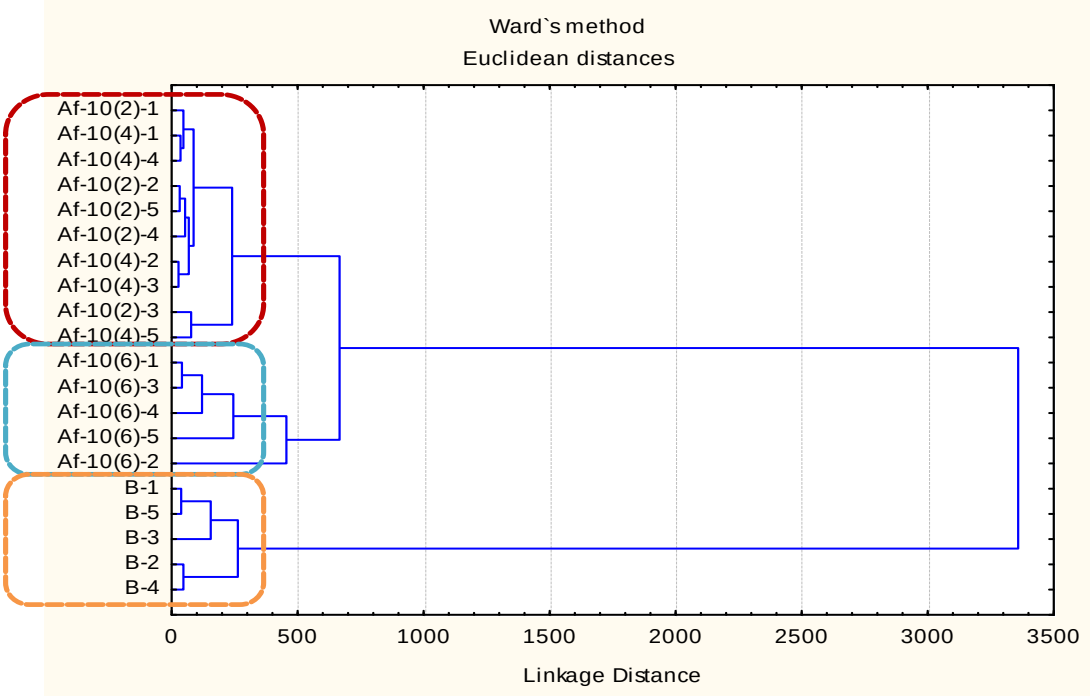



**Figure 3.6:** Dendrogram of different initial concentrations of *A.fumigatus* after 72 hrs incubation. (Key: same as Figure 3.5).

For the yeast, *C.albicans*, there was no discrimination between the negative controls and the other test concentrations. Only $10^6$ cells $ml^{-1}$ samples were clustered separately after 24 hrs incubation. After 48 hrs, $10^2$ cell $ml^{-1}$ samples were classified separated from blank and control samples but together with the rest of inoculated groups. (Figure C.2, Appendix C).

The PC analyses of the data from both Gram-negative bacteria showed different behaviour when compared to the yeast. After 24 hrs incubation it was possible to distinguish between the controls, $10^2$ cells $ml^{-1}$ and the higher concentration treatments ($10^4$ and $10^6$ cells $ml^{-1}$). Figure 3.7 shows the PCA score plot of *E.cloacae* after 24 hrs incubation and Figure 3.8 the dendrogram. Similar patterns of discrimination were obtained for *K.pneumoniae* (Figure C.3, Appendix C).

Finally, after 72h, all the microorganisms showed the same distinct clusters with all of the concentrations grouped together, and the negative control samples in a separate group.

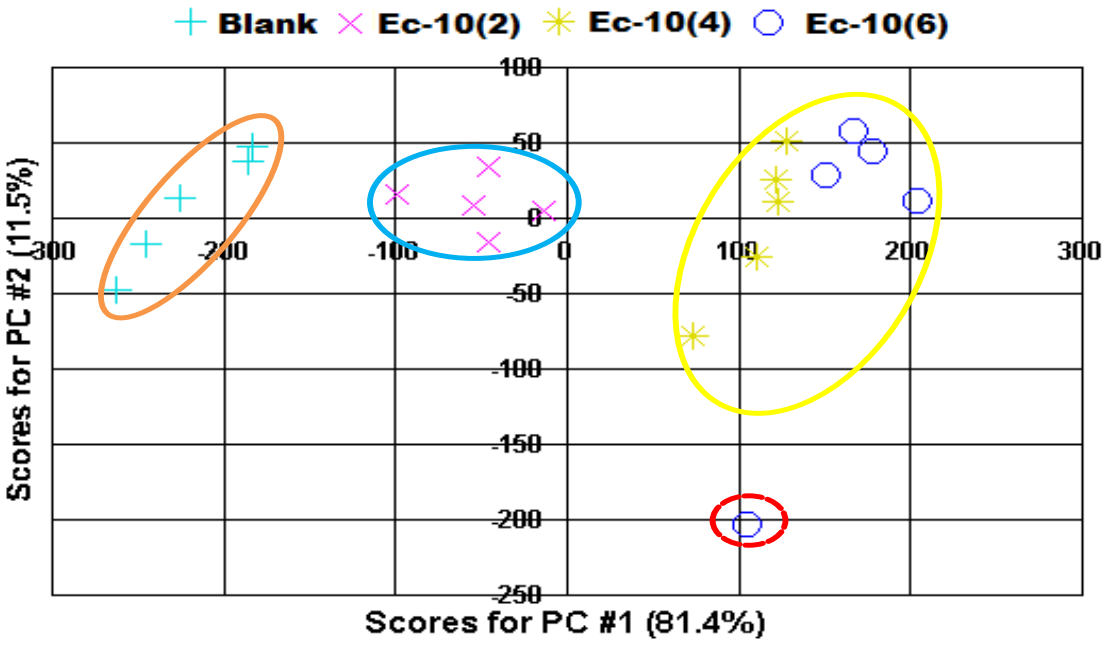



**Figure 3.7:** PCA score plot of *E.cloacae* after 24 hrs incubation at 37°C in NB. Red dotted line shows an outlier. (Key: Ec: *E.cloacae*: 10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ cells $ml^{-1}$).

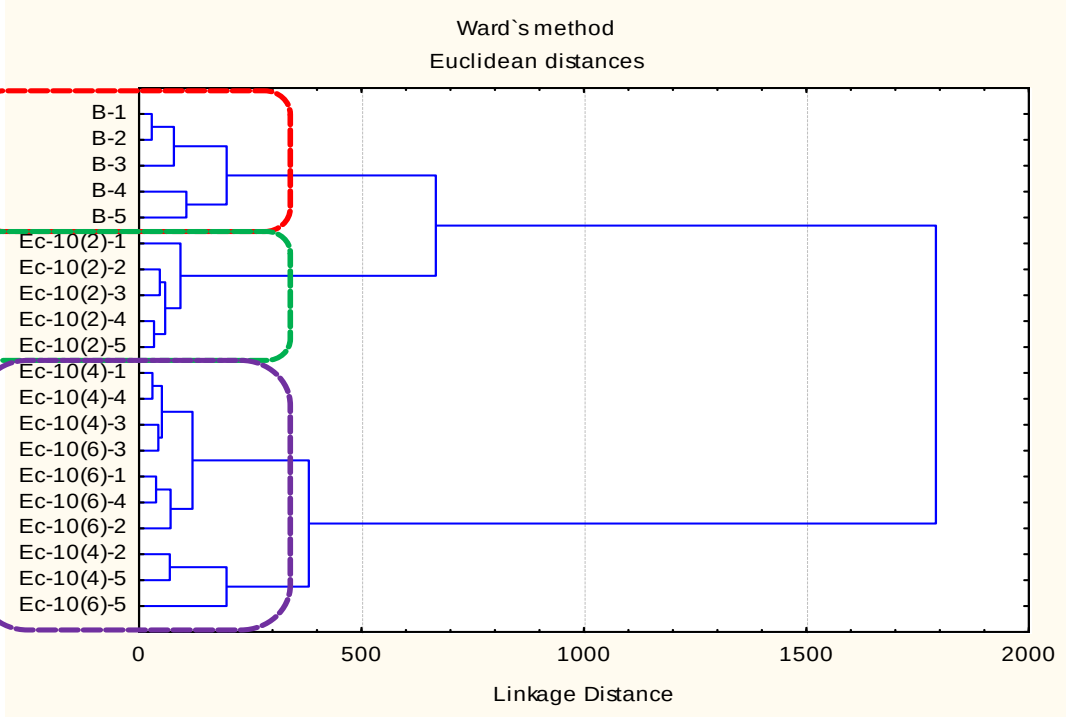



**Figure 3.8:** Dendrogram of different concentrations of *E.cloacae* after 24 hrs incubation at 37°C in NB. (Key: same as in Figure 3.7).

It should be noted that the *Staphylococcus* strains demonstrated very poor growth. After 48 hrs incubation, the PCA plots showed three clusters, one with the negative controls and a second with the $10^2$ cells $ml^{-1}$ samples, very close to each other.

## 3.3 DISCRIMINATION USING VOLATILES IN MIXED CULTURES

Attempts were made to discriminate between mixed populations of cells of two different VAP microorganisms using qualitative volatile production patterns. The first set of experiments included a mixture of *Staphylococcus* strain and *E.cloacae* in different concentrations. The analyses of the head space from the cultures were taken every 24 hours for 72 hrs. At each sampling time the same pattern of clustering was obtained: one cluster of negative control samples (NB, without any inoculum), one cluster with the *Staphylococcus* strain grown alone, and another cluster with the rest of the mixtures and also *E.cloacae* alone. This can be seen in the PCA plot after 24 hrs incubation (Figure 3.9). This experiment was repeated with the same results.

The second experiment involved using a mixture of *C.albicans* and *E.cloacae.* The same pattern of volatile production clusters was observed over the 72 hrs incubation period. The PCA plot is shown in Figure 3.10. This shows that the negative control samples were in one cluster, *C.albicans* growing alone, in a second cluster and the rest of the mixed microbial treatments in a third cluster, including *E.cloacae* alone.

In both PCA plots, the red dotted circles include the outliers which correspond to the first measurement performed per cycle.

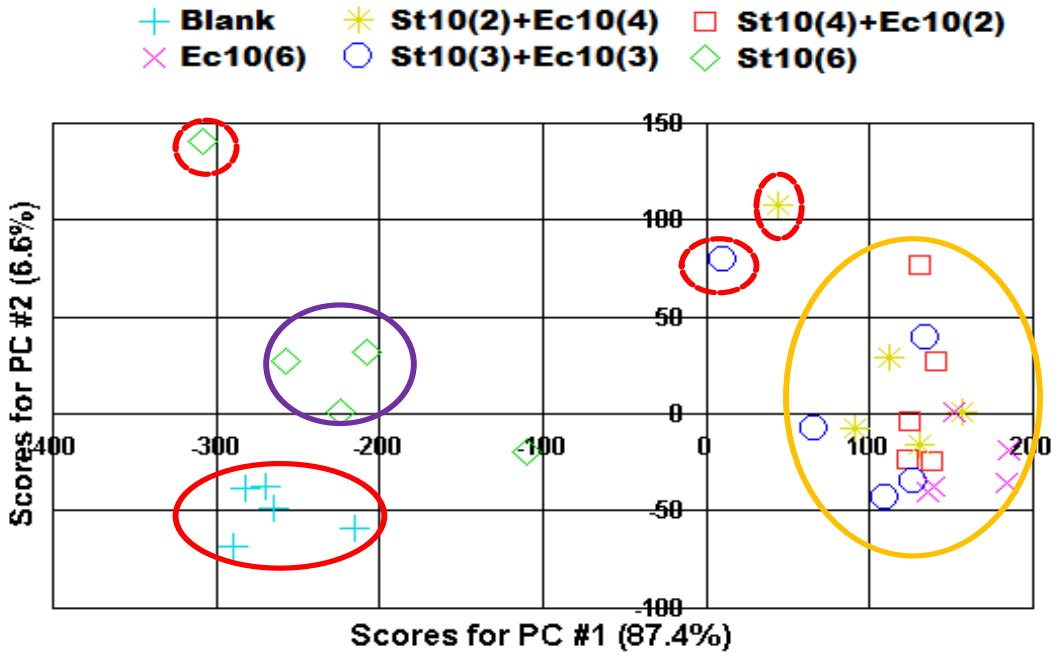



**Figure 3.9:** PCA score plot a mixture of two microorganisms at 24 hrs incubation at 37°C in NB. (Key: Ec: *E.cloacae*; St: *Staphylococcus* species; 10(2), 10(3), 10(4), and 10(6): $10^2$, $10^3$, $10^4$, and $10^6$ cells $ml^{-1}$). Red dotted circles: outliers.

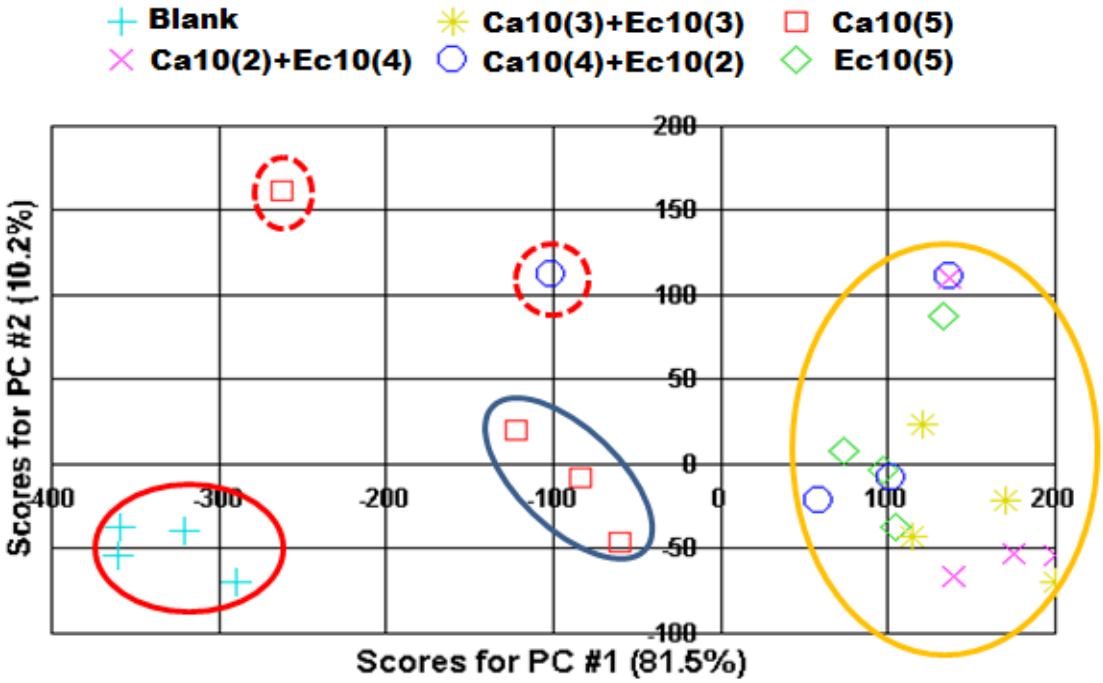



**Figure 3.10:** PCA score plot of different mixtures of two microorganisms at 24 hrs of incubation at 37°C in NB. (Key: Ca: *C.albicans*; Ec: *E.cloacae*; 10(2), 10(3), 10(4), and 10(6): $10^2$, $10^3$, $10^4$, and $10^6$ cells $ml^{-1}$). Red dotted circles: outliers.

In order to assess the proportion of each microorganism present after the incubation period, the number of CFUs for each species in the mixture was determined after 72 hrs using two different solid media (NA and McConkey). It was found that in all the mixtures the Gram-negative bacterium *E. cloacae* was the predominant microorganism even when it was inoculated with the lowest initial concentration (Figure 3.11).

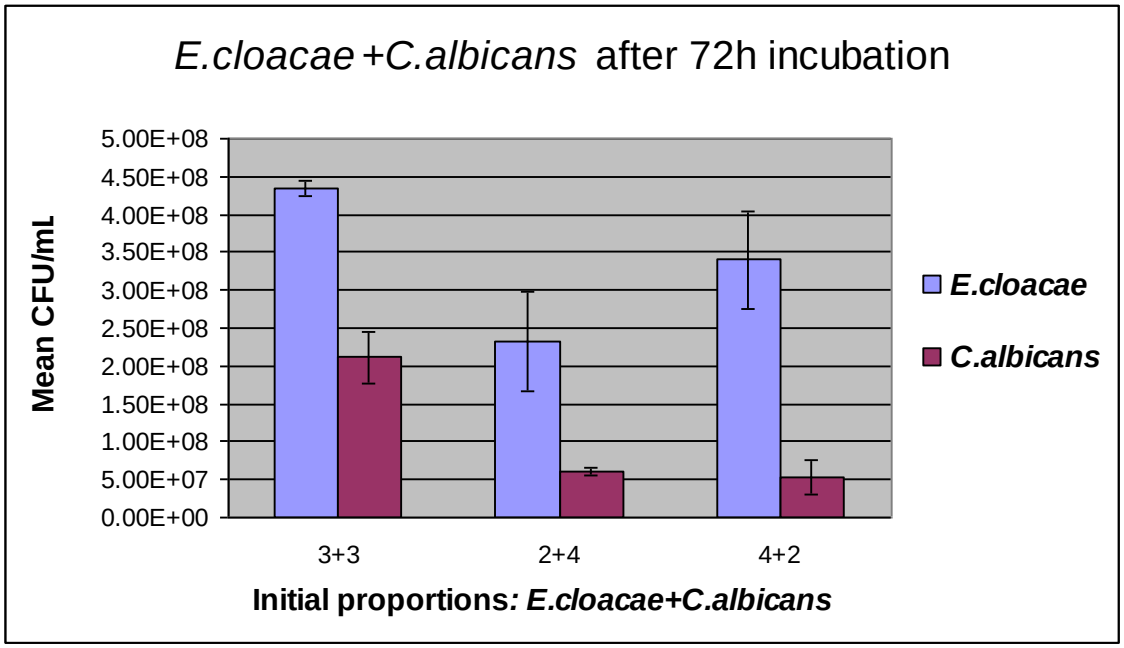



**Figure 3.11:** Histogram of three different mixtures of *E.cloacae* and *C.albicans* after 72 hrs incubation in NB at 37°C. (Key: 3+3: $10^3$+$10^3$ cells $ml^{-1}$; 2+4: $10^2$+$10^4$ cells $ml^{-1}$; 4+2: $10^4$+$10^2$ cells $ml^{-1}$; I: Standard error bars).

## 3.4 USE OF VOLATILE PRODUCTION PATTERNS TO DISCRIMINATE BETWEEN DIFFERENT ANTI-FUNGAL TREATMENTS AGAINST *A.FUMIGATUS* AND *C.ALBICANS*

### (a) Effect of $ED_{50}$/$ED_{90}$ anti-fungals on growth

Initial studies were carried out to obtain dose response curves for the efficacy of different concentrations of tebuconazole, fluconazole and benomyl when used against *C.albicans* and *A.fumigatus*. These could be used to determine the $ED_{90}$ and $ED_{50}$ concentrations to use in subsequent experiments to evaluate volatile production patterns for discriminating between treatments.

Figure 3.12 shows, as an example, the effect of different concentrations of tebuconazole on temporal growth of *C.albicans* over periods of 160 hrs at 37°C in saline solution containing a low concentration of additional glucose and peptone. Similar curves were obtained for the other fungicides examined. For *A.fumigatus,* the effect of different concentrations of tebuconazole on growth rates is shown in Figure 3.13 which shows that a higher concentration of this fungicide was needed to control this fungal pathogen (5-10ppm). Similar experiments were carried out with benomyl and fluconazole (see Figures C.4 and C.5 respectively, in Appendix C).

That data set was used to calculate the $ED_{50}$ and $ED_{90}$ concentrations required to control these two pathogens. Table 3.1 summarises all the $ED_{50}$ and $ED_{90}$ values of the different anti-fungals required for the two species at 25°C and 37°C. It should be noted that benomyl was not effective against *C.albicans* and fluconazole had no efficacy against *A.fumigatus* at 25°C/37°C or *C.albicans* at 37°C.

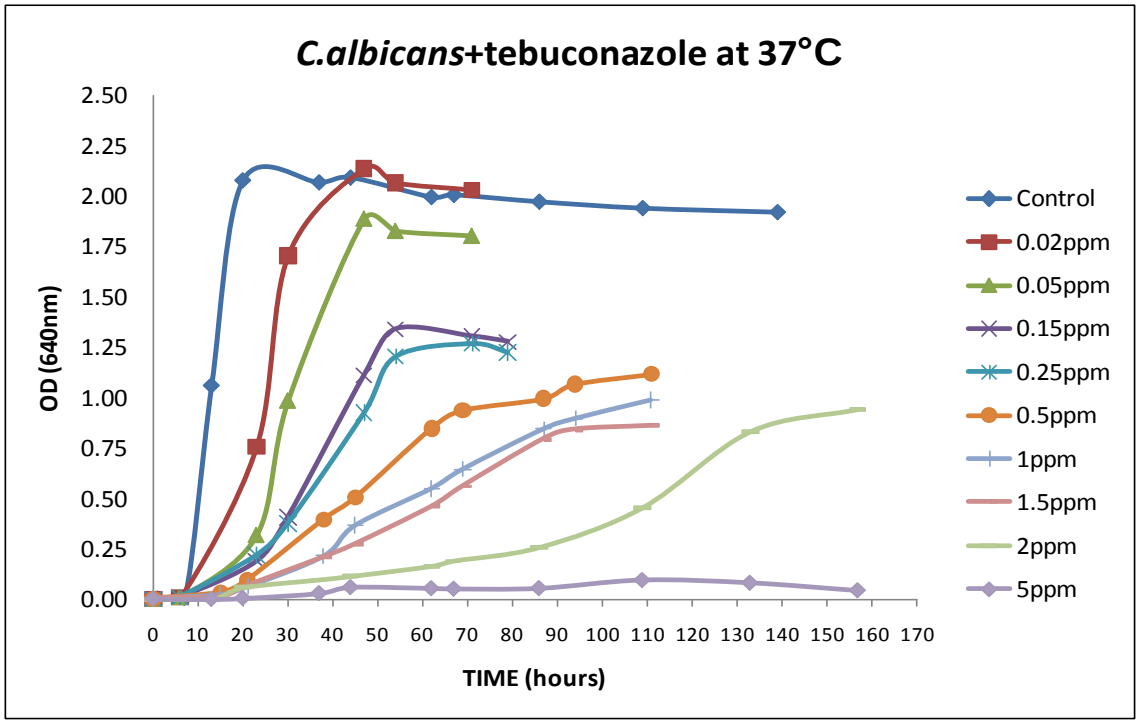



**Figure 3.12:** Growth rates of *C.albicans* at 37°C with different concentrations of tebuconazole in saline solution+0.2% glucose and 0.2% peptone over a period of 6 days.

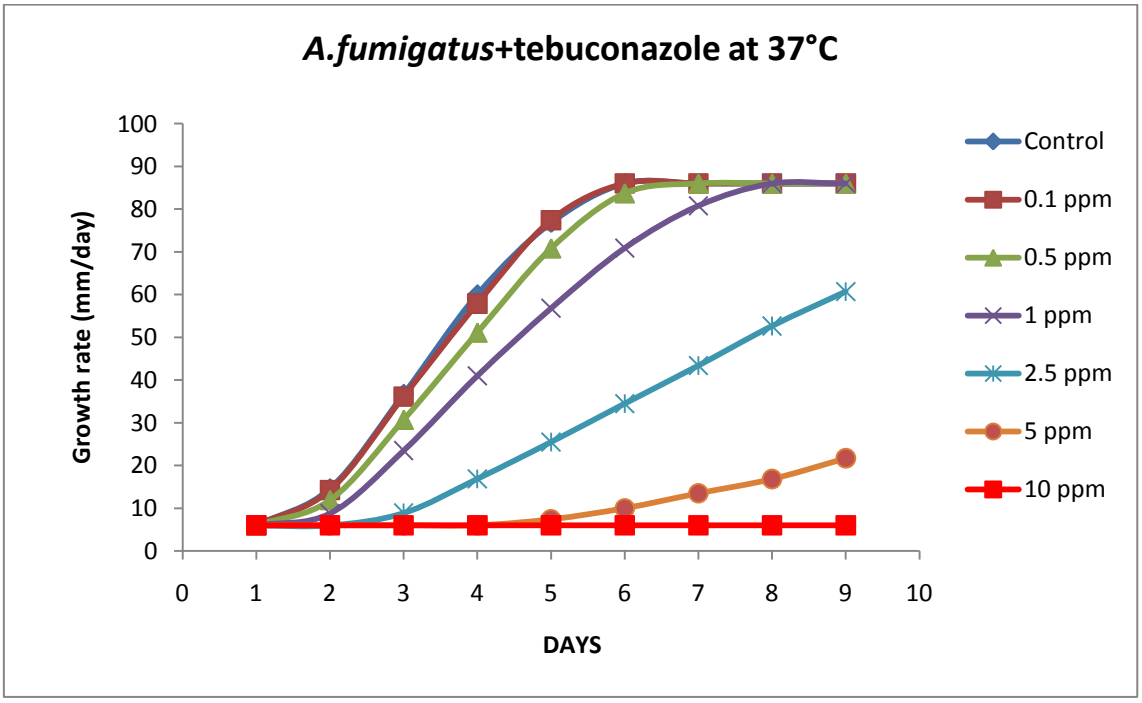



**Figure 3.13:** Growth rates of *A.fumigatus* at 37°C with different concentrations of tebuconazole on MEA over a period of 9 days.

**Table 3.1:** Summary of effective doses for different anti-fungals, species, media and temperatures. (Key: MEA: Malt extract agar; NB: Nutrient broth; SS+0.2%G-P: saline solution 0.2% Glucose-0.2% Peptone).

| Anti-fungals | Species | $ED_{50}$ | $ED_{90}$ | Temp | Medium |
|---|---|---|---|---|---|
| Tebuconazole | *A.fumigatus* | 1.553 µg $ml^{-1}$ | 4.240 µg $ml^{-1}$ | 25°C | MEA |
| | *A.fumigatus* | 1.934 µg $ml^{-1}$ | 6.850 µg $ml^{-1}$ | 37°C | MEA |
| | *C.albicans* | 0.175 µg $ml^{-1}$ | 1.985 µg $ml^{-1}$ | 25°C | NB |
| | *C.albicans* | 0.135 µg $ml^{-1}$ | 1.893 µg $ml^{-1}$ | 37°C | SS+0.2%G-P |
| Benomyl | *A.fumigatus* | 0.451 µg $ml^{-1}$ | 1.012 µg $ml^{-1}$ | 25°C | MEA |
| | *A.fumigatus* | 1.239 µg $ml^{-1}$ | 2.248 µg $ml^{-1}$ | 37°C | MEA |
| | *C.albicans* | Not effective | Not effective | 25°C | NB |
| Fluconazole | *A.fumigatus* | Not effective | Not effective | 25°C | MEA |
| | *A.fumigatus* | Not effective | Not effective | 37°C | MEA |
| | *C.albicans* | 0.078 µg $ml^{-1}$ | 3.829 µg $ml^{-1}$ | 25°C | NB |
| | *C.albicans* | Not effective | Not effective | 37°C | SS+0.2%G-P |

**(b) Effect of $ED_{50}$/$ED_{90}$ of anti-fungals on volatile production patterns and discrimination between treatments**

The $ED_{50}$ and $ED_{90}$ concentrations were then used to carry out fully replicated experiments with which included positive and negative blanks using the e-nose to evaluate the volatile production patterns. This showed that discrimination between treatments could be observed after 48 hrs incubation for *A. fumigatus* in the presence of two fungicides (benomyl, tebuconazole) at 37ºC. PCA plot is shown in Figure C.6 (Appendix C). A radar plot (Figure 3.14) has been included to show the different responses from MOSFET sensors for each treatment. No good discrimination was achieved at 25ºC at any time (data not shown).

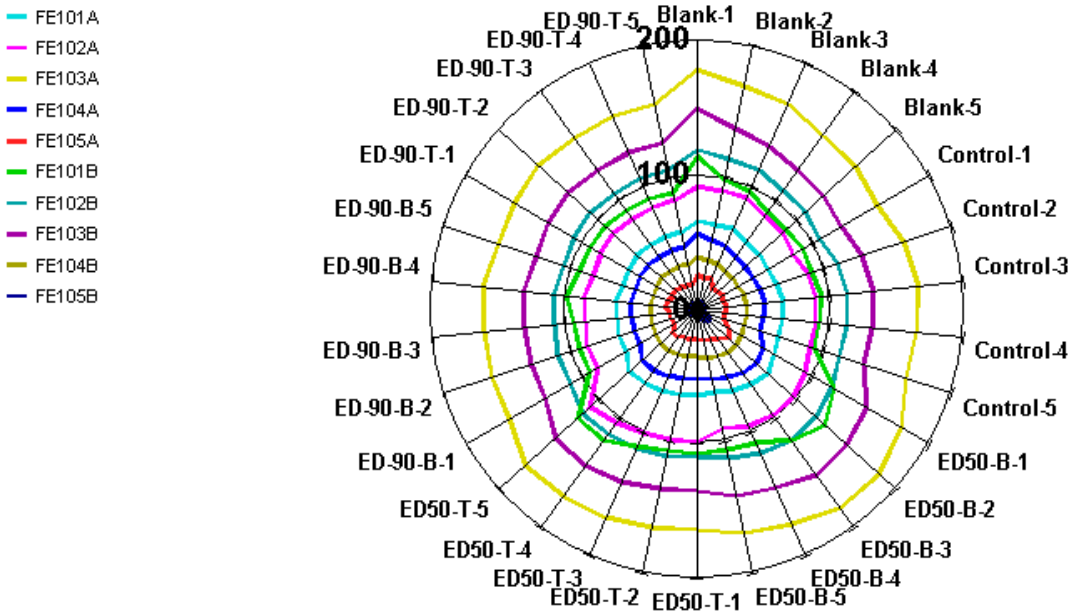



**Figure 3.14:** Radar plot of MOSFET sensors showing the distinctive responses for different samples of *A.fumigatus* with benomyl and tebuconazole effective doses of 50% and 90% of growth inhibition ($ED_{50}$, $ED_{90}$) after 48 hrs incubation on MEA cultures at 37°C. (Key: ED50-T/B and ED90-T/B: $ED_{50}$/ $ED_{90}$ of tebuconazole/benomyl).

DA was performed for *A.fumigatus* experiment to assess the accuracy of a possible model. Figure 3.15, shows discrimination between controls, $ED_{50}$ treatments, $ED_{90}$ treatments gathered with blank samples. A cross-validation method, LOO, was used to assess the accuracy of the training set in this study. However, more sets of measurements would be needed in order to remove a complete set of replicates each time instead of one single sample which might have inflated the accuracy of the model. Table 3.2 shows the classification matrix resulting from the cross-validation test. A total of seven samples were misclassified (23.33%). This misclassification occurs between the blanks and $ED_{90}$ treatments.

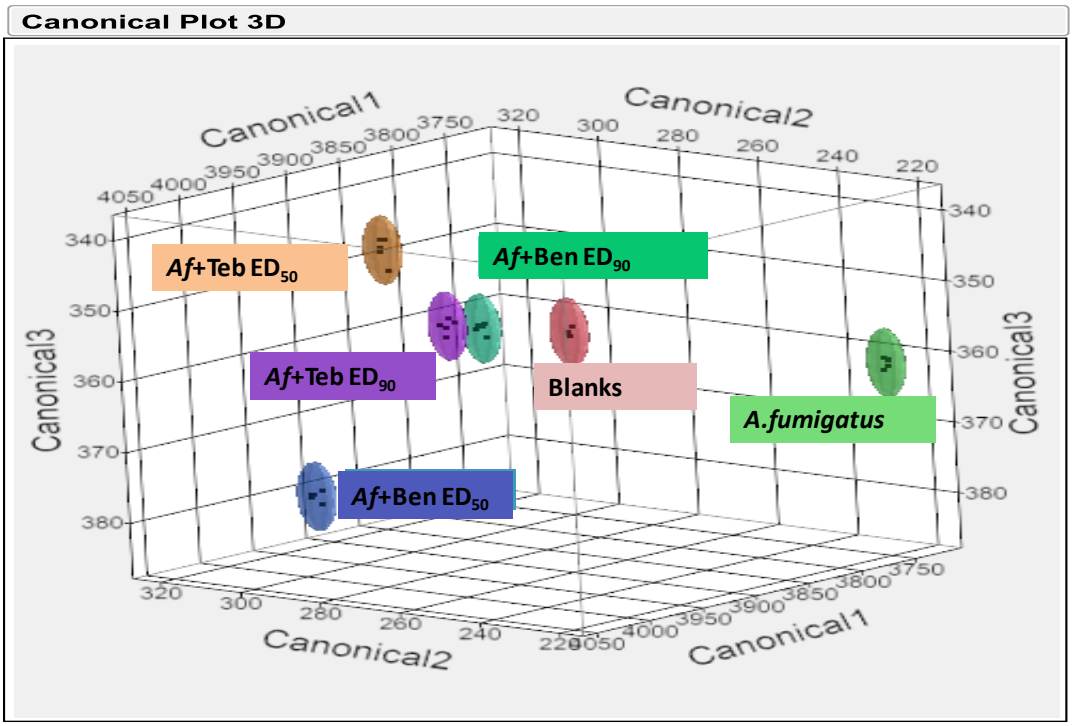



**Figure 3.15:** 3D canonical plot of *A.fumigatus* after 48 hrs incubation on MEA. Key: *Af*: *A.fumigatus*; Ben ED50/ED90: Benomyl $ED_{50}/ED_{90}$; Teb ED50/ED90: Tebuconazole $ED_{50}/ED_{90}$.

**Table 3.2:** Classification matrix after cross-validation test based on the DA model. *A.fumigatus* at 37°C on MEA after 48 hrs incubation. Accuracy prediction of 76.66% (23/30). (Key: B: benomyl; T: tebuconazole).

| **Actual Rows by Predicted Columns** | **Blank** | **Control** | ***Af*+$ED_{50}$-B** | ***Af*+$ED_{50}$-T** | ***Af*+$ED_{90}$-B** | ***Af*+$ED_{90}$-T** |
|---|---|---|---|---|---|---|
| **Blank** | **2** | 0 | 0 | 0 | 3 | 0 |
| **Control** | 0 | **5** | 0 | 0 | 0 | 0 |
| ***Af*+$ED_{50}$-B** | 0 | 0 | **5** | 0 | 0 | 0 |
| ***Af*+$ED_{50}$-T** | 0 | 0 | 0 | **5** | 0 | 0 |
| ***Af*+$ED_{90}$-B** | 0 | 0 | 0 | 0 | **3** | 2 |
| ***Af*+$ED_{90}$-T** | 0 | 0 | 0 | 0 | 2 | **3** |

Regarding *C.albicans* with fluconazole at 25°C in NB, there was no discrimination between control samples and $ED_{50}$ values at any time. Data showed in Figure C.7 (Appendix C). The experiments with *C.albicans* amended with tebuconazole were chronologically performed, initially, at 25ºC in NB and then at 37º C in saline solution+0.2% glucose-0.2% peptone. In NB, there was slight discrimination between treatments (control, $ED_{50}$ and $ED_{90}$) after 48 hrs incubation as it can be seen in Figure 3.16, also Figure C.8 (Appendix C) depicts the CA for this analysis. However, in the saline solution there was no discrimination at any time. Figure 3.17 shows the CA after 24 hrs incubation in saline solution where three clusters could be distinguished: a control group, one for all the blanks (negative controls) and a third for both anti-fungal treatments together.

These studies were complimented with analyses of volatiles using GC-MS and SIFT-MS to identify compounds might be related to the differences found.

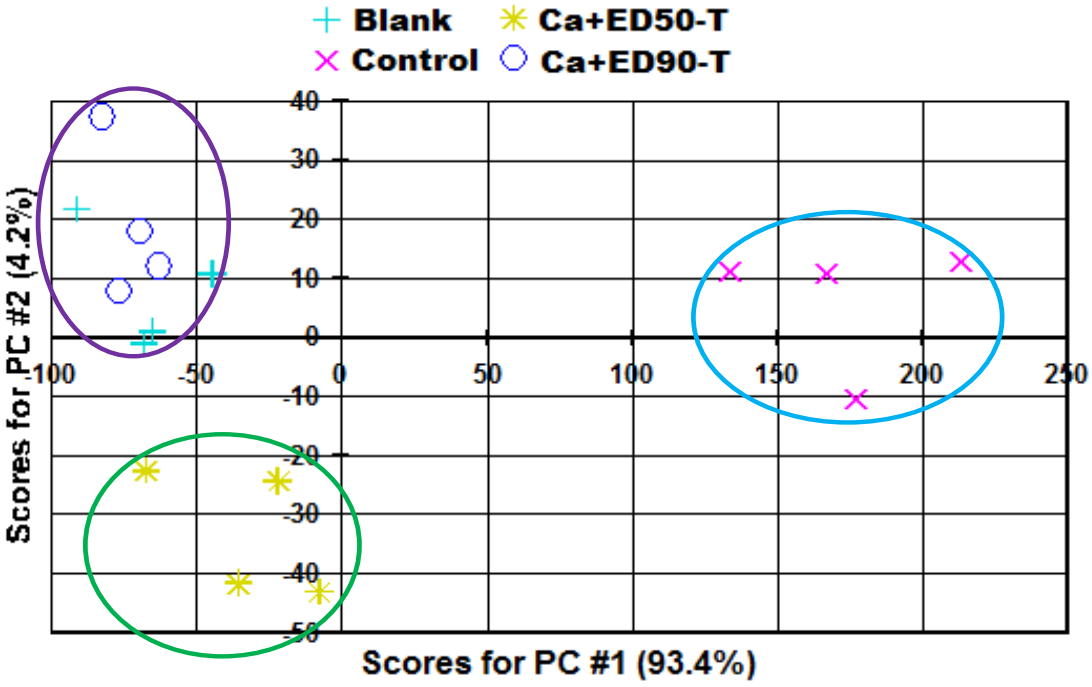



**Figure 3.16:** PCA score plot PCA *C.albicans* after 48 hrs incubation in NB at 25°C. (Key: Blank: NB without inoculum; Control: *C.albicans* without anti-fungal; Ca+ED50/90-T: C.albicans+$ED_{50/90}$-Tebuconazole).

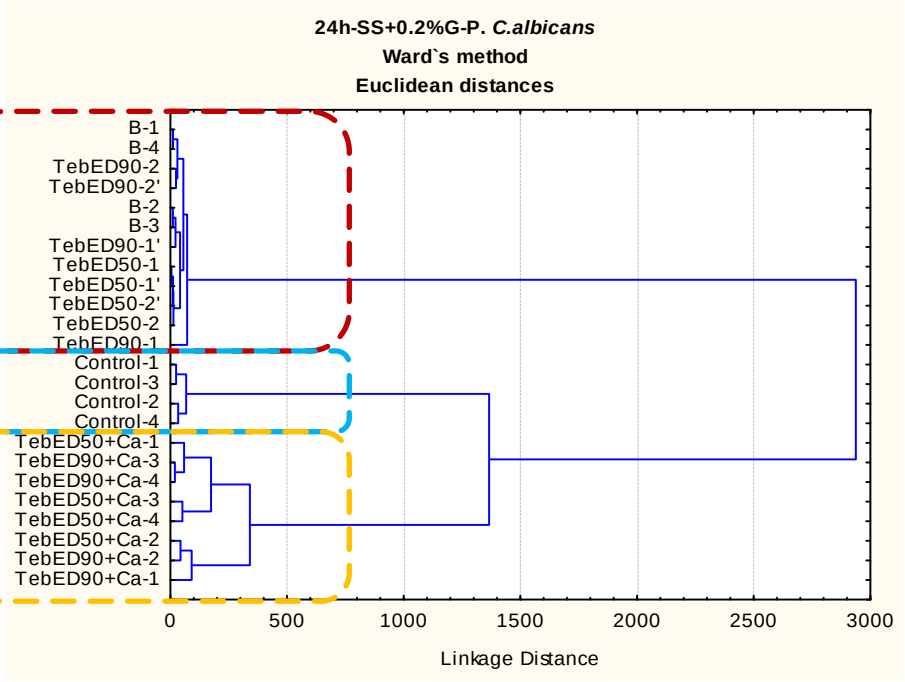



**Figure 3.17:** Dendogram of *C.albicans* after 24 hrs incubation at 37°C in saline solution + 0.2%G-P. (Key: B: Blanks; Control: *C.albicans* without anti-fungal; TebED90/50: $ED_{90}/ED_{50}$ concentrations of tebuconazole; TebED90/ED50+Ca: $ED_{90}/ED_{50}$ concentrations of tebuconazole+*C.albicans*).

# 3.5 IDENTIFICATION OF VOLATILES USING GC-MS AND SIFT-MS SYSTEMS

## 3.5.1 ANALYSIS OF VOLATILES OF 5 MICROORGANISMS GROWN ON NUTRIENT AGAR

Initially, studies were carried out to identify the MVOCs produced by all five VAP microbial species (*A.fumigatus*, *C.albicans*, *K.pneumoniae*, *E.cloacae* and *Staphylococcus* strain). One replicate of all the microbial species were grown on NA in Petri dishes at 37°C for 24 hrs in nalophan bags before head space sampling was carried out. Firstly, SIFT-MS system was used to identify key substances and afterwards, analysis of the headspace by TD-GC/MS (Thermal Desorption GC-MS) was performed.

As part of the TD-GC/MS method, AMDIS (Automated mass spectral deconvolution and identification system software) and NIST (National Institute of Standards and Technology) library data bases were used to search for the substance identification. Results were obtained in order of increasing retention time (RT) with one worksheet for each sample. For each peak detected on the chromatogram, the three most likely substances were listed in decreasing order of probability. Semi-quantitative estimation of the substances was made using the internal standard, D8-Toluene, added to all the TD tubes at a concentration of 50 ng . Table 3.3 summarises the key substances in samples obtained from the reports using the AMDIS software. Please note that substance such as styrene ($C_8H_8$) might have originated from the polystyrene Petri dishes as it is present in all the samples, although in different amounts. From Table 3.3 it can be seen that various sulphur-containing substances were found only in *A.fumigatus* such as 2,4-dithiapentane ($C_3H_8S_2$); or in larger concentration, such as dimethyl disulphide ($C_2H_6S_2$) also found in all the samples. However, the TD methodology used in this study is not particularly suited to sulphur compounds so the quantification data must be interpreted with caution. Other substances appeared to discriminate between samples, *e.g.*, 1,4-pentadiene ($C_5H_8$) present only in *A.fumigatus* and *E.cloacae*; 2-methyl-3-isopropylpyrazine ($C_8H_{12}N_2$) and 4,4-dimethyl-1-pentene

($C_7H_{14}$) in *A.fumigatus*; mercaptoacetone ($C_3H_6OS$) in *C.albicans*; 2-methyl-1-propanol ($C_4H_{10}O$) in *K.pneumoniae* and *E.cloacae* cultures. The ketone, 2-pentanone ($C_5H_{10}O$) was found in all the species except in the *Staphylococcus* strain.

3-methyl-butanal ($C_5H_{10}O$) was not observed in *A.fumigatus* and 3-methyl-1-butanol ($C_5H_{12}O$) was found in high concentrations in *E.cloacae* but not in blank medium and *A.fumigatus*. Benzaldehyde ($C_7H_6O$) was found in *C.albicans* and *Staphylococcus* species, also in blank medium. 3-methylfuran ($C_5H_60$) was only found in small amounts in cultures of *A.fumigatus* and *E.cloacae*.

**Table 3.3:** Summary of identified susbtances from GC-MS analyses. Microbial species were grown on NA at 37°C for 24 hrs. (Key: +++: 3,606-2,405 ng/l; ++: 2,404-1,203 ng/l; +: 1,202-2 ng/l; -: not found).

| Compound ID | Blank Medium | *Aspergillus fumigatus* | *Enterobacter cloacae* | *Candida albicans* | *Staph* species | *Klebsiella pneumoniae* |
|---|---|---|---|---|---|---|
| Sulphur dioxide | - | + | - | + | + | - |
| 2,4-dithiapentane | - | + | - | - | - | - |
| 1,4-Pentadiene | - | + | + | - | - | - |
| Furan, 3-methyl- | - | + | + | - | - | - |
| 1-Propanol, 2-methyl- | - | - | + | - | - | + |
| 1-Butanol, 3-methyl | - | - | +++ | ++ | ++ | + |
| Butanal, 3-methyl- | + | - | + | + | + | + |
| 2-Pentanone | - | + | + | + | - | + |
| Mercaptoacetone | - | - | - | + | - | - |
| Styrene | + | +++ | +++ | ++ | +++ | + |
| Dimethyl disulphide | + | +++ | ++ | + | + | + |
| Benzaldehyde | + | - | - | + | + | - |
| 2-Methyl-3-isopropylpyrazine | - | + | - | - | - | - |
| 1-Pentene, 4,4-dimethyl- | - | + | - | - | - | - |

SIFT-MS analysis was also performed. Only a few compounds were found distinctive between samples: ammonia ($NH_3$) was present in larger amount in *C.albicans*; methanethiol, also known as methyl mercaptan ($CH_4S$) in *A.fumigatus*; acetone was mainly found in *K.pneumoniae*, *A.fumigatus* and *C.albicans*; and propanol which was found in blank medium and bacterial species and barely found in fungal species. See Appendix C (Figure C.9) for the bar chart for this analysis.

### 3.5.2 IDENTIFICATION OF VOLATILES PRODUCED DURING EXPOSURE TO ANTI-FUNGALS

#### (a) *A.fumigatus* grown in presence of $ED_{50}$ concentrations of tebuconazole and benomyl

Volatile fingerprints were also analysed from *A.fumigatus* cultures on MEA, with and without $ED_{50}$ concentrations of tebuconazole and benomyl. MIM mode was used to extract the data on selected compounds: hydrogen sulphide, dimethyl disulphide, methanethiol, isoprene, methyl pentadiene, methanol, ethanol, propanol, acetonitrile, hydroxide cyanide, formaldehyde, acetaldehyde, acetone and ammonia. These substances were selected because most of them are considered common metabolites. Isoprene is an intermediate in the synthesis of cholesterol in animal cells but it is also produced by several bacterial and fungal species. Sulphur-containing compounds such as sulphides can be detected by SIFT-MS but not by the GC-MS method used which is not especially suited for those substances; also ammonia can be detected by the SIFT-MS technique. Microorganisms can produce acetaldehyde, ethanol and propanol in fermentative conditions.

Only the most abundant and most distinctive compounds extracted from the analysis were selected to build the bar graphs in Figures 3.18 and 3.19 for 48 hrs and 72 hrs incubation, respectively. In these bar graphs some compounds such as: dimethyl disulphide ($C_2H_6S_2$), isoprene ($C_5H_8$), methanol ($CH_4O$), acetone ($C_3H_6O$), methyl pentadiene (isomer not known) and ethanol ($C_2H_6O$) were found as possibly

discriminating compounds. There were no differences between the rest of the compounds targeted in the SIFT-MS analysis.

This experiment was repeated three times in total. However, the results obtained were not consistent. During the second set of experiments the capillary column was blocked, and during the third a significant decrease of the number of counts. This was found to be related to the overheating of the SIFT-MS device.

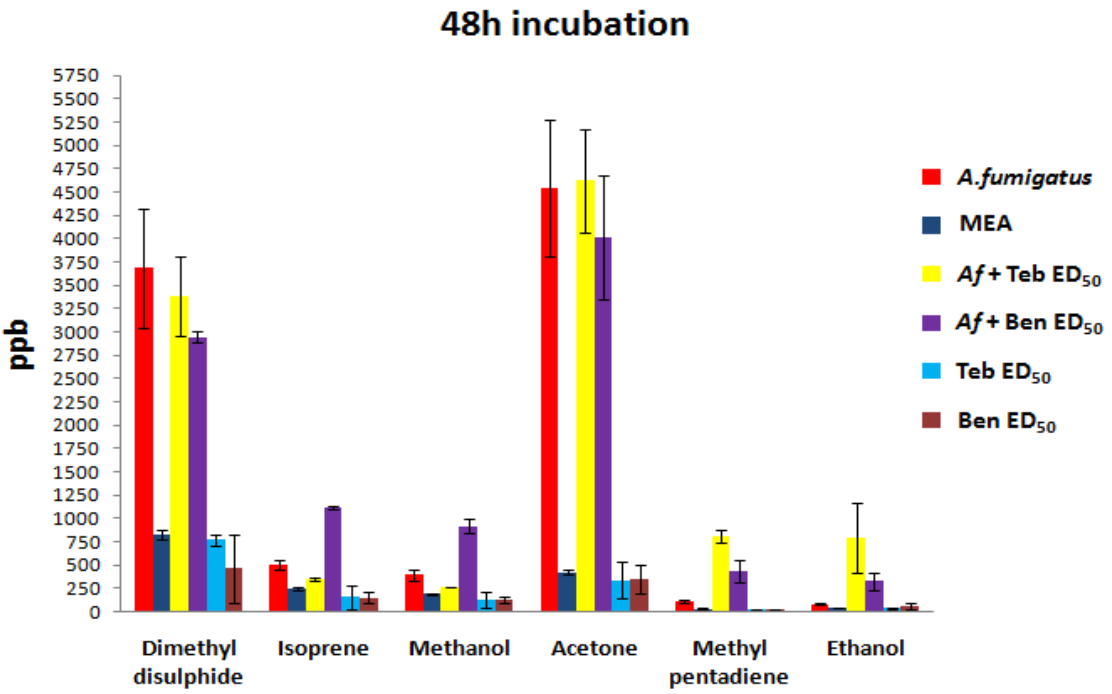



**Figure 3.18:** Average amount of different compounds detected after 48 hrs incubation using SIFT-MS. (Key: *A.fumigatus*: positive control; Af+TebED50/BenED50: *A.fumigatus* with $ED_{50}$ of tebuconazole/benomyl; Teb/Ben $ED_{50}$: negative control (MEA+ Teb/Ben $ED_{50}$); I: standard error bars).

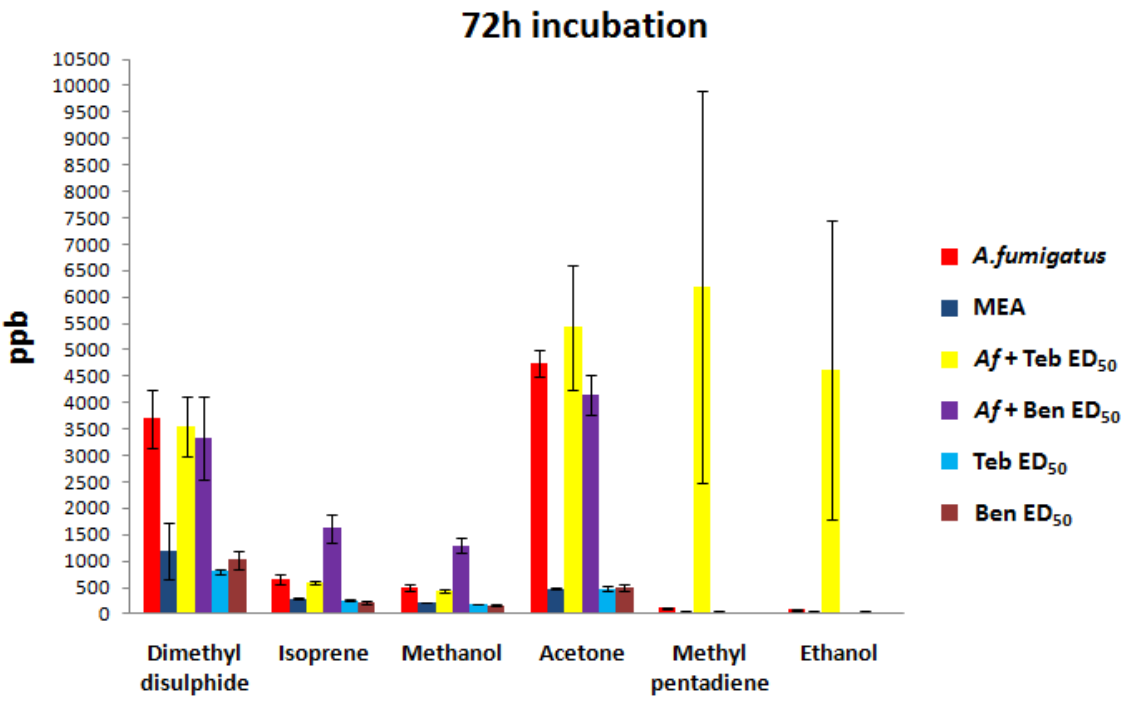



**Figure 3.19:** Average amount of different compounds detected after 48 incubation using SIFT-MS. (Key: same as Figure 3.18; I: standard error bars).

Chromatograms obtained from the TD-GC/MS system were also analysed using the data base AMDIS and NIST MS programmes. The analysis was firstly qualitative as visual inspection was made and quantitative of each treatment by using the internal standard as a calibration factor. The quantitative data analysis is not shown because it was not conclusive due to the differences between replicates in some treatments, the large number of peaks, the noise in the chromatograms and the lack of identification of the internal standard in some samples. However, the chromatograms of all treatments after 48 hrs incubation were compared in terms of treatments and over time (48, 72 hrs). The names and the RT of some compounds and, especially the internal standard (D8-toluene; 13.9-14 min), have been indicated in the chromatograms.

Figure 3.20 shows the chromatograms resulting from GC-MS analysis of all the treatments (control, *A.fumigatus* +tebuconazole and benomyl $ED_{50}$ concentrations) after 48 hrs incubation. Overall, the chromatograms are quite similar. The component 2-methyl-1,3-pentadiene ($C_6H_{10}$) appeared to be present with a larger peak in the *Af*+Teb

$ED_{50}$ treatment than in the others. This would correspond with what was observed in the SIFT-MS analyses.

Figure 3.21 compares the volatiles obtained from an empty bag, one replicate for Ben $ED_{50}$ (Teb $ED_{50}$ was omitted because of the high background) and one negative control (MEA without any anti-fungal). In these cases some compounds had evident peaks in the blank samples (MEA and Ben $ED_{50}$) which did not appear in the empty bag head space such as the aldehydes 2 and 3-methyl-butanal ($C_5H_{10}O$) and dimethyl disulphide ($C_2H_6S_2$). Comparing both sets of chromatograms it is possible to identify different compounds from the head space of *A.fumigatus* such as 2-methyl-1-propanol ($C_4H_{10}O$) and 3-methyl-1-butanol ($C_5H_{12}O$) which were not found in the blank samples. Components such as dimethyl disulphide, sulphur dioxide ($SO_2$), acetaldehyde ($C_2H_4O$), pentane ($C_5H_{12}$), acetone ($C_3H_6O$), isopropanol ($C_3H_8O$), benzene ($C_6H_6$) among others were all found in the blanks and fungal treatments. The chromatograms from all treatments after 72 hrs incubation showed the same trend as was found at 48 hrs incubation (data no shown).

Unfortunately not many differences were found between treatments to explain the good discrimination achieved using the e-nose when the quantitative analysis of the GC-MS studies was performed. Some compounds targeted with SIFT-MS system were not identified in the chromatograms such as isoprene, methanol and ethanol and the rest did not show the same pattern. This was probably due to the difficulty of the GC-MS system to identify lower molecular weight species. Compounds such as 3 and 2-methyl butanal, 2-methyl propanal ($C_4H_8O$) were predominantly found in blank media, whereas 3-methyl-butanol, 2-methyl-1-propanol and pentadiene were found exclusively in control and treatment samples. Acetone, sulphur dioxide and dimethyl disulfide were found in all samples, negative controls and treatments. The analysis of the results was partially inconclusive due to the large variance between replicates especially for *Af*+Ben $ED_{50}$.

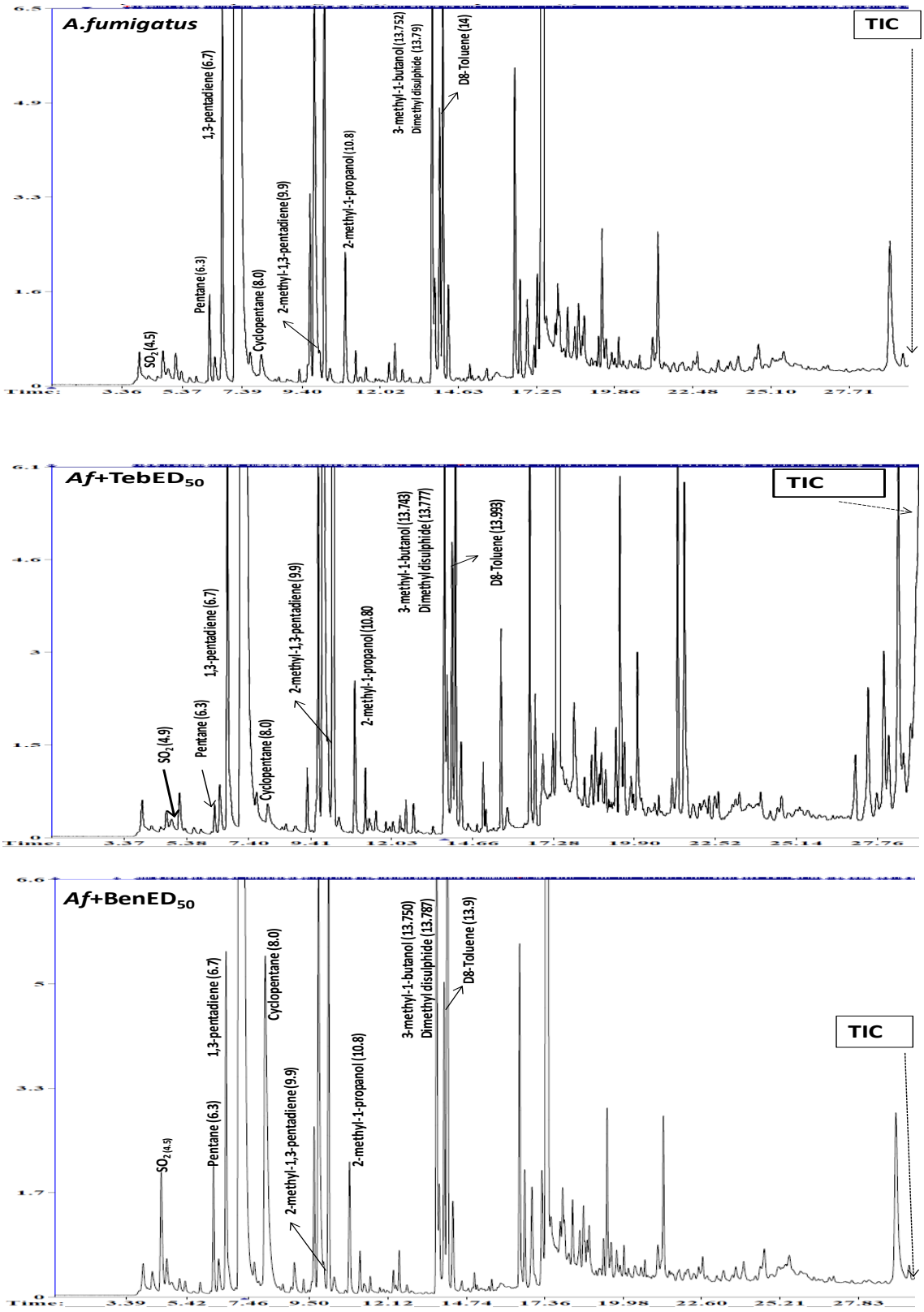



**Figure 3.20:** GC-MS ion chromatograms after 48 hrs incubation for three types of samples (Key: TIC: Total ion current).

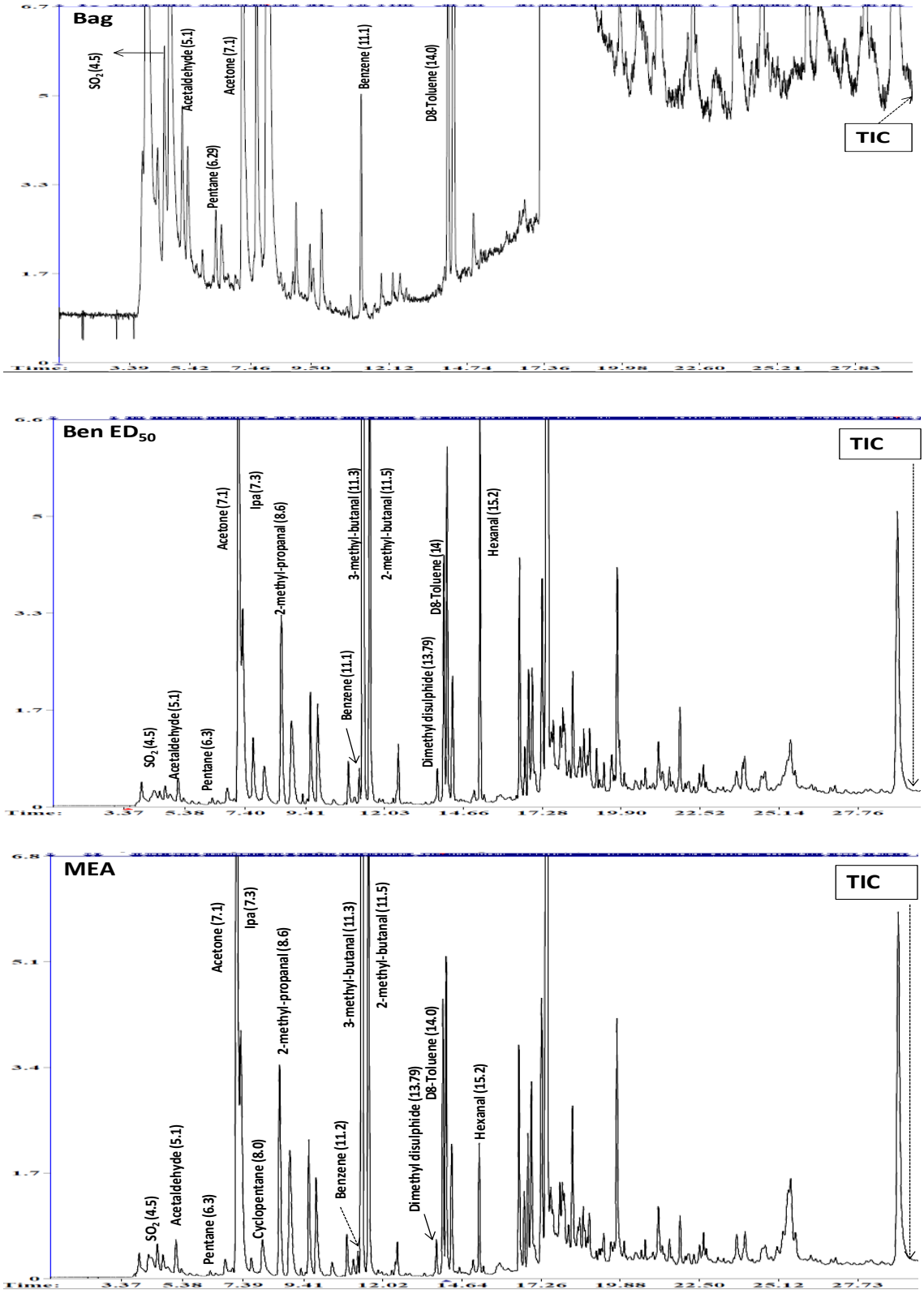



**Figure 3.21:** GC-MS ion chromatograms after 48 hrs incubation for one blank of each type. (Key: TIC: Total ion current).

**(b) *C.albicans* grown in presence of $ED_{50}$/$ED_{90}$ concentrations of tebuconazole**

This experiment involved *C.albicans* with $ED_{50}$ and $ED_{90}$ concentrations of tebuconazole in saline solution amended with 0.2% glucose and 0.2% peptone. Three replicates of the control and for both anti-fungal treatments were analysed using the GC-MS as detailed in Section 2.7b. Two replicates of the same medium without inoculum were used as negative controls.

The chromatograms showed that some alcohols were more abundant in the controls such as ethanol ($C_2H_6O$), 2-methyl-1-propanol ($C_4H_{10}O$), 2 and 3-methyl-1-butanol ($C_5H_{12}O$), whereas some aldehydes such as 3 and 2-methyl-butanal ($C_5H_{10}O$) and 2-methyl-propanal ($C_4H_8O$) were found in slightly higher amounts in the $ED_{50}$ treatments (Figure 3.22). In order to compare between treatments, two GC-MS spectra were combined in the same graph. Figure 3.23 shows the comparison between one control and one $ED_{50}$ treatment and in Figure 3.24 between both treatments ($ED_{50}$ and $ED_{90}$).

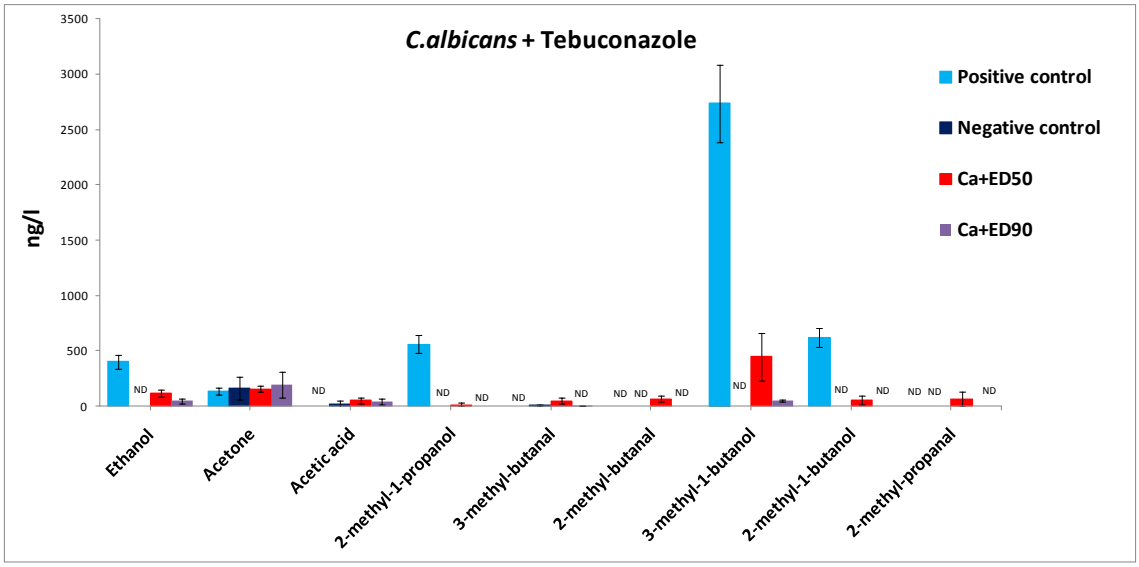



**Figure 3.22:** Mean values of some compounds found in cultures of *C.albicans* with tebuconazole. (Key: Ca+ED50/90: *C.albicans* with $ED_{50}$/$ED_{90}$; ND: no detected values).

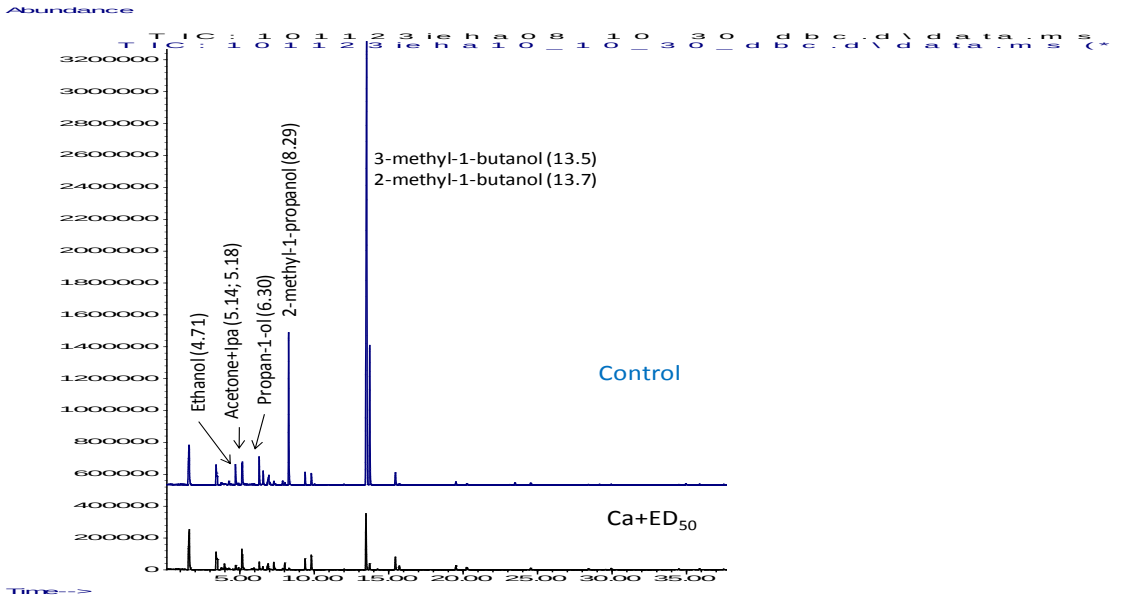



**Figure 3.23:** GC-MS chromatograms for *C.albicans* control in blue and for *C.albicans* treated with $ED_{50}$ of tebuconazole ($Ca+ED_{50}$) in black after 24 hrs incubation. (Both chromatograms on same scale).

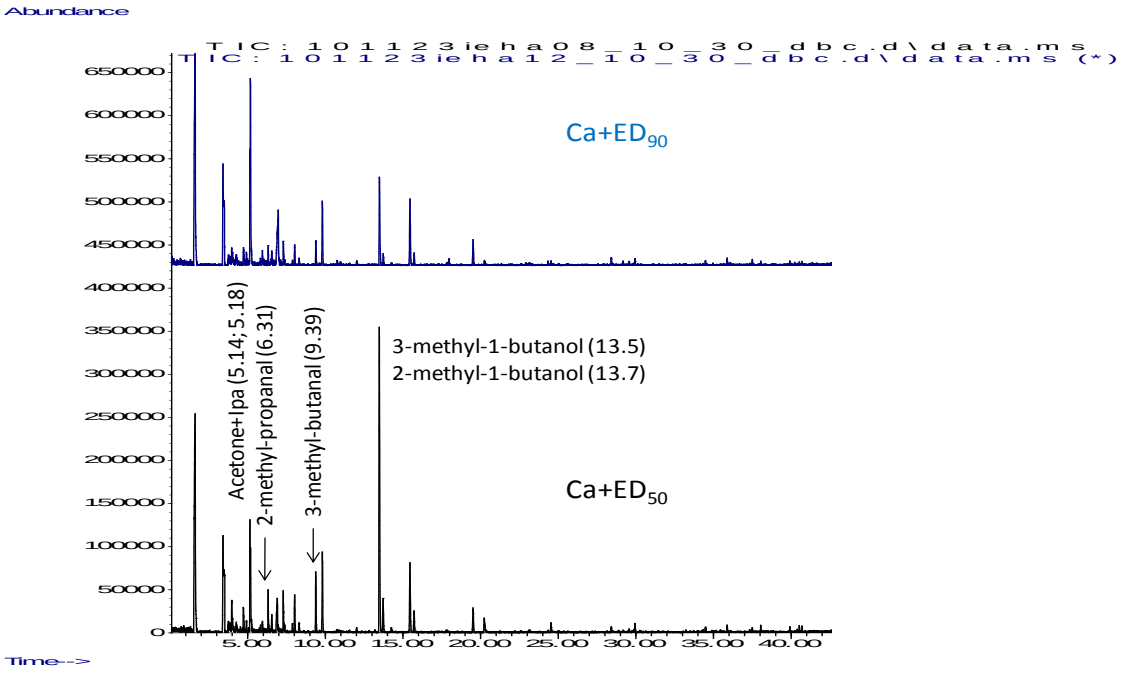



**Figure 3.24:** GC-MS chromatograms for *C.albicans* treated with $ED_{50}$ /$ED_{90}$ of tebuconazole ($Ca+ED_{50}$/ $Ca+ED_{90}$) in black and blue respectively after 24 hrs incubation. (Both chromatograms on same scale).

Others volatile compounds were not significantly different between treatments: acetone and acetic acid were also detected in the blank samples (Figure 3.25). Note that compounds found in unused tubes were not included. An example is shown in Figure 3.26. These included trifluorobenzene ($C_6H_3F_3$), benzene ($C_6H_6$), internal standard, heptamethyl cyclotetrasiloxane (HMCTS; $C_7H_{22}O_4Si_4$), acetone ($C_3H_6O$), acetaldehyde ($C_2H_4O$).

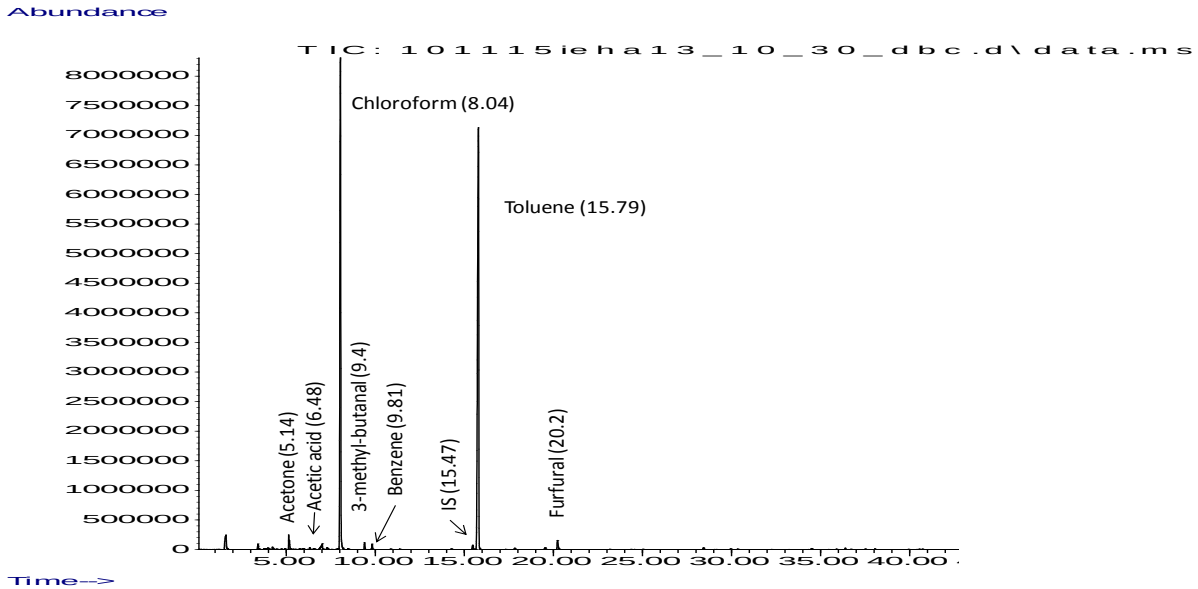



**Figure 3.25:** GC-MS chromatogram for one blank sample after 24 hrs incubation.

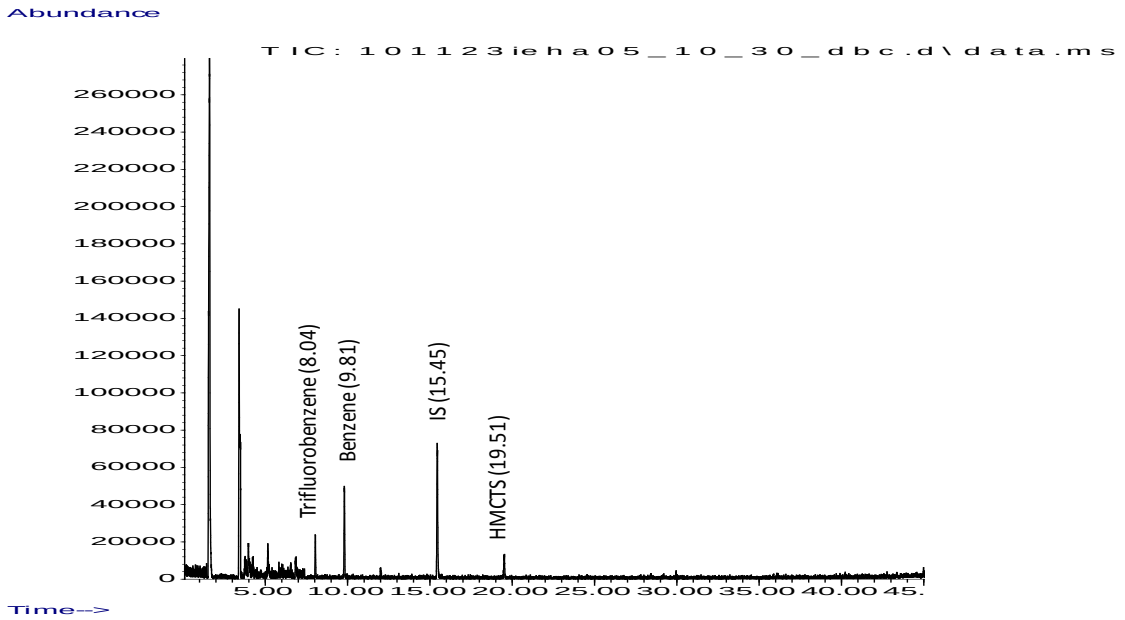



**Figure 3.26:** GC-MS chromatogram for one unused tube.

## 3.6 COMPARISON OF DISCRIMINATION BETWEEN 5 MICROORGANISMS USING NUTRIENT BROTH AND SALINE SOLUTION MEDIUM

Because clinical samples are obtained from patients in saline solution it was necessary to compare the discrimination based on the best nutritional medium with saline solution for evaluating the ability to directly use saline samples for analyses.

Experiments with NB were carried out a number of times with very similar results in terms of VAP microorganism discrimination using the e-nose. Thus after 24 hrs incubation with an initial cell/spore concentration of $10^4$ $ml^{-1}$, it was possible to clearly differentiate between four different clusters: one with the two Gram-negative bacteria, a second one with *C.albicans*, a third one with the *Staphylococcus* strain and a fourth one with the blanks and the *A.fumigatus* treatments. Figure 3.27 shows the PCA obtained and Figure 3.28 the dendrogram based on the volatile fingerprints obtained. After 48 hours, *A. fumigatus* could be distinguished from the blank samples but was placed very close to the *Staphylococcus* strain and *C.albicans* clusters. The discrimination did not improve after 72 or 96 hrs incubation (data not shown).

Since saline solution is relatively poor nutritionally, it was decided to examine the addition of two concentrations of glucose and peptone, 0.1% and 0.2% and examine the potential for discrimination between three microorganisms: *C.albicans, K.pneumoniae* and *S.aureus*. The final concentration for all the microorganisms was $10^4$ cells/spores $ml^{-1}$. The analysis of the head space of samples and treatments using the e-nose after 24 hrs incubation at 37°C showed better discrimination between microorganisms utilising 0.2% glucose and 0.2% peptone. Figures 3.29 and 3.30 show the PCA score plots for both concentrations.

Subsequently, the head space of the five VAP microbial species grown in saline solution amended with 0.2% of glucose and 0.2% peptone at 37°C were analysed daily with the e-nose. After the first 24 hrs incubation, the PC analysis showed four clusters (Figure 3.31): one for both Gram-negative bacteria, a second one for *C.albicans*, a third

one for *S.aureus* and a fourth one for *A.fumigatus* very close to blank samples. Figure 3.32 shows the clustering obtained for this data set. The experiment was repeated with very similar results (Figures 3.33 and 3.34). These measurements were used later to build a laboratory based model (Section 3.7).

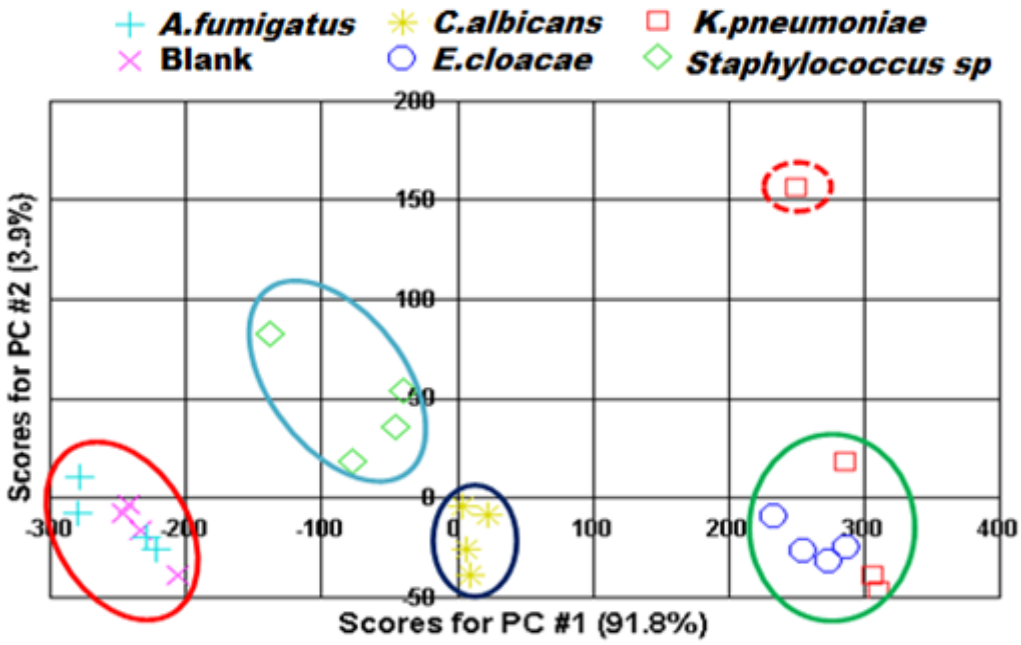



**Figure 3.27:** PCA score plot after 24 hrs incubation at 37°C of five microorganisms in NB media. Dotted line circle indicates one probable outlier.

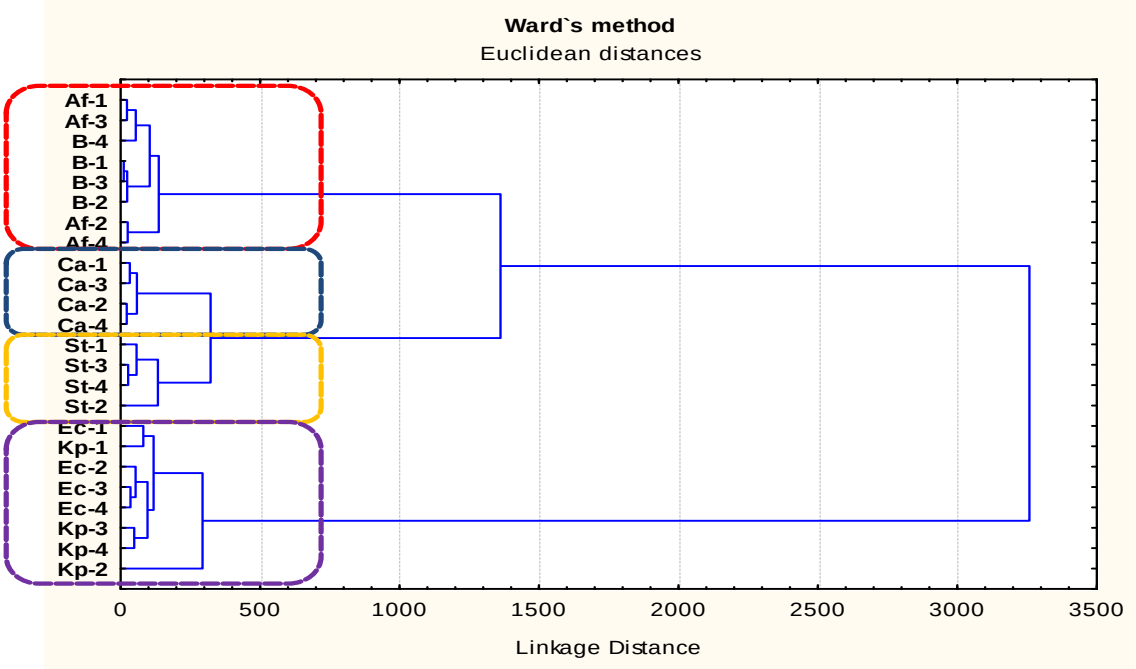



**Figure 3.28:** Dendrogram of five different microorganisms after 24 hrs incubation at 37°C in NB. (Key: Af: *A.fumigatus*; Ca: *C.albicans*; Ec: *E.cloacae; Kp*: *K.pneumoniae*; St: *Staphylococcus* species).

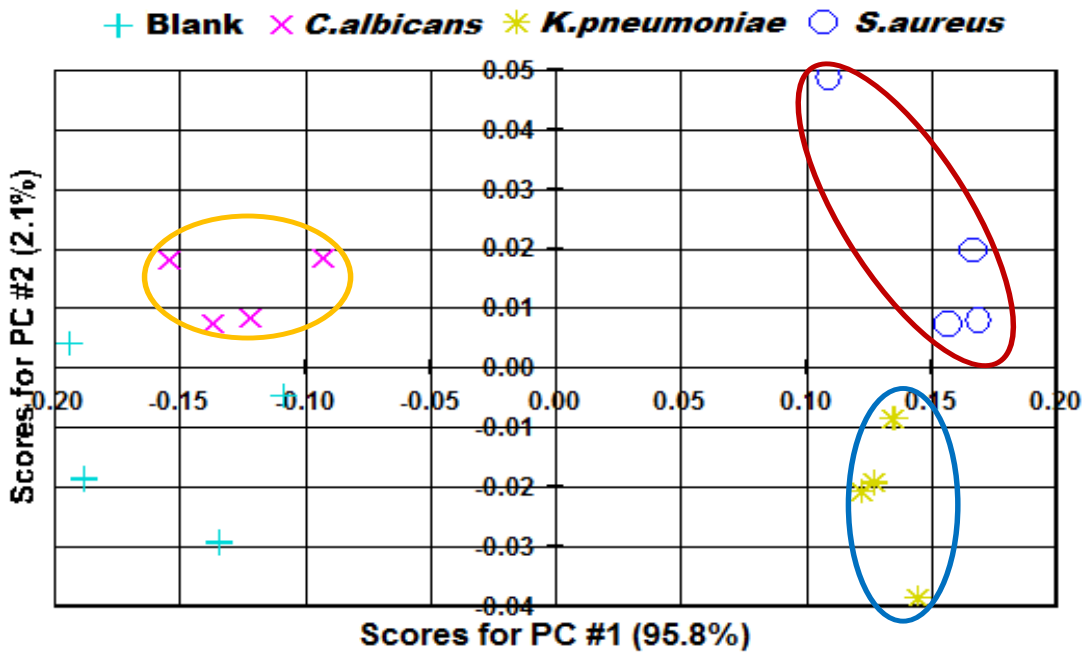



**Figure 3.29:** PCA score plot after 24 hrs incubation at 37°C in saline solution+0.1% Glucose-0.1% Peptone. Data was normalised.

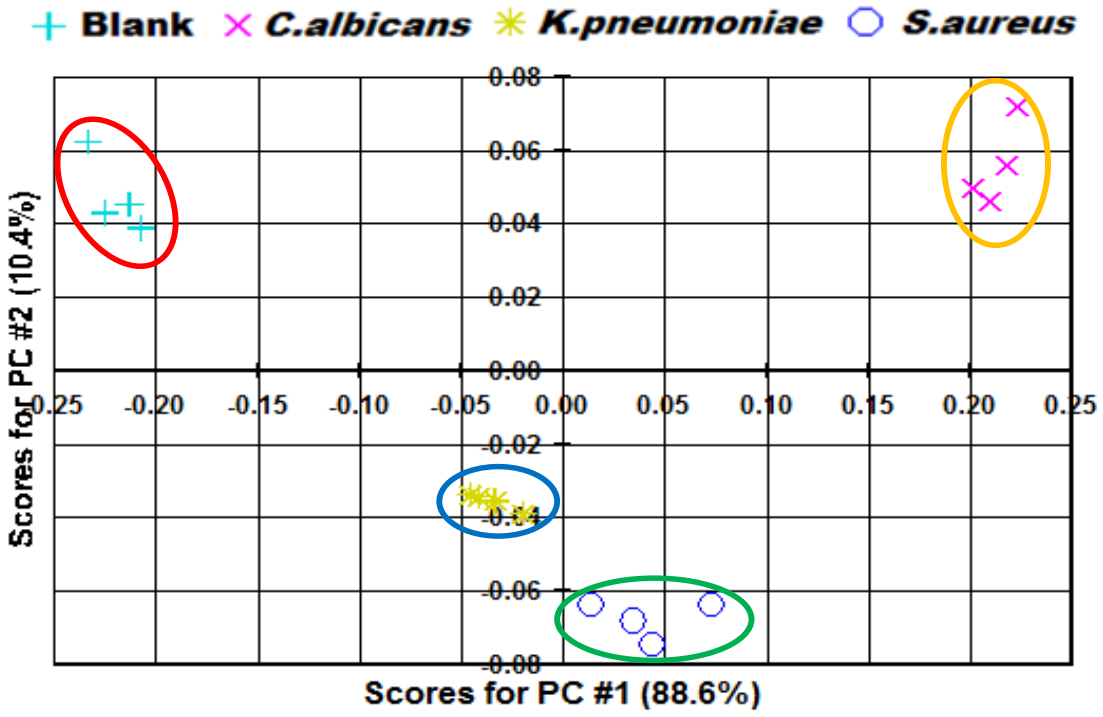



**Figure 3.30:** PCA score plot after 24 hrs incubation at 37°C in saline solution+0.2% Glucose-0.2% Peptone. Data was normalised.

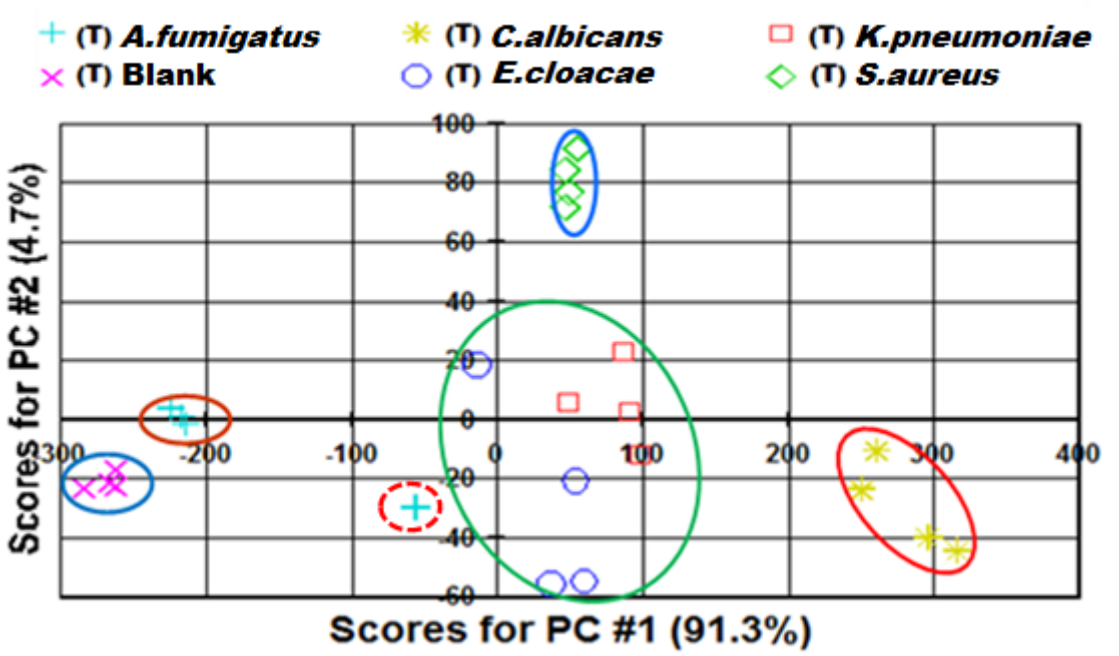



**Figure 3.31:** PCA score plot after 24 hrs incubation at 37°C in saline solution+0.2% Glucose-0.2% Peptone. Red dashed line: outlier.

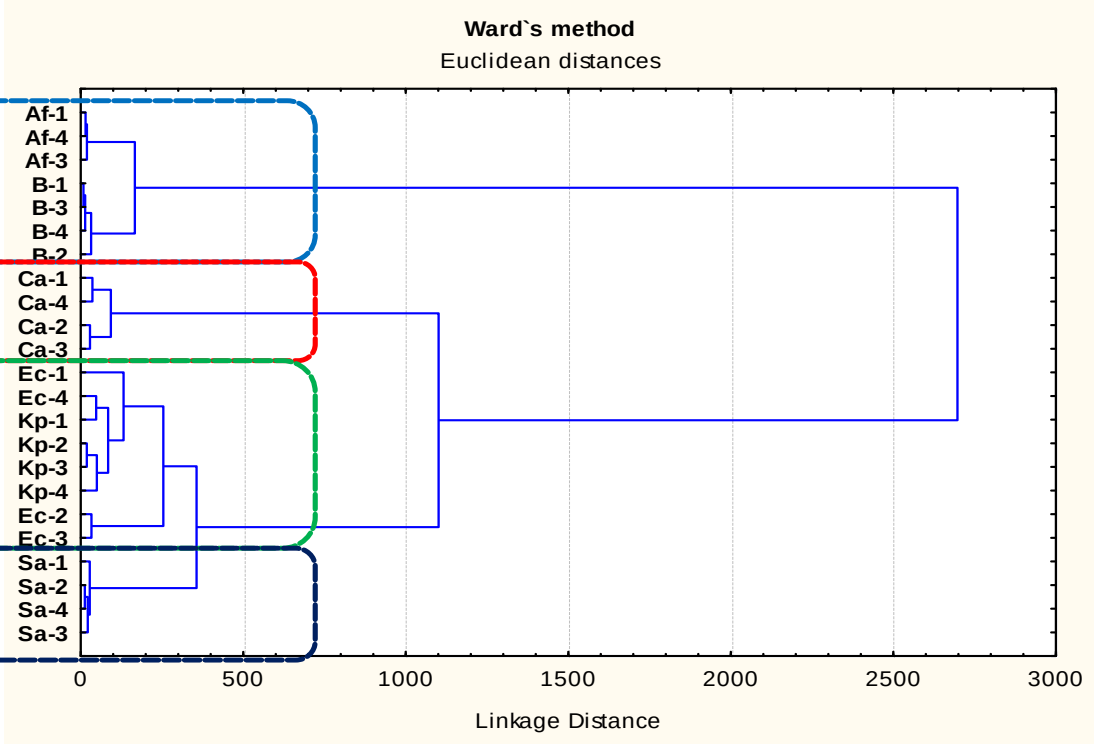



**Figure 3.32:** Dendrogram after 24 hrs incubation of five microorganisms in saline solution with 0.2% glucose and 0.2% peptone. (Key: Af: *A.fumigatus*; Ec: *E.cloacae*; Ca: *C.albicans*; Kp: *K.pneumoniae*; Sa: *S.aureus*). One outlier was removed.

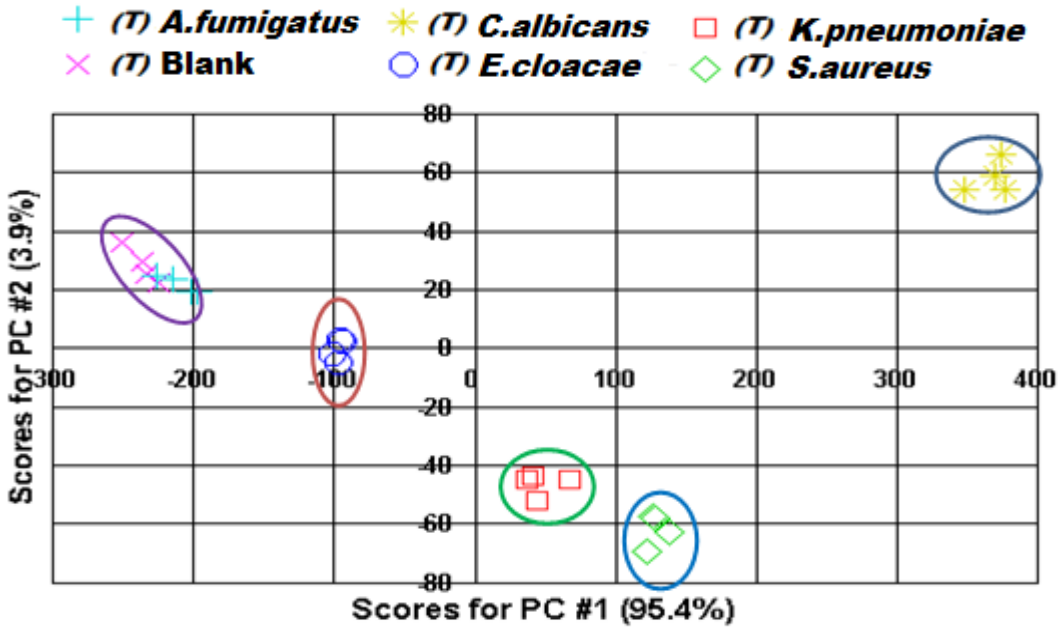



**Figure 3.33:** PCA score plot after 24 hrs incubation at 37°C in saline solution+0.2% Glucose-0.2% Peptone. This was a repeated experiment.

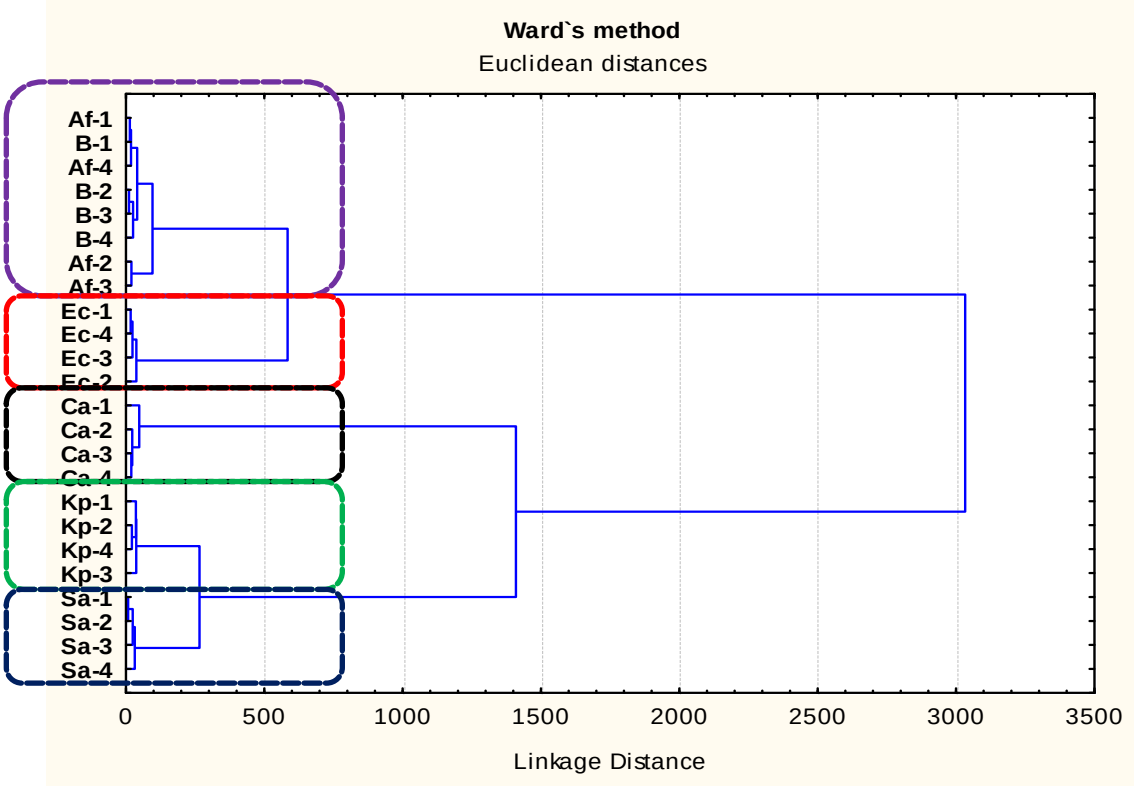



**Figure 3.34:** Dendrogram after 24 hrs incubation of five microorganisms in saline solution with 0.2% glucose and 0.2% peptone. (Key: B: Blanks; Af: *A.fumigatus*; Ec: *E.cloacae*; Ca: *C.albicans*; Kp: *K.pneumoniae*; Sa: *S.aureus*). This was a repeated experiment.

## 3.7 USE OF DATA FROM NUTRIENT BROTH AND SALINE SOLUTIONS ON THE VAP MICROORGANISMS TO BUILD A MODEL FOR PREDICTION OF CLINICAL SAMPLES

The data sets obtained above were used to develop and build a model to enable unknown clinical samples to be evaluated based on volatile fingerprints. DA was the method chosen to select a reduced number of variables (sensors) with a training set of samples in order to build the model for classifying a validation set. Once the model was built it was necessary to assess the accuracy of the predictive model using a cross-

validation method. For this, leave-one-out (LOO) was used for the validation of the model due to the number of samples needed to perform this cross-validation method.

Besides the mean-centred scale already performed by the software on the e-nose measurements, other scale methods were examined including range-scale and normalisation. It was also necessary to perform some kind of standardisation between sets of samples because they were taken on different days. To do this, the measurements from blank samples (nutrient broth; saline solution amended with 0.2% of glucose and peptone) were used to compensate for the measurement variations between different sets using MDC, explained in Section 2.8.3.

For the NB model, two different days of measurements made after 24 hrs incubation at 37°C, were used to perform the multivariate analysis. One day for the training set (n=30), the second one for validation purposes (n=24). A set of selected sensors (listed below) was found the best for "unknown" sample prediction. Three samples out of the 24 "unknown" samples were misclassified; this gave 87.5% accuracy prediction. Table 3.4 shows the confusion matrix for this model. Cross-validation LOO was used to check the accuracy of the training set. Every time one sample was removed and identified using the rest of the training set for classification. The mean of the conditional probability of misclassification (-2loglikelihood) was 2.3E-06. Figures 3.35 and 3.36 depict the canonical plots in 2D and 3D respectively for the DA performed with the data obtained in NB. A total of 8 sensors were selected to build the model as they were most discriminating: MOSFET 102A, 104A, 102-3B and MOS 113-5, 117.

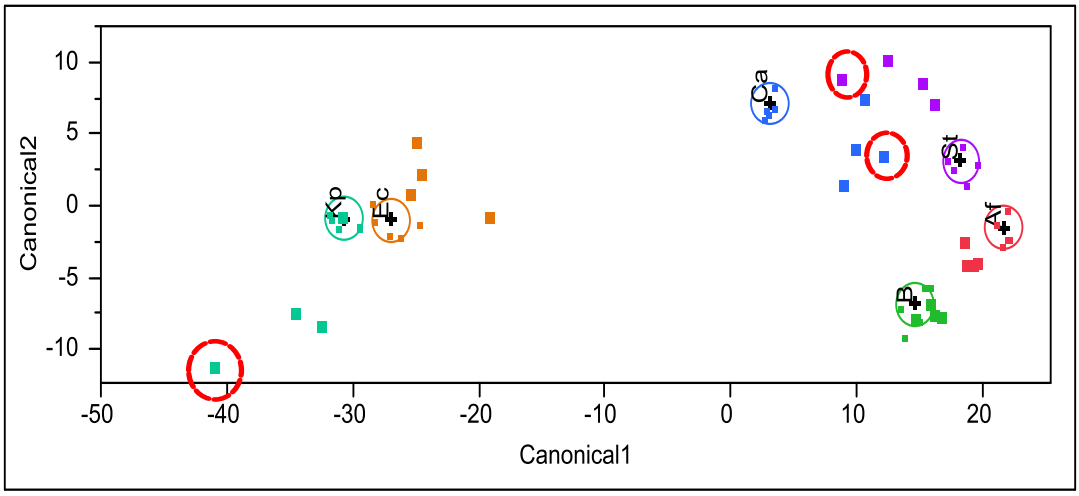



**Figure 3.35:** Canonical plot for DA of training and validation set (highlight squares) in NB after 24 hrs incubation. Dotted circles show misclassified samples. Selected sensors: MOSFE102A, 104A, 102-3B, MOS113-5, 117. (Key: Af: *A.fumigatus*; Ec: *E.cloacae*; Ca: *C.albicans*; Kp: *K.pneumoniae*; St: *Staphylococcus* species; B: Blanks).

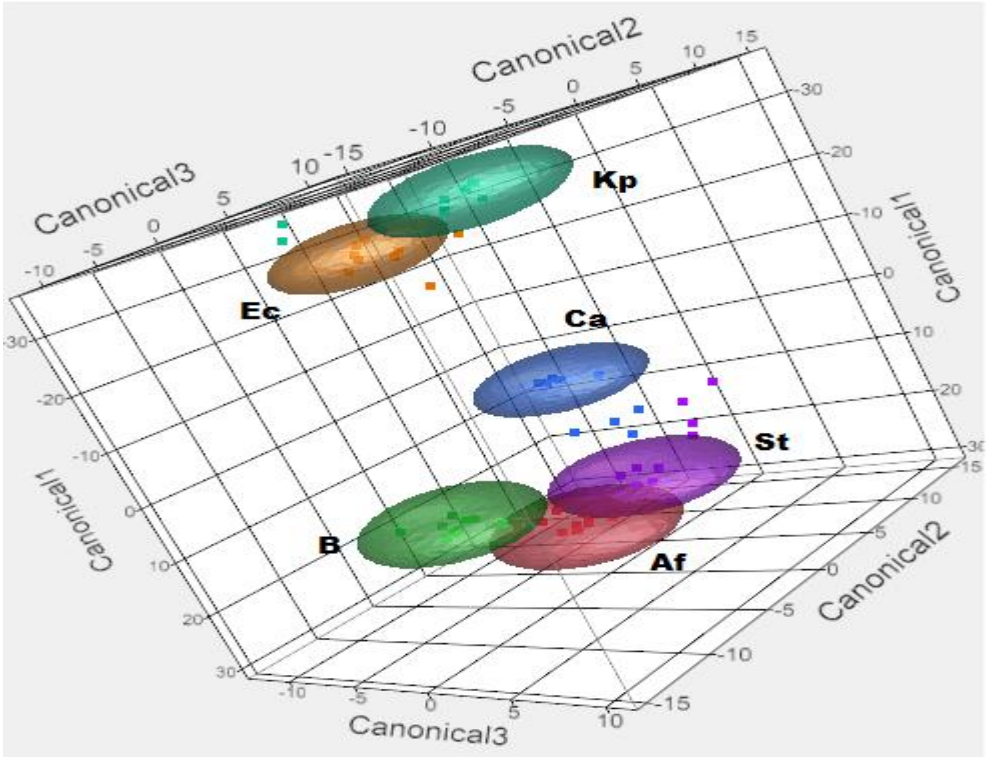



**Figure 3.36:** Canonical 3D plot for DA of training and validation set (highlight squares) in NB after 24 hrs incubation. (Key: same as Figure 3.35).

**Table 3.4:** Confusion matrix showing the performance of the model in NB at 37°C after 24 hrs incubation. Accuracy of 87.5% (21/24). (Key: Af: *A.fumigatus*; B: Blanks; Ca: *C.albicans*; Ec: *E.cloacae*; *K.pneumoniae*; St: *S.aureus*).

| Actual Rows by Predicted Columns | Af | B | Ca | Ec | Kp | St |
|---|---|---|---|---|---|---|
| **Af** | **4** | 0 | 0 | 0 | 0 | 0 |
| **B** | 0 | **4** | 0 | 0 | 0 | 0 |
| **Ca** | 0 | 0 | **3** | 0 | 0 | 1 |
| **Ec** | 0 | 0 | 0 | **4** | 0 | 0 |
| **Kp** | 0 | 0 | 0 | 1 | **3** | 0 |
| **St** | 0 | 0 | 1 | 0 | 0 | **3** |

The analysis of volatiles from microbial cultures in saline solution amended with glucose and peptone by using the e-nose was done three times on three different days. Several models were tried after the collection of that data. Table 3.5 summarises all these models with their classification results and the number of samples per set, training and validation sets. For models C, D, E and F, one replicate of *A.fumigatus* was removed from all the analyses.

With model A used as a training set, a group of measurements done with 5 microorganisms as a training set and only 3 microorganisms as "unknown samples", the percentage of misclassified was zero. Blank sample replicates were also analysed. A second model was investigated (model B) with 5 microorganisms in the validation set. The prediction accuracy was 83.3%. Four samples over twenty four were misclassified. Table 3.6 shows the confusion matrix and Figure 3.37 shows the 3D score plot for this analysis. The selected sensors for this model were the following seven: MOSFET102B, MOS102, 104, 112, 114-6.

The accuracy of the results obtained from the DA were checked using the LOO cross-validation method which might be considered an overly optimistic method as only one sample is being removed each time. To assess the accuracy of a training set by removing a complete set of replicates instead of one single replicate every time it is necessary to have at least two sets of replicates from the same class. This was only

possible with model G. In this model the accuracy was not good as only 50% of the samples were classified, although the misclassification occurred between *A.fumigatus* and blank samples after 24 hrs incubation. Please note that for the attempt at clinical sample prediction one of the *in vitro* models was used and a more robust cross-validation method such as Monte Carlo simulation was utilised.

**Table 3.5:** Summary of results for different models analysed by DA in saline solution.

| Model | Training set | | Validation set | | % misclassified | -2loglikelihood (mean) LOO method for cross validation |
|---|---|---|---|---|---|---|
| | Number of samples | Total nr. microorg | Number of samples | Total nr. microorg | | |
| **A** | **24** | **5** | **16** | **3** | **0** | **17.7** |
| **B** | **24** | **5** | **24** | **5** | **16.6 (4/24)** | **0** |
| **C** | **23** | **5** | **40 (16+24)*** | **5** | **27.5 (11/40)** | **0** |
| **D** | **24** | **5** | **39 (23+16)*** | **5** | **20.5 (8/39)** | **8.33e-18** |
| **E** | **39 (23+16)*** | **5** | **24** | **5** | **20.8 (5/24)** | **1.16e-02** |
| **F** | **40 (24+16)*** | **5** | **23** | **5** | **39.1 (9/23)** | **2.15e-06** |
| **G** | **48 (24+24)*** | **5** | **16** | **3** | **50 (8/16)** | **6.44** |

* When sets are composed of two different days of measurements.

**Table 3.6:** Confusion matrix showing the performance of model B in saline solution at 37°C after 24 hrs incubation. Accuracy of 83.3% (20/24). (Key: Af: *A.fumigatus*; B: Blanks; Ca: *C.albicans*; Ec: *E.cloacae*; *K.pneumoniae*; St: *S.aureus*).

| **Actual Rows by Predicted Columns** | ***Af*** | **B** | ***Ca*** | ***Ec*** | ***Kp*** | ***Sa*** |
|---|---|---|---|---|---|---|
| ***Af*** | **3** | 0 | 0 | 1 | 0 | 0 |
| **B** | 2 | **2** | 0 | 0 | 0 | 0 |
| ***Ca*** | 0 | 0 | **4** | 0 | 0 | 0 |
| ***Ec*** | 0 | 0 | 0 | **4** | 0 | 0 |
| ***Kp*** | 0 | 0 | 0 | 0 | **4** | 0 |
| ***Sa*** | 0 | 0 | 0 | 0 | 1 | **3** |

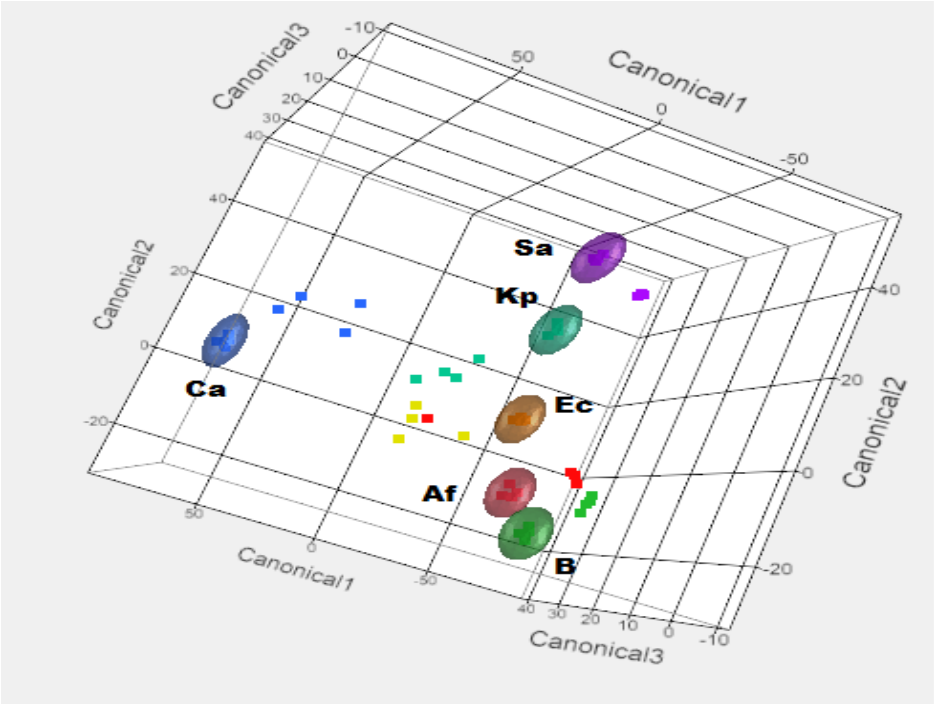



**Figure 3.37:** Canonical 3D plot for model B. Clouds correspond to training set samples. Highlighted squares correspond to validation set samples. Selected sensors for this model: MOSFET102B, MOS102, 104, 112, 114-6 (Key: Ca: *C.albicans*; Sa: *S.aureus*; Kp: *K.pneumoniae*; Ec: *E.cloacae*; Af: *A.fumigatus*; B: Blanks).

Unfortunately, no promised patient samples materialised during the course of this PhD for analyses. Thus necessitated the use of a previous data set from patients which was originally analysed by Sahgal (2008). Based on the microbiological results obtained from the hospital laboratory (standard microbiological culturing), attempts were made to predict which VAP microorganisms may be present in the clinical samples using the laboratory-based models and DA. In this previous study, blind BAL samples were taken by the clinician from patients with mechanical ventilation for more than 72 hrs at Gloucestershire Hospitals NHS Foundation Trust. Ethical approval was obtained at the time of the study. Every sample was divided in two parts, for microbiological and e-nose analyses. The sampling procedure was described by Sahgal (2008) and it is in the recent publication by Humphreys *et al*. (2011).

*In vitro* data sets from both NB and saline solution were utilised to attempt the prediction of clinical samples (validation set; n=88). The clinical data set was split into four groups: Gram-positive (n=11), Gram-negative (n=8), fungi (n=13) and blanks (no growth; n=56). Samples with mixed cultures were removed for the analysis. For NB, three sets of data obtained on different days done on two e-noses were gathered to complete a training set of ninety four samples. With no standardisation between e-noses performed, forty one samples were misclassified, giving an accuracy of 53.41%. For saline solution two sets of data measured in the same e-nose were checked for the prediction (n=40) of clinical samples analysed in a different e-nose. Thirty two samples were wrongly predicted, giving an accuracy of 63.63%. Table 3.7 summarises these results.

Subsequently, the PLS-DA technique in collaboration with Dr. Michael Cauchi from Bioinformatics Group was attempted to classify the set of clinical samples with a single set of laboratory-based model. First of all it was necessary to standardise the two e-nose systems using a technique called piecewise direct standardisation (PDS) or direct standardisation (DS). The same set of standards was measured on both instruments. A mathematical model is then generated (different window sizes were investigated for PDS) and then the mathematical model used to predict what responses the samples would have attained on the instrument being standardised. These responses are then used to classify the clinical samples which were divided into four groups instigating a 4-

way classification: Gram-positive, Gram-negative, Fungi and No Growth; a fifth group comprising mixed microorganisms was omitted because there were an insufficient number of samples. Different scale methods were tested and the percentage of correctly classified samples was calculated in each case. Table 3.8 summarises all these results.

**Table 3.7:** Summary of results for two *in vitro* models for clinical samples prediction using DA.

| CLINICAL SAMPLES (n=88) | | |
|---|---|---|
| Data base with *in vitro* samples in SS+0.2%G-P (n=40) | | Data base with *in vitro* samples in NB (n=94) |
| Number of misclassified | 32/88 | 41/88 |
| Accuracy of prediction | 63.63% | 53.41% |

The maximum overall of classification success was 63.6% when some selected sensors (MOSFET 101A, 103A, 101-2 B, MOS 104, 111, 113 and 118) were used for the analysis. However, this approach failed in terms of sensitivity, *i.e.*, only the no growth samples were 100% predicted whereas the rest of the groups were misclassified. The rest of the analyses show very variable percentages of prediction with a loss of classification in some of the groups.

**Table 3.8:** Summary of the attempts for classifying clinical samples using different standardisation methods, scale, latent variables (LV) with an *in vitro* model built in saline solution+0.2%G-P cultures after 24 hrs incubation at 37°C.

| Standardisation between devices | % Overall classification success | LV | Scale | % Gram pos classif. success | % Gram neg classif. success | % Fungi classif. success | % No growth classif. Success |
|---|---|---|---|---|---|---|---|
| PDS: Window 1 | 27.3 | 3 | MC | 45.4 | 0 | 61.5 | 19.6 |
| | 60.2 | 1 | None | 0 | 0 | 0 | 94.6 |
| | 35.2 | 5 | MC | 72.7 | 0 | 0 | 41.0 |
| | 63.6 | 1 | None | 0 | 0 | 0 | 100 |
| PDS: Window 5 | 61.4 | 3 | MC | 0 | 0 | 38.4 | 87.5 |
| | 35.2 | 7 | None | 0 | 0 | 53.8 | 42.8 |
| PDS: Window 11 | 60.2 | 3 | MC | 0 | 0 | 23.0 | 89.2 |
| PDS: Window 17 | 60.2 | 3 | MC | 0 | 0 | 23.0 | 89.2 |
| DS: Direct only | 9.0 | 3 | MC | 0 | 100 | 0 | 0 |
| | 42 | 4 | MC | 0 | 0 | 46.1 | 55.3 |

Also PLS-DA was used to attempt the prediction of clinical samples using the data generated in NB. Standardisation between devices was necessary. The results are summarised in Table 3.9. The best overall accuracy prediction was 52.27% with 13 or 14 LV with normalisation of the values. Only no growth group achieved a respectable 73.2% of accuracy prediction.

**Table 3.9:** Summary of results after PLS-DA assessment in NB with different latent variables (LV) and scales.

| Samples | LV | Scale | Overall | Gram positive | Gram negative | Fungi | No growth |
|---|---|---|---|---|---|---|---|
| NB | 12 | RS1* | 12.5% | 0 | 87.5% | 30.8% | 0 |
| NB | 13 | Norm | 52.27% | 0 | 0 | 38.56% | 73.2% |
| NB | 14 | Norm | 52.27% | 0 | 0 | 38.56% | 73.2% |
| NB | 11 | None | 38.64% | 0 | 0 | 0 | 60.7% |

* RS1: Range scale equation 2 (Section 2.8.3).

Monte Carlo simulation technique was utilised in all the cases to assess the significance of the results in order to ensure that the overall classified was not due to chance. Figure C.10 in Appendix C shows as a matter of example the simulation results displayed in a histogram obtained with one model in saline solution used to classify clinical samples.

# CHAPTER 4: GENERAL DISCUSSION

## 4.1 ANALYSES OF VOLATILES OF VAP MICROORGANISMS IN DIFFERENT MEDIA

Initially studies were carried out to determine the impact that different nutritional media would have on volatile production patterns produced by VAP microorganisms. This was done to optimise a medium which would give better discrimination under conditions which are preferential for growth of these species. Furthermore, media used to isolate the microbial species from different medical matrices may not be best for volatile fingerprint production. For example, Sahgal (2008) found earlier discrimination (72 hrs incubation) between different dermatophytes using Sabouraud Brain Heart Infusion Agar (SBHIA) which is less commonly used in medical microbiology laboratories than Sabouraud Dextrose Agar (SDA).

The studies in this thesis demonstrated that for an individual bacterial or fungal species markedly different volatile fingerprints were produced depending on the substrate. NB was better for early discrimination of *C.albicans* and *K.pneumoniae* and this might be due to more active growth and utilisation of nutrients than in the other media tested. The aim was to have a medium which would enable differentiation between VAP species within 48-72 hrs based on volatile fingerprints by analyses of the head space. Previous studies, predominantly on food matrices or food analogues have tried to simulate nutritional make up of the food type. For example, Needham *et al*. (2005) used flour or bread-based media to discriminate between spoilage moulds and bacteria within 48-72 hrs which are important in bakery products. Bread analogue was also prepared by mixing flour with others components for early detection of two *Eurotium* spp. and *Penicillium chrysogenum* (Keshri *et al*., 2002). Needham and Magan (2003) used wheat and maize-based media to try and discriminate between mycotoxin and non-mycotoxin producing strains of mycotoxigenic fungi. A similar approach was used successfully to taxonomically discriminate between ochratoxin A production by strains from the *Aspergillus* section *Nigri* group on grape juice medium (Cabañes *et al*., 2009) and for

*Penicillium nordicum* strains and ochratoxin A production in ham-based media (Camardo *et al*., 2011). In the latter case a much longer incubation time (>7 days) was required because of the slow growth rate to use volatile production patterns to be successfully used to discriminate between them. Other mycotoxigenic fungal strains such as *Fusarium* spp. were successfully discriminated from the uninoculated controls and from the non-mycotoxigenic strains after 48 hrs incubation in wheat meal agar before using the analysis of volatile fingerprints (Keshri and Magan, 2000). Other mycotoxins such as patulin were investigated by Karlshøj *et al*. (2007a) using an e-nose. They collected and analysed the head space of apples infected with *Penicillium expansum* used in the apple juice industry and built a model with PLS regression analysis for patulin prediction. Magan *et al*. (2001) found that by using milk-based media it was possible to discriminate between 5 different spoilage microbial species (three bacteria, two *Pseudomonas* spp. and *Bacillus cereus*; two yeasts, *Candida pseudotropicalis* and *Kluyveromyces lactis*) within 72 hrs. Others studies have investigated the differentiation of spoilage fungal species in more general agar media. Malt peptone agar was used to discriminate between seven species of *Agaricus* and between others homobasidiomycetes mushrooms (Keshri *et al*., 2003) after 14 days incubation at 25°C using a CP sensor array. A variety of food substrates have been investigated during the ripening/maturation process. Trihaas and Nielsen (2005), for example, assessed the quality and state of ripening of two types of Danish blue cheese by using an e-nose and multivariate analysis.

Laboratory media have been used for detection of medically important pathogens, including *H. pylori*, *P.mirabilis* and *S.aureus* (Pavlou *et al*., 2000). Pavlou *et al*. (2004) used Tween albumin medium for discriminating four bacteria (*M.avium, M.tuberculosis*, *M.scrofulaceum* and *P.aeruginosa*) and successfully classified 96% of the 15 randomly selected samples from the *in vitro* analysis using a CP sensor array and multivariate analyses and neural network methods. More complex media have been prepared by mixing BHIB, cooked meat broth, urea, some sugars and several amino acids to discriminate between four groups of UTI pathogens and correctly classify seven "unknowns" samples by using DA and cross-validation (Pavlou *et al*., 2002a).

In environmental applications, the analysis of volatile fingerprints has permitted the detection of water contaminants (Canhoto and Magan, 2005) and different soils environmental conditions such as temperature, moisture and nutrient addition (Bastos and Magan, 2007) in water and soils samples, respectively.

## 4.2 SENSITIVITY OF DETECTION OF VAP MICROORGANISMS

In the present study it was shown that the time necessary for achieving the minimum threshold of detection of specific microorganisms was achieved either earlier or later depending on the species and the length of incubation. For *A.fumigatus* ($10^2$ spores $ml^{-1}$) it was possible after 72 hrs growth for discrimination from blank samples. Previously, Sahgal (2008) using four different concentrations ($10^1$, $10^3$,$10^5$ and $10^7$ spores $ml^{-1}$) of two dermatophytes and found that only after 72 hrs growth and for the two highest concentrations was there discrimination. This is probably due to the much slower rate of growth of dermatophytes when compared to species such as *A.fumigatus.*

For *C.albicans* cultures, the discrimination between blank samples and $10^2$ cells $ml^{-1}$ was achieved after 48 hrs incubation. For *K.pneumoniae* and *E.cloacae* it was found that discrimination could be obtained at $10^2$ cells $ml^{-1}$ after 24 hrs, while the volatiles produced by $10^4$-$10^6$ cells $ml^{-1}$ were grouped together. However, results for the *Staphylococcus* species were not possible to interpret because of the very slow growth of this strain, regardless of concentration used.

Previous studies on a milk-based medium by Magan *et al.* (2001) examined the potential for discrimination between three very close but high concentrations ($10^6$, $3.5x10^8$ and $8x10^8$ cells $ml^{-1}$) of *Pseudomonas aureofaciens* after 60 min incubation. They also showed discrimination between butanol-skimmed milk control, skimmed milk control and *Candida pseudotropicalis* ($8x10^8$ cells $ml^{-1}$) in skimmed milk medium after 60 min incubation and for *S. aureus* and *Kluyveromyces lactis* (yeast) after 2 and 5 hrs incubation respectively using a 14 CP sensor array. This discrimination was achieved with a very high initial concentration of $10^8$ in milk which is unrealistic and

would have significantly enhanced the volatile production and contributed to the discrimination after subsequently short incubation time. Furthermore, only two microorganisms were tested.

Probably, concentrations of cells/spores in the range of $10^3$-$10^4$ $ml^{-1}$ over the incubation period is enough to produce volatiles which can be detected by e-nose systems. This range has been corroborated in studies of different microorganisms in food substrates. Keshri *et al.* (2002) found discrimination between moulds in bread analogues after 48 hrs incubation with initial spore concentration of about $10^3$ CFU $g^{-1}$. Although during this study the population, in terms of CFUs $ml^{-1}$, was not enumerated for each e-nose measurement, it was similar to the range of sensitivity that was found in the present studies. However, it would be useful to take into account and check the growth phase of individual microorganisms. Table 4.1 summarises and compares the available information on relative sensitivity of microorganisms in different matrices.

There is also evidence that volatile production patterns may be modified temporally as a microbial species grows. Thus, the volatile fingerprints may be different depending on the growth phase, influencing the threshold of detection of the e-nose system. Gardner *et al*. (1998) investigated the classification of bacterial growth phases. Classification of the lag phase had the lowest prediction rate (14.3%) compared with more successful prediction of log (95.1%) and stationary (73.1%) phases. Pavlou *et al*. (2002b) achieved good discrimination between anaerobic bacterial strains (*Clostridium* spp. and *Bacteroides fragilis*) from blank samples grown in blood agar medium with an initial inoculum of $10^6$ cells $ml^{-1}$ initially in the lag phase bacteria, using an electronic nose system and PCA and neural networks for data analysis. However, they reported good discrimination after 16 hrs incubation in anaerobic conditions at 37°C. Unfortunately, measurements in subsequent growth phases were not reported. Pavlou *et al*. (2004) reported good discrimination after 5-6 hrs incubation at 37°C between *Mycobacterium* spp. and *P.aeruginosa* using cultures in the stationary phase, but not based on CFUs $ml^{-1}$.

It is important to obtain knowledge of the relative thresholds of sensitivity of different VAP causing microorganisms, based on volatile fingerprint discrimination, to enable

information to be obtained on whether the different bacteria/fungi can be detected at levels similar to which they may be isolated from patients. The threshold values for cultured clinical samples with significant infection is normally around $10^3$-$10^5$ CFU $ml^{-1}$ which is similar to the observed threshold of sensitivity using the e-nose systems used in *in vitro* experiments.

**Table 4.1:** Summary of the sensitivity achieved in different matrices from the literature.

| Matrix/Microbial spp. | Microbial population CFUs $ml^{-1}$ | Incubation period (hours) | References |
|---|---|---|---|
| **Skimmed milk medium** *P.aureofaciens, P.fluorescens, B.cereus, C.pseudotropicalis, K.lactis* | $10^3$-$10^4$ | 2-5 | Magan *et al*. (2001) |
| **Bread analogue** ***Eurotium* spp**., *P.chrysogenum* | $10^3$ CFU $g^{-1}$ | 40 | Keshri *et al*. (2002) |
| **Water sample** *E. aerogenes, E.coli, P.aeruginosa* | $10^2$-$10^4$ | 24 | Canhoto and Magan (2003) |
| **Saline solution** *S.aureus, H.influenzae, S.pneumoniae, E.coli, P.aeruginosa, M.catarrhalis* | $10^4$ | No incubation in saline solution | Boilot *et al*. (2002) |
| **SBHI agar*** *Trychophyton* spp. | $10^5$-$10^7$ | 96 | Sahgal (2008) |
| **Blood agar** *Clostridium* spp., *B.fragilis* | $10^6$ | 16 (anaerobic incubation) | Pavlou *et al*. (2002b) |

* SBHI: Sabouraud brain heart infusion.

## 4.3 DISCRIMINATION BETWEEN MICROORGANISMS WHEN PRESENT IN MIXED CULTURE USING VOLATILE PRODUCTION PATTERNS

In the present study, mixtures of bacteria (*E.cloacae* and a *Staphylococcus* strain) and of a bacteria + yeast (*E.cloacae* + *C.albicans*) mixture were examined. These studies showed that one of the species, *E.cloacae*, was predominant in all the mixed groups after 24 hrs incubation at 37°C in NB. This was supported by the clustering pattern seen in the PCA analyses where control samples of *E.cloacae* were grouped with the rest of mixed concentrations.

The detection and discrimination of species in mixtures is a complex issue which is still being studied. It is important to identify the causative species assuming that the organisms recovered from the cultures in large numbers are the presumed etiological agent (Baltimore, 2003). In the present studies it appeared that interactions between microorganisms in mixed culture are complex. The initial concentration of individual species in a mixture is critical and the competitiveness of an individual species varies depending on this. Thus relative concentrations may result in dominance by certain species over others. This suggests that because of the competitiveness of *E.cloacae*, identifying key volatile biomarkers of this more competitive species is important to assist in effective discrimination using volatile fingerprints. Very few studies have been done using natural mixtures of microorganism. However, one previous study which examined sputum samples from patients with a CP sensor array has been done (Pavlou *et al.* 2004). Samples consisted of three different bacteria individually (*Mycobacterium avium*, *M.tuberculosis* and *P.aeruginosa*), a mixture of these microorganisms and controls, all treated with lipase enzymes. They found clear discrimination between treatments after 6 hrs incubation at room temperature with the mixed group being separated from the others. In this case it was not possible to establish any relationship between any of the controls with the mixed group.

Hence mixed infections are not rare; the identification of mixture cultures in the analyses of natural samples is important where groups of positive, negative bacteria and fungi may be present in different concentrations. Thus, background data may well be critical in developing models which can be reliably used to assist with choosing the right treatment. However, in this study clinical samples with a mixture of microbes were removed from the statistical analyses which led to the need for further studies on the actual volatile profiles from microbial mixtures where both antagonistic and perhaps additive/synergistic effects may occur.

## 4.4 USE OF VOLATILE PRODUCTION PATTERNS TO DISCRIMINATE BETWEEN DIFFERENT ANTI-FUNGAL TREATMENTS AGAINST *A.FUMIGATUS* AND *C.ALBICANS*

The present study suggests that it is possible to discriminate between effective and non-effective concentrations of anti-fungal compounds for *A.fumigatus* using VOCs. Volatile fingerprints appear to be a good indicator for differentiation between treatments. Thus, regardless of the anti-fungal compound investigated, differentiation was found after 48 hrs between positive (control) growing cultures of *A.fumigatus*, those treated with the $ED_{50}$ and $ED_{90}$ concentrations. The rapid discrimination achieved for *A.fumigatus* at 37ºC contrasts with the poorer discrimination achieved at 25ºC. This species is a thermophile and thus grows much slower at 25 than 37$^{o}$C. Because all the pathogens investigated were isolated from human specimens, 37°C was the selected temperature to work with in most of the experiments using the e-nose. Another important factor is the age of the culture and the anti-fungal effect as reported by Naraghi *et al*. (2010). They found better discrimination between treatments after 120 rather than 96 hrs when the dermatophytes were treated with griseofulvin instead of itraconazole.

This approach of using volatile fingerprints to try and discriminate between the efficacy of different concentrations of anti-microbials has not been examined extensively

previously. Certainly no information is available with regard to *A.fumigatus*. Recently Naraghi *et al*. (2010) examined and compared the effect of anti-fungal and anti-oxidant compounds for efficacy against dermatophytic *Trichophyton* species. They were able to discriminate between the effects of $ED_{50}$ and $ED_{90}$ concentrations of itraconazole on *Trichophyton* species based on volatile production patterns using a similar hybrid sensor array e-nose as used in the present study. Indeed, other studies by Sahgal and Magan (2008) demonstrated the use of volatile production patterns to discriminate, at both a species and strain level, dermatophyte species causing human and animal skin diseases.

Fluconazole among others triazoles, has been widely used to treat aspergillosis disease but it has been observed, as found in this study, a high percentage of failures in the treatments not always due to the drug resistance itself but to the underlying disease (Verweij *et al*., 2007). The mechanisms of the resistance acquisition are not yet clear; patients may be infected by triazole-resistant strains due to prolonged therapies or due to azole exposure in the environment (Snelders *et al*., 2009).

Good performance of classification was found when DA was performed on the data sets for *A.fumigatus* treatments. However, it was not tested against other groups of *in vitro* samples as validation sets. This study has been an attempt to evaluate the ability of the e-nose to monitor fungal therapies but more studies are needed. This approach could be complimentary to existing standard susceptibility tests currently used, which often does not give reliable results (Sohnle and Hahn, 2002). Another important area where this approach could be useful would be in detection of a build up of resistance to existing or new drugs. Background information could be kept on individual strains of specific species and compared with existing data on volatile fingerprints and type strain information to identify which and how many strains may be developing resistance.

The experiments with *C.albicans* and anti-fungals (tebuconazole and fluconazole) were performed in liquid broth medium. Probably, the volatile profiles production was not different enough for treatment discrimination using the e-nose. It may be interesting to examine volatile production by *A.fumigatus* in liquid broth cultures. However, in this case, the best discrimination between treatments may be delayed because *A.fumigatus*

grows in biomass pellets in an agitated liquid medium and contact with anti-fungals may be different from that in solid media.

The lack of good discrimination between *C.albicans* treatments at 37°C (in saline solution) may be due to the relative interaction between the yeast cells and the anti-fungal treatments resulting in very little difference in VOCs produced in the head space of such cultures. The e-nose was capable of distinguishing between the controls, blank medium and "a third group of samples" (*C.albicans* + $ED_{50}$, $ED_{90}$) but no discrimination within the latter group. An attempt has been made to relate these to GC-MS volatile production patterns. These studies have shown that potential does exist to use volatile fingerprints as a rapid screening method to identify compounds which might have capacity as anti-fungal treatments.

## 4.5 IDENTIFICATION OF VOLATILES PRODUCED DURING EXPOSURE TO ANTI-FUNGALS USING GC-MS AND SIFT-MS SYSTEMS

The aim of the GC-MS and SIFT-MS studies was to investigate the pattern of volatile metabolites that could explain the differences between species or groups of microorganisms and between anti-fungal treatments rather than specifically isolated compounds, then to link the e-nose results with possible volatile biomarkers. In the initial experiments, 5 VAP microorganisms grown on NA standard medium were used. In a second experiment, *A.fumigatus* was grown on MEA cultures amended with the $ED_{50}$ concentrations of two anti-fungals (tebuconazole and benomyl). A third experiment was done to examine the head space of cultures of *C.albicans* in saline solution (with 0.2% glucose and 0.2% peptone) amended with tebuconazole $ED_{50}$ and $ED_{90}$ values.

Some of the VOCs reported in different studies (Table 1.3), regardless of the medium used for culturing, were found in the present GC-MS and SIFT-MS analyses such as:

dienes (1,4-pentadiene), methyl pentadienes, isoprene, amyl alcohols (2-methyl-1-butanol, 3-methyl-1-butanol) and in small concentrations some alkanes (heptane, undecane) in fungal cultures; and dimethyl disulphide, acetone, 2-butanone in bacterial cultures. As the interest was to find those compounds significantly different between species/treatments, only the most distinctive substances have been specifically mentioned.

In the first experiment with 5 VAP microorganisms some compounds were found which were characteristic of specific species which might allow discrimination for the others. SIFT-MS data showed differences mainly with regard to ammonia, acetone and methanethiol between species. In the GC-MS analyses, there were some distinctive substances: 1,4-pentadiene which was only present in *A.fumigatus* and *E.cloacae*; 4,4-dimethyl-1-pentene, 2-methyl-3-isopropylpyrazine in *A.fumigatus*; mercaptoacetone in *C.albicans*; 2-methyl-1-propanol only present in *K.pneumoniae* and *E.cloacae* cultures. Using SIFT-MS analysis a few compounds were found distinctive between samples: ammonia was present in larger amount in *C.albicans*; methanethiol in *A.fumigatus*; acetone was mainly found in *K.pneumoniae*, *A.fumigatus* and *C.albicans*; and propanol which was found in blank medium and bacterial species and barely found in fungal species.

This first study using SIFT-MS and GC-MS systems was a preliminary study to the later MVOCs investigations. Further studies should be carried out using liquid instead of solid media for the analysis of volatiles because e-nose analyses were made initially in NB and not in NA. It is difficult to compare both techniques due to their different characteristics and furthermore due to the different media used that probably affected the different volatiles released from the cultures.

Subsequent experiments carried out with *A.fumigatus* on MEA cultures treated with the $ED_{50}$ concentrations of two anti-fungals (tebuconazole and benomyl) at 37°C using SIFT-MS supported the discrimination achieved between treatments and controls using the hybrid e-nose system. Not many differences were detected between treatments and control samples, except in terms of an increase in the production of some volatile compounds (methyl pentadiene, ethanol) when using tebuconanzole as a treatment, especially after 72 hrs growth. Other volatile compounds were more abundant when

benomyl was used to inhibit fungal growth (methanol and isoprene). The complementary data obtained from GC-MS system was examined qualitatively and quantitatively. Some compounds were in all the treatments except blank samples such as 2-methyl-1-butanol and 3-methyl-1-butanol. These were previously found in *A.fumigatus* cultures by Fiedler *et al*. (2001), while 2-methyl-1-propanol also found in cheese-associated fungi (Karlshøj *et al*., 2007b); also dienes (pentadiene and methyl pentadiene) were mostly present in control and treatments samples but no large differences were found between anti-fungal treatments and control samples.

Compounds such as isoprene, ethanol and methanol identified with SIFT-MS system were not clearly identified using GC-MS analysis. This was probably due to the difficulty of the analytical system to identify lower molecular weight species. It is already known that some Gram-negative and Gram-positive bacteria species release isoprene. However, only a few fungi such as *Eurotium amstelodami* have been studied (Kuzma *et al*., 1995). Dienes such as methyl pentadiene and isoprene, present in human exhaled breath samples, have been investigated in fungal and bacterial cultures as well. Wang *et al*. (2004) identified 2-methyl-1-butanol, 4-methyl-1,3-pentadiene and dimethyl polysulphide in the growth of *Pseudomonas* species and related bacteria with SIFT analyses and Fiedler *et al*. (2001) reported isoprene and 2-methyl-1,3-pentadiene in cultures of *A.fumigatus* grown on MEA. Higher amounts of isoprene have been reported in exhaled breath from individuals under psychological and oxidative stress (Smith *et al*., 1999) and compounds such as 1,4-pentadiene have been tentatively identified as one of the breast cancer biomarkers (Phillips *et al*., 2010). This study might open new ways to investigate the presence of such volatile hydrocarbons when fungal species grow under stress conditions as occurs in many human disease states.

The lack of clear key compounds for the discrimination between treatments in *A.fumigatus* did not correspond with the good differentiation achieved with the responses of the e-nose hybrid sensor array to the produced volatile fingerprints. This might be due to the complexity of chromatographic analyses or could be interpreted by the effect of the addition of the anti-fungal compounds. The experiment with an alternative GC-MS system for measuring volatiles produced by *C.albicans* cultures grown in liquid medium for 24 hrs showed some shared compounds with those

produced by *A.fumigatus.* Compounds such as 2 and 3-methyl-1-butanol and 2-methyl-1-propanol were found to be much more abundant in positive controls rather than in the rest of the treatments. However, no specific and important differences were found between treatments which could explain the lack of discrimination using the analysis of the head space for volatile profiles.

Not many studies have analysed which biomarkers are present in fungal cultures when treated with anti-fungal compounds. Willger *et al*. (2008) investigated the metabolism of *A.fumigatus* in low oxygen environments in lung tissues. They found ethanol production when glucose was the main source of carbon indicating that cultures of *A.fumigatus,* which is an obligate aerobic filamentous fungus, switched to alcohol fermentation pathways. They also suggested that *A.fumigatus* used fermentation in order to grow in microaerophylic and hypoxic environments. In the present study, the stress conditions of the anti-fungal treatment might have affected the biosynthetic pathways resulting in alternative pathways being used and resulting in a modified set of volatile compound end products.

The findings of biomarkers with SIFT-MS techniques would permit the acquisition of more knowledge about the volatiles released from each microorganism in normal conditions and under different stressful situation such as drug therapy. This might lead to the discrimination of clinical samples with trained models using the e-nose which may be more user friendly, may not need pre-processing steps to obtain results, perhaps in real time. However, it is necessary to optimise the processing of the clinical samples in order to obtain good identification and discrimination of microbial growth under antibiotic therapies.

GC-MS and SIFT-MS data analysis has been widely used to look for a number of biomarkers in breath samples. The biomarkers could be used to detect disease, monitor disease progression or monitor therapy such as glucose for insulin resistance, methacetin for liver function, ethanol for law enforcement and urea for *H.pylori* infection among others (Risby and Solga, 2006). Breath analysis advantages are various; the lack of invasiveness and the easy collection of samples at the patient's site with the only requirement that the patient must be breathing spontaneously or

mechanically supported. However, breath analysis has shown some disadvantages due to the presence of some confounding factors such as diet, age, smoking habits, patient's cardiopulmonary status and sample contamination from air.

In the current work it appears that some compounds may be present in higher concentrations in some treatments or in some microorganisms when *in vitro* samples were studied. Humphreys (2010) tried to identify specific markers in 58 breath samples from patients with more than 72 hrs intubation. At the same time BAL samples were taken and split into two parts for posterior analyses; for microbiological cultures, and for e-nose analysis. Breath samples from patients with microbial growth, non-microbial growth and healthy volunteers were compared. After appropriate analysis of data using AMDIS and the NIST library no compounds seemed to be clear VAP biomarkers. However, it was found that some were evidently increased in breath samples containing microbial growth, including isoprene; acetone (higher amounts in non-contaminated samples rather than healthy volunteers) and acetaldehyde with respect to the controls. It is difficult to determine which compounds from the breath analyses are exogenous or endogenous and, within these, which ones are indicative of infection/inflammation in body tissues. Bergeron *et al*. (1998) found high levels of pro-inflammatory cytokines and nitric oxide in BAL samples from experimental pneumococcal infections in mice. If some of these compounds are detectable and may form a volatile pattern correlating with the e-nose volatile profiles for specific samples, then these findings could potentially lead to the use of the e-nose system to analyse breath samples from VAP patients in a non-invasive, less time consuming and reliable way of diagnosis.

## 4.6 COMPARISON OF DISCRIMINATION BETWEEN 5 MICROORGANISMS USING NUTRIENT BROTH AND SALINE SOLUTION

The currently but invasive method of sampling procedure for VAP diagnosis is the obtaining of BAL specimens which are performed in sterile saline solution. For this

reason experiments with saline solution were carried out and the results were compared with those carried out in NB medium. All the 5 VAP microorganisms were tested in both media and the discrimination based on the volatile fingerprints was found to be very similar. In both media, with an initial concentration of $10^4$ cells/spores $ml^{-1}$ and after 24 hrs incubation four different clusters were observed: one with the two Gram-negative bacteria, a second one with *C.albicans*, a third one with the *Staphylococcus* strain (or *S.aureus*) and a fourth one with the blanks and the *A.fumigatus* samples.

The capability of the e-nose sensor array system to detect and distinguish between microorganisms has been tested in many studies, using many different media. Urine, sputum or breath samples have been analysed either directly or after culturing in commercial or more complex media. For example, Gardner *et al.* (1998) distinguished between *E.coli* and *S.aureus*, a Gram-negative and a Gram-positive bacterium in standard nutrient medium (blood agar) in 12 hrs incubation but the initial concentration used was not reported.

No attempt had previously been made to use saline solution as a basal laboratory medium for growing microorganisms for head space volatile analyses. Boilot *et al*. (2002) showed that using a CP e-nose it was possible to discriminate between six different bacteria (*S.aureus, H.influenzae, E.coli, P.aeruginosa, M.catarrhalis, S.pneumoniae*) responsible of eye infections in saline solution. However, in this case, all the bacterial strains were grown in commercial agar media overnight and then suspended in saline solution for analysis.

In this study, saline solution as a culture medium was successfully used for microbial discrimination. It could be possible that initial culture on agar media facilitated the discrimination at earlier stages. Casalinuovo *et al*. (2006) suggested that bacterial agar cultures could produce more volatiles than broth cultures. In their experiment, they analysed the head space from four microorganisms (*K.pneumoniae, C.albicans, P.aeruginosa, E.coli*) cultured on agar and in broth media, after 24 hrs incubation with an initial concentration of $10^6$ CFU $ml^{-1}$. They were able to discriminate between bacteria in agar cultures and yeasts in both cultures but they concluded that the volatile production patterns from bacterial broth cultures were not detectable by the e-nose used, an acoustic wave sensor which can display the volatiles in chromatograms. In my study,

agar media were only used to grow fresh cultures for inoculation of saline solution treatment media. I obtained good discrimination in broth cultures, NB and saline solution with five very similar microorganisms to the ones used in this previous study and with a different e-nose. However, they used a higher initial concentration than in my studies on saline and NB broth. Previously, Sahgal (2008) investigated the discrimination between twelve microorganisms distributed in eight different genera using NB and a similar e-nose. After the analysis with PCA and DA, she found that not all of the microbial species could be distinguished. She was able to split them into four groups: Gram-positive, Gram-negative, fungi and controls. This was achieved with a classification accuracy of 82.6%.

The grouping distribution by microorganism found in the current work was modified with longer incubation periods. There was poorer discrimination between microorganisms as the incubation period was extended. Volatile fingerprints may vary depending not only on the initial number of microorganisms, but also on the time of growth. Presumably, the analysis of head space at 24 hrs incubation occurred when the most of the bacterial species and the yeast, reached the stationary phase of growth. Some authors (Gardner *et al*., 1998) investigated the discrimination between two bacteria and its growth phase by using an e-nose with six metal-oxide sensors and ANN. They found that 80.7% of all tests were correctly classified and only bacterial growth in the lag phase failed in terms of prediction with 14.3% of good classification. Clearly, the best discrimination between microorganisms has to be reached when they are actively growing, *i.e.*, during log and stationary phases, when more volatiles are present in the head space. These results are consistent with what was found in the current studies.

## 4.7 USE OF VOLATILE DATA SETS FROM NUTRIENT BROTH AND SALINE SOLUTIONS ON THE VAP MICROORGANISMS TO BUILD A MODEL FOR PREDICTION OF CLINICAL SAMPLES

Two statistical models were built and then used to try and predict the groupings for the clinical samples using DA: one for NB data and another for saline solution (amended with 0.2% glucose and 0.2% peptone). Both models used the e-nose data analysed from 24 hrs incubation at 37°C for the 5 VAP microorganisms. These models showed good accuracy of prediction when they were tested as a validation set. Later, these models were used to predict a set of clinical samples which had been analysed using a different e-nose. Furthermore, PLS-DA was used to predict the groupings into which the clinical samples fell, based on the *in vitro* model.

Alternative scaling methods were investigated although the raw data provided by the e-nose software was already scaled by the mean-centred method. Jurs *et al*. (2000) suggested there is no guideline for choosing the most appropriate method and it is necessary to explore and choose the most suitable pre-processing method for the later analysis with multivariate techniques. Also, the fact that different data sets were measured on different days led to the use of a simple and quick standardisation process. Haugen *et al*. (2000) suggested that if changes in the measurements are observed over time, the responses of calibration substances closely related to real samples might be used to correct the differences between measured samples on different days. Multiplicative drift correction (MDC) was used in the present study to correct the signal differences over time using the closest calibration substance available, the blanks of each medium.

After all these considerations, the models for clinical classification were applied. The results were not very good for clinical samples resulting in an overall prediction of 63.6% and 53.4% in NB and saline solution respectively, using DA and 63.6% and 52.3% for each medium when using PLS-DA and standardisation of the e-nose devices.

Most importantly, looking at the % of classification success per each group in which the clinical samples were divided, the models with highest overall classification, *i.e.* > 60%, had good prediction only in "no growth" group. Certainly, the results with saline solution using DA failed in terms of sensitivity. Only the large group of "no growth" samples was successfully predicted. The different number of training vectors for each group might have made tighter associations between some vector responses with the largest classes (Gardner *et al*., 1998). This is especially true for non-linear methods such

as ANNs where it is necessary to have more samples than variables (Sahgal, 2008) but even more importantly than the number of samples is having an adequate number of representative and reproducible samples for each class (Kodogiannis *et al*., 2002), especially in neural network models. The use of non-parametric methods such as ANN might have improved the classification of unknown samples in this study if more data for the training set was available and if it had been more homogeneous.

The relative success of using an *in vitro* model for clinical sample prediction might be due to the use of very heterogeneous data sets. Most of the clinical samples were taken from patients under antibiotic therapy which might have affected the accuracy of the e-nose results compared with the microbiological outcomes. As Sahgal (2008) found, false negative cultures may be identified as positive by the analysis of the volatiles with the e-nose.

It is also important to ascertain whether the discrimination between samples is due to the microbial metabolism or due to the products of the host's immune system. If it is due to the microbial activity *per se*, there is the need of improving the liberation of volatiles with biochemical precursors as Pavlou *et al*. (2002b) suggest or with specific enzymes (Pavlou *et al*., 2004).

It was unfortunate that during this project no new clinical sample sets could be provided by clinicians to facilitate the testing of the *in vitro* models more extensively, to enable the outcomes of this being tested against what was found microbiologically using microbiological analyses. This would have even been more interesting if these could have been divided based on whether they had antibiotic or anti-fungal treatments.

# CHAPTER 5: CONCLUSIONS AND FUTURE WORK

## 5.1 CONCLUSIONS

- Due to the high importance of the control of the sensor responses, it is necessary to monitor the consistency of the sensors over the period of study. Good consistency of the responses of the sensors was observed. It is also important to analyse a set of reference samples prior to each experiment.

- Three enriched commercial media were tested for discrimination of two microorganisms, a yeast and a Gram-negative bacterium. NB showed earlier discrimination between both species after 24 hrs incubation. This medium was selected for carrying out subsequent experiments.

- The sensitivity for the detection of three different initial concentrations of five VAP microorganisms was tested. $10^2$ CFU $ml^{-1}$ as initial concentration may be discriminated from blank samples after: 24 hrs for bacteria; 48 hrs for yeast; and 72 hrs for filamentous fungus.

- In mixtures of two microorganisms: There was predominant growth of one of the microorganisms (*E.cloacae*) when two are mixed together; the volatile pattern production might permit the identification of the predominant species.

- $ED_{50}$ and $ED_{90}$ concentrations of different anti-fungal compounds were determined for control of *A.fumigatus* and *C.albicans* based on growth studies on agar and liquid broth media, respectively.

- Good discrimination between fungal samples treated with anti-fungal (*A.fumigatus*+ $ED_{50}$ and $ED_{90}$ Benomyl/Tebuconazole) at 37°C on solid medium after 48h of incubation) but not at 25°C; poor results for the yeast grown in liquid medium (*C.albicans*+ $ED_{50}$ and $ED_{90}$ Tebuconazole) at 37°C in saline solution.

- SIFT-MS analyses showed that some compounds were present in higher amount in certain treatments when the fungus *A.fumigatus* was grown on solid medium amended with different concentrations of anti-fungal which could explain the good discrimination in the e-nose.

- The head space from 5 VAP microorganisms grown on NA was analysed using SIFT-MS system and some key components were found: 1,4-pentadiene present only in *A.fumigatus* and *E.cloacae*; 4,4-dimethyl-1-pentene, 2-methyl-3-isopropylpyrazine and methyl mercaptan in *A.fumigatus*; mercaptoacetone and phenylethyl alcohol in *C.albicans*; aletamine in *E.cloacae*; and 2-methyl-1-propanol in *K.pneumoniae* cultures.
- Similar analyses in liquid medium with *C.albicans* showed no differences between treatments which were confirmed by the lack of discrimination achieved with the e-nose.
- Very good discrimination between 5 VAP microorganisms grown in NB and saline solution (amended with 0.2% glucose and 0.2% peptone) laboratory-based model. Potential exists for using the laboratory-based model for examining clinical samples to try to classify the contamination with Gram-positive, Gram-negative and fungal infections.
- To build a laboratory-based model to predict microbial contents of clinical samples using volatile fingerprints it was necessary to: (1) standardise measurements when several sets of samples are analysed at different times; (2) develop a scaling approach to reduce the differences between groups.
- Two *in vitro* models were tested, *i.e.,* NB and saline solution amended with 0.2% of glucose+0.2% peptone for clinical samples prediction divided in 4-groups. Similar accuracy was obtained using DA and PLS-DA methods: 63.6% for both techniques in saline solution; 52.3% with PLS-DA and 53.4% with DA in NB.
- The maximum overall accuracy classification was 63.6% using *in vitro* model in saline solution. The sensitivity achieved was very poor, only the largest group of "no growth" was mostly well predicted.

## 5.2 FUTURE WORK

- To use the e-nose analyses to build two models for prediction of unknown samples using ANNs and compare their results, one with clinical samples of patients, another with *in vitro* samples.

- To investigate the improvement of volatile profiles released from clinical samples either by adding specific compounds or filtering and adding little amounts of biochemical precursors as sources of nitrogen and energy and incubate for a short period of time.

- Finding which enzymes might be added to clinical samples in order to intensify volatile fingerprint patterns also in samples from patients who are being treated with antibiotics.

- To analyse more combinations of microbial mixtures using the e-nose and chromatographic systems, also with microbiological techniques in order to find their interactions and results in numbers and in volatile profiles.

- To investigate the effect of antibiotic therapy on the volatile production using the e-nose in liquid media with filamentous fungus, also with bacteria in order to correlate with clinical samples.

- To analyse VAP patients' breath and compare with other results such as the analysis of the BAL samples with the e-nose and BAL cultures. These analyses have not been done before and might eventually give some information about volatiles.

- Determining the possible biomarkers for discrimination in liquid media from microbial cultures using GC-MS, this could explain the discrimination between groups of microorganisms and the lack of distinction within same type of microbes, *e.g.* Gram-negative.

# REFERENCES

**Álvarez-Lerma, F. (1996).** Modification of empiric antibiotic treatment in patients with pneumonia acquired in the intensive care unit. *Intensive Care Medicine* **22**, 387-394.

**American Journal Respiratory Critical Care Medicine (2005).** Guidelines for the management of Adults with Hospital-acquired, Ventilator-associated and Healthcare-associated pneumonia. *American Journal of Respiratory and Critical Care Medicine* **171**, 388-416.

**Baltimore, R.S. (2003).** The difficulty of diagnosing Ventilator-Associated Pneumonia. *Pediatrics* **112**, 1420-1421.

**Baranyi, J., Roberts, T.A., McClure, P. (1993).** A non-autonomous differential equation to model bacterial growth. *Food Microbiology* **10**, 43–59.

**Baranyi, J., Roberts, T.A. (1994).** A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology* **23**, 277-294.

**Bastos, A.C., Magan, N. (2006).** Potential of an electronic nose for the early detection and differentiation between *Streptomyces* in potable water. *Sensors and Actuators B: Chemical* **116**, 151-155.

**Bastos, A.C., Magan, N. (2007)**. Soil volatile fingerprints: Use for discrimination between soil types under different environmental conditions. *Sensors and Actuators B: Chemical* **125**, 556-562.

**Baughman, R.P. (2005).** Microbiologic diagnosis of ventilator-associated pneumonia. *Clinics in Chest Medicine* **26**, 81-86.

**Bergeron, Y., Ouellet, N., Deslauriers, A.M., Simard, M., Olivier, M., Bergeron, M.G. (1998).** Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infection and Immunity* **66**, 912-922.

**Boilot, P., Hines, E.L., Gardner, J.W., Pitt, R., John, S., Mitchell, J., Morgan, D.W. (2002).** Classification of bacteria responsible for ENT and eye infection using the Cyranose System. *IEEE Sensors Journal* **2**, 247-253.

**Boudonck, K.J., Mitchell, M.W., Német, L., Keresztes, L., Nyska, A., Shinar, D., Rosenstock, M. (2009).** Discovery of metabolomics biomarkers for early detection of nephrotoxicity. *Toxicologic Pathology* **37**, 280-292.

**Bouza, E., Brun-Buisson, C., Chastre, J., Ewig, S., Fagon, J.Y., Marquette, C.H., Muñoz, P., Niederman, M.S., Papazian, L., Rello, J., Rouby, J.J., Van Saene, H., Welte, T. (2001).** Ventilator-associated pneumonia. *European Respiratory Journal* **17**, 1034-1045.

**Buratti, S., Benedetti, S., Cosio, M.S. (2005).** An electronic nose to evaluate olive oil oxidation during storage. *Italian Journal of Food Science* **17**, 203-210.

**Cabañes, F.J., Sahgal, N., Bragulat, M.R., Magan, N. (2009).** Early discrimination of fungal species responsible of ochratoxin A contamination of wine and other grape products using an electronic nose. *Mycotoxin Research* **25**, 187-192.

**Camardo Leggieri, M., Planas Pont, N., Battilani, P., Magan, N. (2011).** Detection and discrimination between ochratoxin producer and non-producer strains of *Penicillium nordicum* on a ham-based medium using an electronic nose. *Mycotoxin Reearch* **27**, 29-35.

**Canhoto, O., Magan, N. (2003).** Potential for detection of microorganisms and heavy metals in potable water using electronic nose technology. *Biosensors and Bioelectronics* **18**, 751-754.

**Canhoto, O., Magan, N. (2005).** Electronic nose technology for the detection of microbial and chemical contamination of potable water. *Sensors and Actuators B: Chemical* **106**, 3-6.

**Casalinuovo, I.A., Di Pierro, D., Bruno, E., Di Francesco, P., Coletta, M. (2006).** Experimental use of a new surface acoustic wave sensor for the rapid identification of bacteria and yeasts. *Letters in Applied Microbiology* **42**, 24-29.

**Chastre, J., Fagon, J.Y. (2002)**. Ventilator-associated pneumonia. *American Journal of Respiratory and Critical Care Medicine* **165**, 867-903.

**D'Amico, A., Pennazza, G., Santonico, M., Martinelli, E., Roscioni, C., Galluccio, G., Paolesse, R., Di Natale, C. (2010).** An investigation on electronic nose diagnosis of lung cancer. *Lung Cancer* **68**, 170-176.

**Demyttenaere, J.C.R., Moriña, R.M., Sandra, P. (2003).** Monitoring and fast detection of mycotoxin-producing fungi based on head space solid-phase microextraction and head space sorptive extraction of the volatile metabolites. *Journal of Chromatography A* **985**, 127-135.

**Dennesen, P.J.W., Van der Ven, J.A.M., Kessels, A.G.H., Ramsay G., Bonten M.J.M. (2001).** Resolution of infectious parameters after antimicrobial therapy in patients with ventilator-associated pneumonia. *American Journal of Respiratory Critical Care Medicine* **163**, 1371-1375.

**Di Natale, C., Macagnano, A., Davide, F., D'Amico, A., Paolesse, R., Boschi, T., Faccio, M., Ferri, G. (1997).** An electronic nose for food analysis. *Sensors and Actuators B* **44**, 521-526.

**Dutta, R., Morgan, D., Baker, N., Gardner, J.W., Hines, E.L. (2005).** *Sensors and Actuators B: Chemical* **109**, 355-362.

**Evans, P., Persaud, K.C., McNeish, A.S., Sneath, R.W., Hobson, N., Magan, N. (2000).** Evaluation of a radial basis function neural network for the determination of wheat quality from electronic nose data. *Sensors and Actuators B: Chemical* **69**, 348-358.

**Fábregas, N., Ewig, S., Torres A., El-Ebiary, M., Ramírez, J., Puig de la Bellacasa, J., Bauer, T., Cabello, H. (1999).** Clinical diagnosis of ventilator associated pneumonia revisited: comparative validation using immediate post-mortem lung biopsies. *Thorax* **54**, 867-873.

**Fagon, J.Y., Chastre, J. (2005).** Antimicrobial Treatment of Hospital-Acquired Pneumonia. *Clinics in Chest Medicine* **26**, 97-104.

**Fiedler, K., Schütz, E, Geh, S. (2001).** Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. *International Journal of Hygiene and Environmental Health* **204**, 111-121.

**Gardner, J.W., Bartlett, P.N. (1994)**. A brief history of electronic nose. *Sensors and Actuators B: Chemical* **18**, 210-211.

**Gardner, J.W., Bartlett, P.N. (1999)**. *Electronic noses: principles and applications*. Oxford: Oxford University Press.

**Gardner, J.W., Craven M., Dow, C., Hines, E.L. (1998)**. The prediction of bacteria type and culture growth phase by an electronic nose with a multi-layer perceptron network. *Measurement Science and Technology* **9**, 120–127.

**Gardner, J.W., Shin, H.W., Hines, E.L. (2000a).** An electronic nose system to diagnose illness. *Sensors and Actuators B: Chemical* **70**, 19-24.

**Gardner, J.W., Shin, H.W., Hines, E.L., Dow, C.S. (2000b).** An electronic nose system for monitoring the quality of potable water. *Sensors and Actuators B: Chemical* **69**, 336-341.

**Garner, J.S., Jarvis, W.R., Emori, T.G., Horan, T.C., Hughes, J.M. (1988).** CDC definitions for nosocomial infection, 1988. *American Journal of Infection Control* **16**, 128-140.

**Garner, C.E., Smith, S., Costello, B, White, P., Spencer, R., Probert, C.S.J., Ratcliffe, N.M. (2007).** Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease. *FASEB Journal* **21**, 1675-1688.

**Haugen, J.E., Tomic, O., Kvaal, K. (2000).** A calibration method for handling the temporal drift of solid state gas-sensors. *Analytica Chimica Acta* **407**, 23-29.

**Humphreys, M.L. (2010).** Volatile diagnostic techniques for ventilator associated pneumonia. *DM Thesis*. Cranfield University.

**Humphreys, L., Orme, R.M.L.E, Moore, P., Charaklias, N., Sahgal, N., Planas Pont, N., Magan, N., Stone, N., Kendall, C.A. (2011).** Electronic nose analysis of bronchoalveolar lavage fluid. *European Journal of Clinical Investigation* **41**, 52-58.

**Hunter, J.D. (2006)**. Ventilator associated pneumonia. *Postgraduate Medical Journal* **82**, 172-178.

**Ibrahim, E.H., Ward, S., Sherman, G., Kollef, M.H. (2000).** A comparative analysis of patients with early-onset vs late-onset nosocomial pneumonia in the ICU setting. *Chest* **117**, 1434-1442.

**Johanson, W.G., Pierce, A.K., Sanford, J.P., Thomas, G.D. (1972).** Nosocomial Respiratory Infections with Gram-Negative Bacilli. *Annals of Internal Medicine* **77**, 701-706.

**Jonsson, A. Winquist, F., Schnürer, J., Sundgren, H., Lundström, I. (1997).** Electronic nose for microbial quality classification of grains. *International Journal of Food Microbiology* **35**, 187-193.

**Jurs, P.C., Bakken, G.A., McClelland, H.E. (2000).** Computational Methods for the Analysis of Chemical Sensor Array Data from Volatile. *Chemical Reviews* **100**, 2649-2678.

**Karlshøj, K., Nielsen, P.V., Larsen, T.O. (2007a).** Prediction of *Penicillium expansum* spoilage and patulin concentration in apples used for apple juice production by electronic nose analysis. *Journal of Agricultural and Food Chemistry* **55**, 4289-4298.

**Karlshøj, K., Nielsen, P.V., Larsen, T.O. (2007b).** Differentiation of closely related fungi by electronic nose analysis. *Food Microbiology and Safety* **72**, 187-192.

**Keshri, G., Magan, N. (2000).** Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of two *Fusaria* spp. using volatile production profiles and hydrolytic enzymes. *Journal of Applied Mycology* **89**, 825-833.

**Keshri, G., Voysey, P., Magan, N. (2002).** Early detection of spoilage moulds in bread using volatile production patterns and quantitative enzyme assays. *Journal of Applied Mycology* **92**, 165-172.

**Keshri, G., Challen, M.P., Elliot, T.J., Magan, N. (2003).** Differentiation of *Agaricus* species and other homodasidiomycetes based on volatile production patterns using an electronic nose system. *Mycological Research **107***, 609-613.

**Kodogiannis, V.S., Chountas, P., Pavlou, A., Petrounias, I., Chowdrey, H.S., Temponi, C. (2002).** Electronic nose: clinical diagnosis based on soft computing methodologies. *In: IEEE International Symposium Intelligent Systems: Methodology, Models, Applications in Emerging Technologies, 10-12 September 2002, Varna, Bulgaria,* 254-259.

**Koenig, S.M., Truwit, J.D. (2006).** Ventilator-associated pneumonia: Diagnosis, treatment and prevention. *Clinical Microbiology Reviews* **19**, 637-657.

**Kollef, M.H., Ward, S. (1998).** The influence of Mini-Bal cultures on patient outcomes: implications for the antibiotic management of ventilator-associated pneumonia. *Chest* **113**, 412-420.

**Kollef, M.H. (2005).** What is ventilator-associated pneumonia and why is it important?. *Respiratory Care* **50**, 714-724.

**Kuzma, J. Nemecek-Marshall, M., Pollock, W.H., fall, R. (1995).** Bacteria produce the volatile hydrocarbon isoprene. *Current microbiology,* **30**, 97-103.

**Larsen, T.O. (1997).** Identification of cheese-associated fungi using selected ion monitoring of volatile terpenes. *Letters in Applied Microbiology* **24**, 463-466.

**Luna, C.M., Vujacich, P., Niederman, M.S., Vay, C., Gherardi, C., Matera, J., Jolly, E.C. (1997).** Impact of BAL data on the therapy and outcome of ventilator-associated pneumonia. *Chest* **111**, 676-685.

**Machado, R.F., Deffenderfer, O., Burch, T., Zheng, S., Mazzone, P.J., Mekhail, T., Jennings, C., Stoller, J.K., Pyle, J., Duncan, J., Dweik, R.A., Erzurum, S.C. (2005).**

Detection of lung cancer by sensor array analyses of exhaled breath. *American Journal of Respiratory and Critical Care Medicine* **171**, 1286-1291.

**Magan, N., Evans, P. (2000).** Volatiles as an indicator of fungal activity and differentiation between species, and the potential use of electronic nose technology for early detection of grain spoilage. *Journal of Stored Products Research* **36**, 319-340.

**Magan, N., Pavlou, A., Chrysanthakis, I. (2001).** Milk-sense: a volatile sensing system recognises spoilage bacteria and yeasts in milk. *Sensors and Actuators B: Chemical* **72**, 28-34.

**Magan, N., Sahgal, N. (2007).** *In Advances in Food Diagnostics*. Wageningen Academic Publishers.

**Mahmoudi, E. (2009).** Electronic nose technology and its applications. *Sensors & Transducers Journal* **107**, 17-25.

**Marshall, A., Young, D., Blyth, J., Kabilan, S., Lowe, C. (2004).** Metabolite-sensitive holographic biosensor. *Analytical Chemistry* **76**, 1518-1523.

**Meijerink, J., Braks, M.A.H., Brack, A.A., Adam, W., Dekker, T., Posthumus, M.A., Van Beek, T.A., Van Loon, J.J.A. (2000).** Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. *Journal of Chemical Ecology* **26**, 1367-1382.

**Morehead, R.S., Pinto, S.J. (2000)**. Ventilator-associated pneumonia. *Archives of Internal Medicine* **160**, 1926-1936.

**Naraghi, K., Sahgal, N., Adriaans, B., Barr, H., Magan, N. (2010).** Use of volatile fingerprints for rapid screening of anti-fungal agents for efficacy against dermatophyte *Trichophyton* species. *Sensors and Actuators B: Chemical* **146**, 521-526.

**Nagle, H. T., Gutiérrez-Osuna, R., Schiffman, S.S. (1998).** The how and why of electronic noses. *Spectrum, IEEE* **35**, 22-31.

**Needham, R., Magan, N. (2003).** Detection and differentiation of toxigenic and non-toxigenic *Penicillium verrucosum* strains on bakery products using an electronic nose. *Aspects of Applied Biology* **68**, 217-222.

**Needham, R., Williams, J., Beales, N., Voysey, P., Magan, N. (2005).** Early detection and differentiation of spoilage of bakery products. *Sensors and Actuators B: Chemical* **106**, 20-23.

**Papazian, L., Bregeon, F., Thirion, X., Gregoire, R., Saux, P., Denis, J.P., Perin, G., Charrel, J., Dumon, J., Affray, J.P., Gouin, F. (1996).** Effect of ventilator-associated pneumonia on mortality and morbidity. *American Journal of Respiratory Critical Care Medicine* **154**, 91-97.

**Pavlou, A.K., Magan, N., Sharp, D., Brown, J., Barr. H., Turner, A.P.F. (2000).** An intelligent rapid odour recognition model in discrimination of *Helicobacter pylori* and other gastroesophageal isolates in vitro. *Biosensors and Biolectronics* **15,** 333-342.

**Pavlou, A.K., Magan, McNulty, C., Jones, J.M., Sharp, D., Brown, J., Turner, A.P.F. (2002a).** Use of an electronic nose system for diagnoses of urinary tract infections. *Biosensors and Biolectronics* **17**, 893-899.

**Pavlou, A.K., Turner, A.P.F., Magan, N. (2002b).** Recognition of anaerobic bacterial isolates *in vitro* using electronic nose technology. *Letters in Applied Microbiology* **35**, 366-369.

**Pavlou, A.K., Magan, N., Jones, J. Brown, J. Klatser, P., Turner, A.P.F. (2004).** Detection of *Mycobacterium tuberculosis* (TB) in vitro and in situ using an electronic nose in combination with a neural network system. *Biosensors and Bioelectronics* **20**, 538-544.

**Pearce, T.C., Schiffman, S.S., Nagle, H.T., Gardner, J.W. (2003).** *Handbook of Machine Olfaction.* Weinheim: Wiley-VCH.

**Peng, G., Hakim, M., Broza Y.Y., Billan, S., Abdah-Bortnyak, R., Kuten, A., Tisch, U., Haick, H. (2010).** Detection of lung, breast, colorectal, and prostate cancers from

exhaled breath using a single array of nanosensors. *British Journal of Cancer* **103**, 542-551.

**Persaud, K., Dodd, G. (1982).** Analysis of discrimination mechanisms in the mammalian olfactory system using a model nose. *Nature* **299**, 352–355.

**Phillips, M., Cataneo, R.N, Saunders, C., Hope, P., Schmitt, P., Wai, J. (2010).** Volatile biomarkers in the breath of women with breast cancer. *Journal of Breath Research* **4**, DOI: 10.1088/1752-7155/4/2/026003.

**Pugin, J., Auckenthaler, R., Mili, N., Janssens, JP., Lew, PD., Suter, PM. (1991).** Diagnosis of ventilator-associated pneumonia by bacteriologic analysis of bronchoscopic and nonbronchoscopic "blind" bronchoalveolar lavage fluid. *American Review of Respiratory Disease* **143 (1)**, 1121-1129.

**Pugin, J. (2002).** Clinical signs and scores for the diagnosis of ventilator-associated pneumonia. *Minerva Anestesiologica* **68**, 261-265.

**Rello, J, Díaz, E., Rodríguez, A. (2005).** Etiology of Ventilator-Associated Pneumonia. *Clinics in Chest Medicine* **26**, 87-95.

**Risby, T.H., Solga, S.F. (2006).** Current status of clinical breath analysis. *Applied Physics B* **85**, 421-426.

**Rumbak, M.J. (2005).** Pneumonia in patients who require prolonged mechanical ventilation. *Microbes and Infection* **7**, 275-278.

**Sahgal, N., Monk, B., Wasil, M., Magan, N. (2006).** *Tricophyton* species: use of volatile fingerprints for rapid identification and discrimination. *British Journal of Dermatology* **155**, 1209-1216.

**Sahgal, N., Needham, R., Cabañes, F.J., Magan, N. (2007).** Potential for detection and discrimination between mycotoxigenic and non-toxigenic moulds using volatile production patterns: A review. *Food Additives and Contaminants* **24**, 1161-1168.

**Sahgal, N. (2008)**. Microbial and non-microbial volatile fingerprints: Potential clinical applications of electronic nose for early diagnoses and detection of diseases. *PhD Thesis*. Cranfield University.

**Sahgal, N., Magan, N. (2008).** Fungal volatile fingerprints: Discrimination between dermatophyte species and strains by means of an electronic nose. *Sensors and Actuators B: Chemical* **131**, 117-120.

**Schaller, E. Bosset, J.O., Escher, F. (1998)**. Electronic nose and their application to food. *Lebensmittel-Wissenschaft und-Technologie* **31**, 305-316.

**Scotter, J.M., Langford, V.S., Wilson, P.F., McEwan, M.J., Chambers, S.T. (2005).** Real-time detection of common microbial volatile organic compounds from medically important fungi by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). *Journal of Microbiology Methods* **63**, 127-134.

**Scotter, J.M., Allardyce, R.A., Langford, V.S., Hill, A., Murdoch, D.R. (2006).** The rapid evaluation of bacterial growth in blood cultures by selected ion flow tube-mass spectrometry (SIFT-MS) and comparison with the BacT/ALERT automated blood culture system. *Journal of Microbiological Methods* **65**, 628-631.

**Smith, D., Španěl, P., Davies, S. (1999).** Trace gases in breath of healthy volunteers when fasting and after a protein-calorie meal: a preliminary study. *Journal of Applied Physiology* **87**, 1584-1588.

**Snelders, E., Huis ins't Veld, R.A.G., Rijs, A.J.M.M., Kema, G.H.J., Melchers, W.J.G., Verweij, P.E. (2009).** Possible Environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Applied and Environmental Microbiology* **75**, 4053-4057.

**Sohnle, P., Hanh, B.L. (2002)**. Effect of prolonged fluconazole treatment on *Candida albicans* in diffusion chambers implanted into mice. *Antimicrobial Agents and Chemotherapy* **46**, 3175-3179.

**Solomkin, J.S. (2005).** Ventilator-associated pulmonary infection: the germ theory of disease remains viable. *Microbes and Infection* **7**, 279-291.

**Španěl, P., Smith, D. (2007a).** Selected ion flow tube mass spectrometry for on-line trace gas analysis in biology and medicine. *European Journal of Mass Spectrometry* **13**, 77-82.

**Španěl, P., Smith, D. (2007b).** The challenge of breath analysis for clinical diagnosis and therapeutic monitoring. *Analyst* **132**, 390-396.

**Spengler, J.D., Wilson, D.G. (2003).** Air quality in aircraft. *Journal of process mechanical engineering* **217**, 323-335.

**Timsit, J.F., Chevret, S. Valcke, J., Misset, B., Renaud, B., Goldstein, F.W., Vaury, P., Carlet, J. (1996).** Mortality of nosocomial pneumonia in ventilated patients: influence of diagnostic tools. *American Journal of Respiratory Critical Care Medicine* **154**, 116-123.

**Trihaas, J, Nielsen, P.V. (2005).** Electronic nose technology in quality assessment: monitoring the ripening process of Danish blue cheese. *Journal of Food Science* **70**, E44-49.

**Trihaas, J., Vognsen, L., Nielsen, P.V. (2005)**. Electronic nose: New tool in modelling the ripening of Danish blue cheese. *International Dairy Journal* **15**, 679-691.

**Trouillet, J.L., Chastre, J., Vuagnat, A., Joly-Guillon, M.L., Combaux, D., Dombret, M.C., Gibert, C. (1998).** Ventilator-associated pneumonia caused by potentially drug-resistant bacteria. *American Journal of Respiratory Critical Care Medicine* **157**, 531-539.

**Turner, A.P.F., Magan, N. (2004).** Electronic noses and disease diagnostics. *Nature Reviews: Microbiology* **2**, 1-6.

**Turner, C., Walton, C., Hoashi, S., Evans, M. (2009).** Breath acetone concentration decreases with blood glucose concentration in type I diabetes mellitus patients during hypoglycaemic clamps. *Journal of Breath Research* **3,** Art. no. 046004.

**Verweij, P.E., Mellado, E., Melchers, W.J., (2007).** Multiple-Triazole-Resistant Aspergillosis. *New England Journal of Medicine* **36**, 1481-1483.

**Wady, L., Bunte, A., Pehrson, C., Larsson, L. (2003).** Use of gas chromatography-mass spectrometry/solid phase microextraction for the identification of MVOCs from moldy building materials. *Journal of Microbiological Methods* **52**, 325-332.

**Wang, T., Smith, D., Španěl, P. (2004).** Selected ion flow tube, SIFT, studies of $H_3O^+$, $NO^+$ and $O_2^+$ with compounds released by *Pseudomonas* and related bacteria. *International Journal of Mass Spectrometry* **233**, 245-251.

**Willger, S.D., Grahl, N., Cramer, R.A. (2008).** *Aspergillus fumigatus* metabolism: Clues to mechanisms of *in vivo* fungal growth and virulence. *Medical Mycology* **47**, S72-S79.

**Wold, S., Esbensen, K., Geladi, P. (1987).** Principal Components Analysis. *Chemometrics and Intelligent Laboratory Systems* **2**, 37-52.

**Zechman, J.M., Aldinger, S., Labows Jr., J.N. (1986)**. Characterisation of pathogenic bacteria by automated head space concentration-gas chromatography. *Journal of Chromatography B: Biomedical sciences and Applications* **377**, 49-57.

# APPENDICES

# APPENDIX A ORIGIN OF THE MICROORGANISMS TESTED

### A.1 Microbial Isolates

COLLECTION: Microbiology Department, Cheltenham General Hospital

SOURCE: Clinical isolates from patients

SITE ORIGIN: Lung, BAL fluid aspirate

a) *Klebsiella pneumoniae* (16129695)*

*b) Staphylococcus* species (16129695)*

c) *Enterobacter cloacae* (16191218)*

d) *Candida albicans* (16191218)*

COLLECTION: American Type Culture collection

ATCC® NUMBER: 6538™

ISOLATION: Human lesion

e) *Staphylococcus aureus* sbsp. *aureus* Rosenbach

COLLECTION: Applied Mycology group, Cranfield Health

f) *Aspergillus fumigatus* (Af293)

* Hospital's microbiology laboratory identification number

# APPENDIX B TABLES

**Table B.1:** Typical formula for three different media (g $l^{-1}$). BHIB: Brain heart infusion broth; TSB: Tryptone soya broth; NB: Nutrient broth.

| BHIB g/l | | TSB g/l | | NB g/l | |
|---|---|---|---|---|---|
| Calf brain infusion | 12.5 | Pancreatic digest of casein | 17 | Meat extract | 1 |
| Calf heart infusion solids | 5 | Enzymatic digest of soya bean (with papain) | 3 | Yeast extract | 2 |
| Proteose peptone | 10 | Di-potassium hydrogen phosphate | 5 | Peptone | 5 |
| Glucose | 2 | Glucose | 2.5 | Sodium chloride | 5 |
| Sodium chloride | 5 | | | | |
| Di-sodium phosphate | 2.5 | | | | |

**Table B.2**: Sensors and detected compounds in NST 3320 instrument

| **Sensor** | **Important detected compounds** |
|---|---|
| MOSFET 101A | Hydrogen, amine |
| MOSFET 102A | Amine, ester |
| MOSFET 103A | Amine, aldehyde, alcohol |
| MOSFET 104A | Hydrogen |
| MOSFET 105A | Hydrogen, amine |
| MOSFET 101B | Amine, aldehyde, ester, alcohol, ketone |
| MOSFET 102B | Hydrogen, amine, alcohol |
| MOSFET 103B | Amine, aromate, aldehyde, ester, alcohol, ketone |
| MOSFET 104B | Hydrogen |
| MOSFET 105B | Hydrogen, amine, aldehyde, ester, alcohol, ketone |
| MOS 101 | Air contaminants (hydrogen, carbon monoxide) |
| MOS 102 | Hydrocarbons |
| MOS 104 | Alcohol, organic solvents |
| MOS 110 | Hydrocarbons |
| MOS 111 | Methane |
| MOS 112 | Propane, butane |
| MOS 113 | Hydrogen |
| MOS 114 | Organic solvents |
| MOS 115 | Alcohol |
| MOS 116 | Freon |
| MOS 117 | Ammonia |
| MOS 118 | Organic solvents, alcohol, hydrogen |
| Humidity sensor | Relative humidity at 70˚ C |
| $CO_2$ (optional) | $CO_2$ |

**Table B.3**: Summary of mean, standard deviations and % of variations of the sensor responses over a period of 24 months.

| | 0.1% acetone | | | 0.1% ipa | | | Distilled water | | |
|---|---|---|---|---|---|---|---|---|---|
| Sensors | Mean | Stdev | % | Mean | Stdev | % | Mean | Stdev | % |
| FE101A | 63.75 | 4.49 | 7.04 | 66.60 | 4.93 | 7.40 | 63.87 | 4.33 | 6.78 |
| FE102A | 90.14 | 7.41 | 8.22 | 93.39 | 7.95 | 8.51 | 89.48 | 7.29 | 8.14 |
| FE103A | 162.71 | 14.85 | 9.13 | 169.80 | 16.15 | 9.51 | 162.99 | 14.81 | 9.09 |
| FE104A | 44.63 | 8.20 | 18.37 | 48.23 | 8.85 | 18.34 | 46.35 | 9.14 | 19.72 |
| FE105A | 20.64 | 5.35 | 25.94 | 23.25 | 6.71 | 28.84 | 21.86 | 4.95 | 22.67 |
| FE101B | 78.40 | 11.02 | 14.05 | 81.84 | 11.89 | 14.53 | 78.01 | 9.32 | 11.95 |
| FE102B | 111.75 | 8.81 | 7.89 | 114.61 | 9.73 | 8.49 | 111.10 | 9.18 | 8.26 |
| FE103B | 133.94 | 13.01 | 9.71 | 140.06 | 14.88 | 10.63 | 135.95 | 13.65 | 10.04 |
| FE104B | 37.48 | 0.83 | 2.20 | 39.01 | 0.65 | 1.66 | 37.90 | 0.85 | 2.23 |
| FE105B | 6.43 | 2.14 | 33.29 | 8.22 | 2.70 | 32.84 | 7.16 | 2.04 | 28.45 |
| MO101 | 415.40 | 7.71 | 1.86 | 390.35 | 8.26 | 2.12 | 126.57 | 22.60 | 17.85 |
| MO102 | 259.03 | 8.23 | 3.18 | 223.90 | 8.11 | 3.62 | 112.58 | 7.99 | 7.09 |
| MO104 | 264.59 | 20.78 | 7.85 | 163.61 | 25.44 | 15.55 | 29.79 | 7.97 | 26.76 |
| MO110 | 237.44 | 15.58 | 6.56 | 198.65 | 14.93 | 7.51 | 106.48 | 6.83 | 6.42 |
| MO111 | 96.09 | 6.98 | 7.26 | 83.36 | 6.01 | 7.22 | 82.36 | 6.25 | 7.59 |
| MO112 | 133.65 | 8.19 | 6.13 | 106.49 | 8.16 | 7.66 | 74.65 | 8.05 | 10.79 |
| MO113 | 14.43 | 2.69 | 18.66 | 6.89 | 1.41 | 20.43 | 1.74 | 0.51 | 29.25 |
| MO114 | 421.09 | 6.49 | 1.54 | 384.64 | 14.08 | 3.66 | 49.10 | 17.05 | 34.73 |
| MO115 | 296.23 | 38.94 | 13.14 | 329.70 | 30.44 | 9.23 | 168.30 | 18.17 | 10.80 |
| MO116 | 396.46 | 9.65 | 2.43 | 394.98 | 10.03 | 2.54 | 168.44 | 20.79 | 12.34 |
| MO117 | 425.61 | 13.57 | 3.19 | 381.81 | 22.13 | 5.80 | 73.24 | 17.19 | 23.47 |
| MO118 | 319.47 | 31.64 | 9.91 | 274.64 | 30.04 | 10.94 | 83.62 | 8.96 | 10.72 |
| Humidity | 12.54 | 0.82 | 6.57 | 12.62 | 0.89 | 7.03 | 12.12 | 0.81 | 6.72 |

# APPENDIX C FIGURES

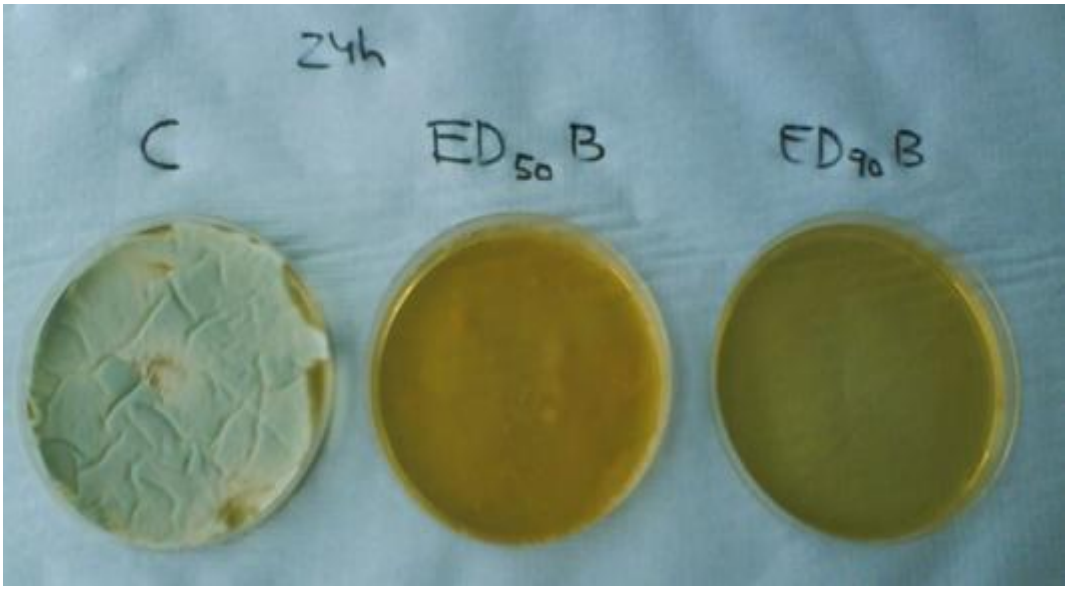



**Figure C.1**: *A.fumigatus* with benomyl $ED_{50}$ and $ED_{90}$ after 24 hrs incubation

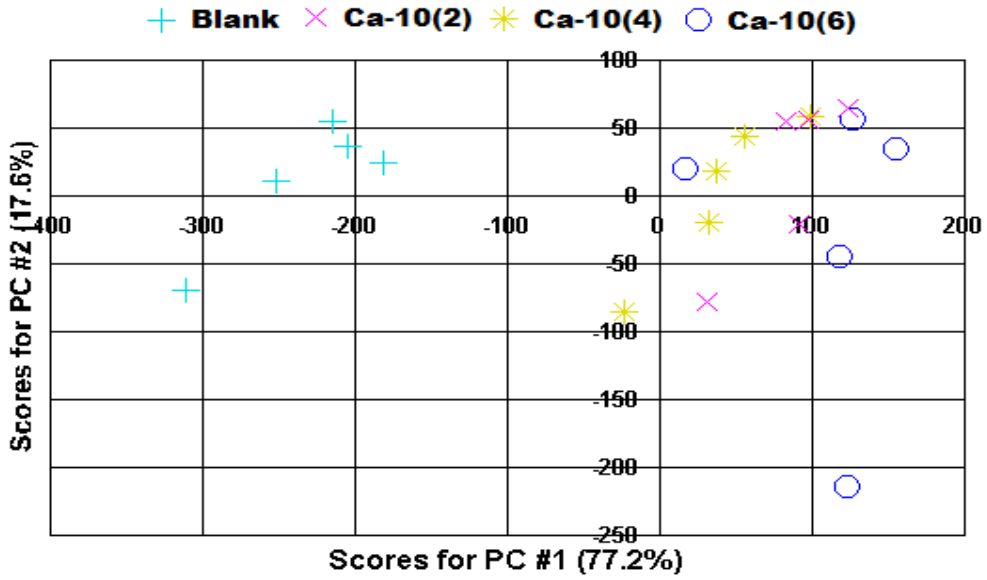



**Figure C.2:** PCA score plot of *C.albicans* 48 hrs incubation at 37°C in NB. (Key: Ca: *C.albicans*: 10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ cells $ml^{-1}$)

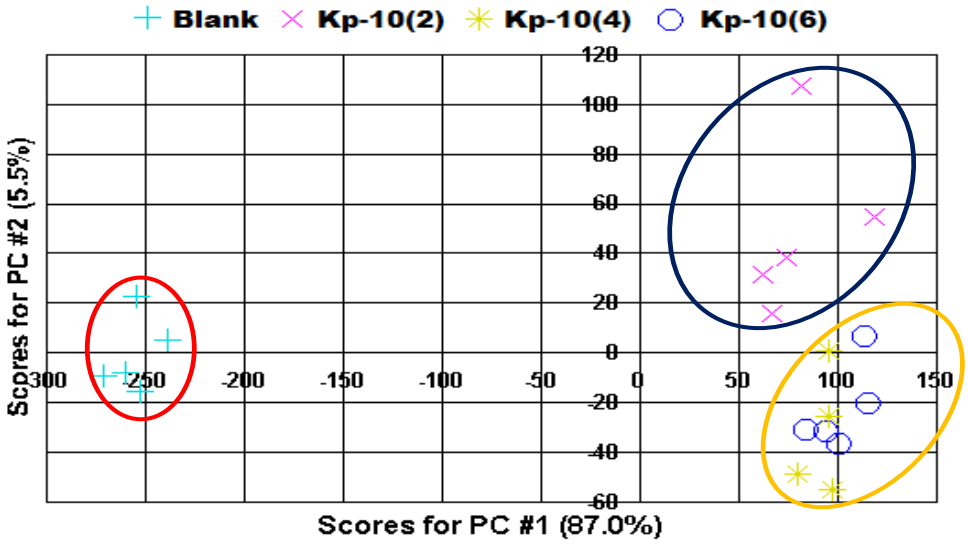



**Figure C.3:** PCA score plot of *K.pneumonaie* after 24 hrs incubation at 37°C in NB. One outlier was removed. (Key: Kp: *K.pneumoniae*: 10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ cells $ml^{-1}$).

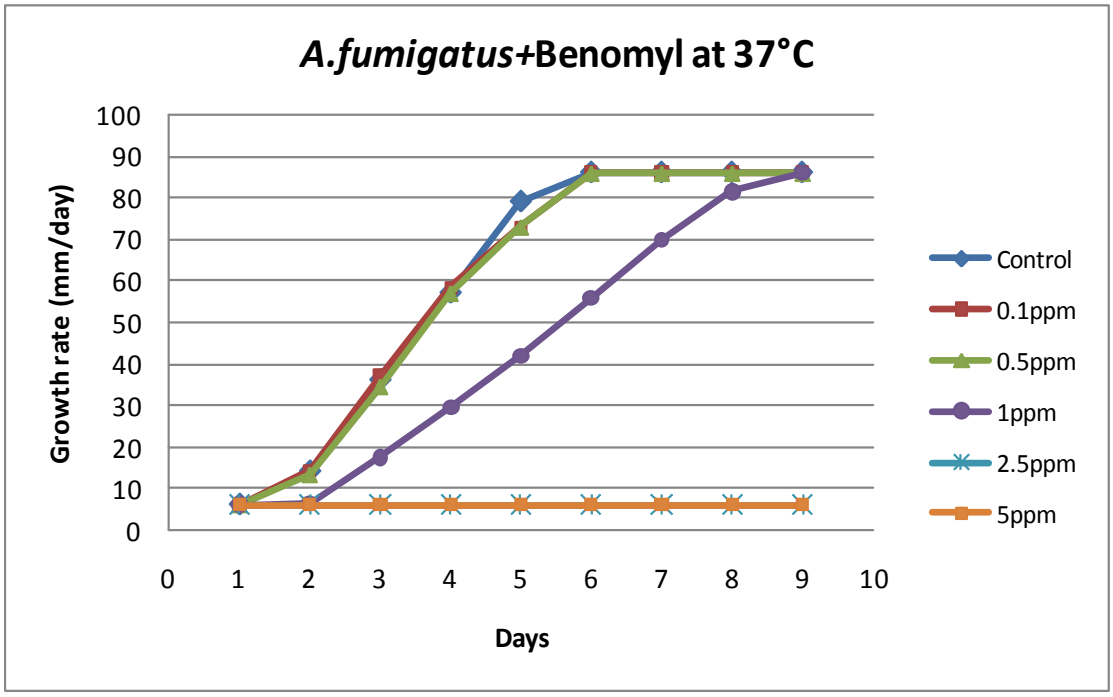



**Figure C.4:** Growth rates of *A.fumigatus* at 37°C with different concentrations of benomyl on MEA over a period of 9 days.

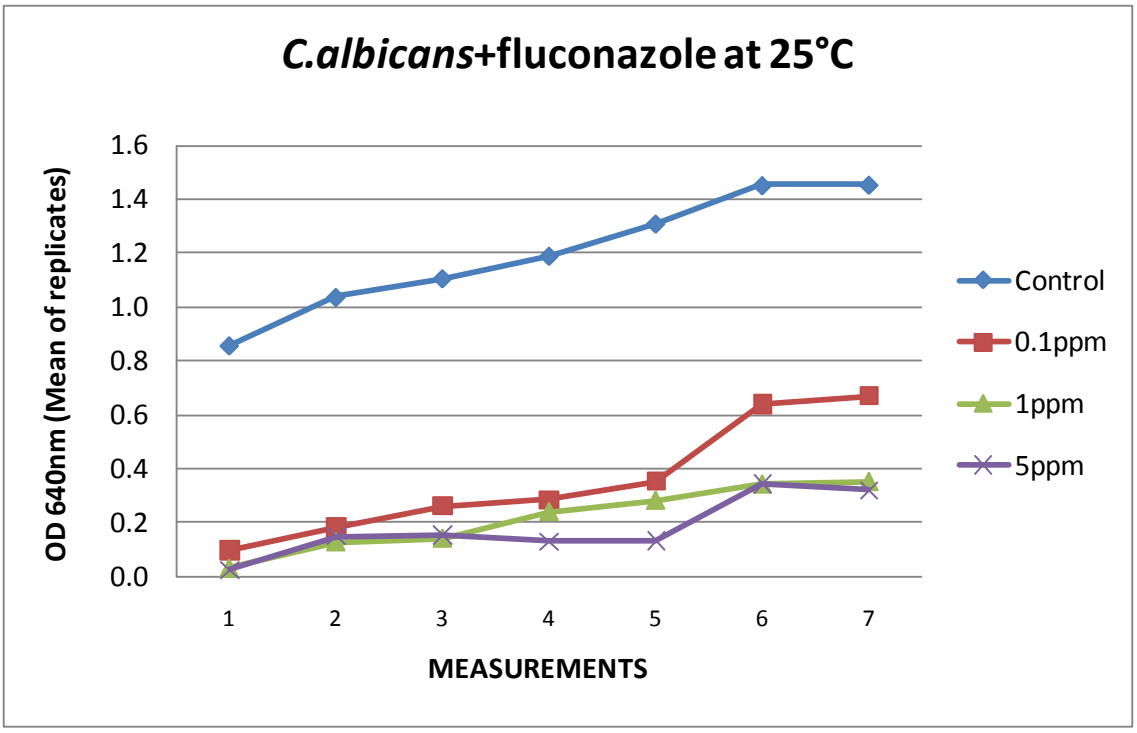



**Figure C.5:** Growth rates of *C.albicans* at 25°C with different concentrations of fluconazole in NB over a period of 6 days.

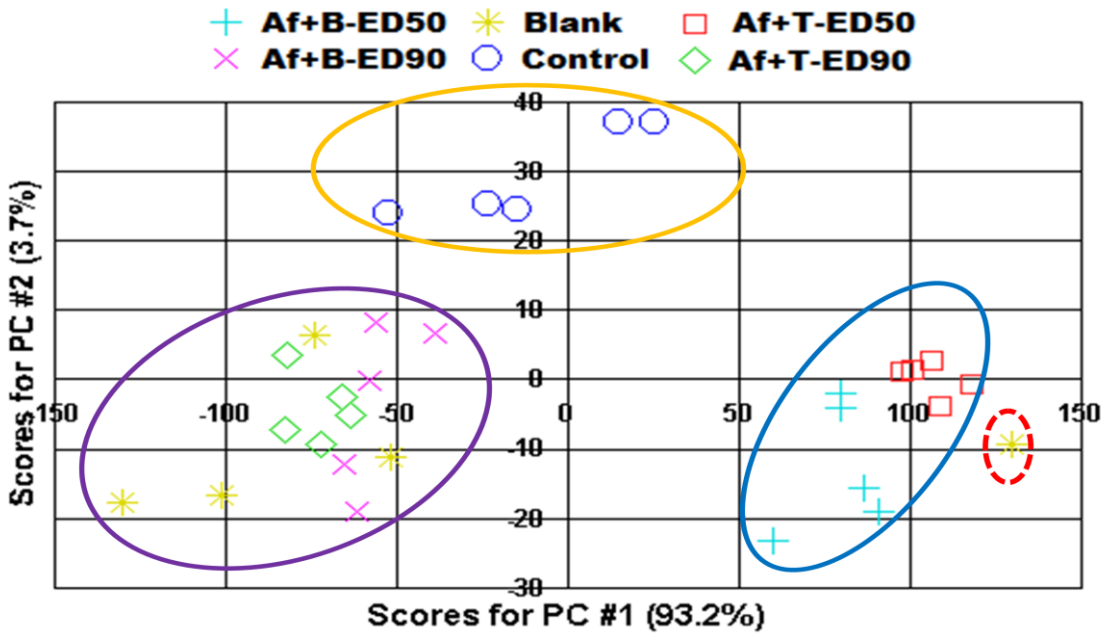



**Figure C.6:** PCA plot after 48 hrs incubation on MEA at 37°C of *A.fumigatus* (Af) with $ED_{50}$/$ED_{90}$ concentrations (ED50/ED90) of tebuconazole (T) and benomyl (B). Red dotted line: outlier.

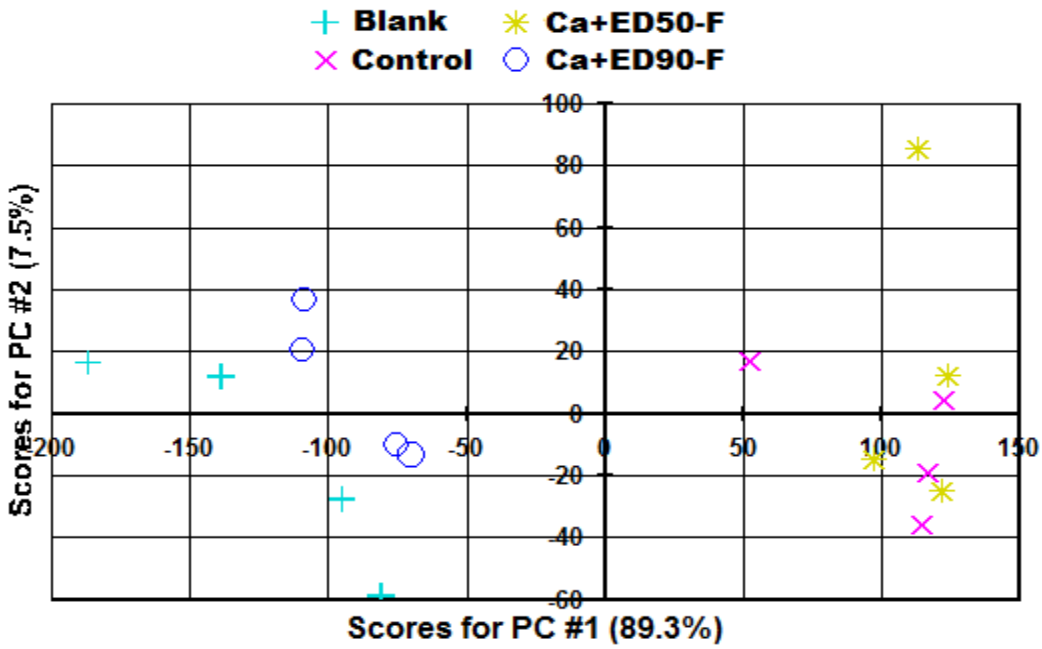



**Figure C.7**: PCA plot of *C.albicans* after 48 hrs incubation at 25°C in NB. (Key: Ca+ED50/90-F: *C.albicans*+$ED_{50}/_{90}$ of fluconazole).

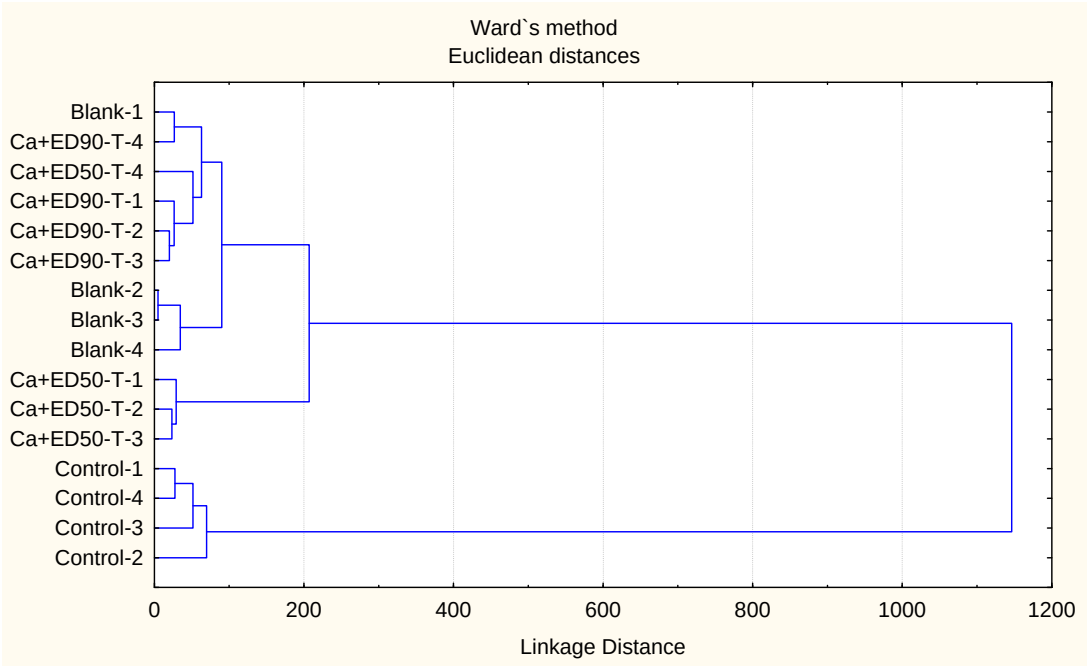



**Figure C.8**: Dendrogram of *C.albicans* after 48 hrs incubation at 25° C in NB. (Key: Blank: NB without inoculum; Control: *C.albicans* without anti-fungal; Ca+ED50/90-T: *C.albicans*+$ED_{50}/_{90}$ of tebuconazole).

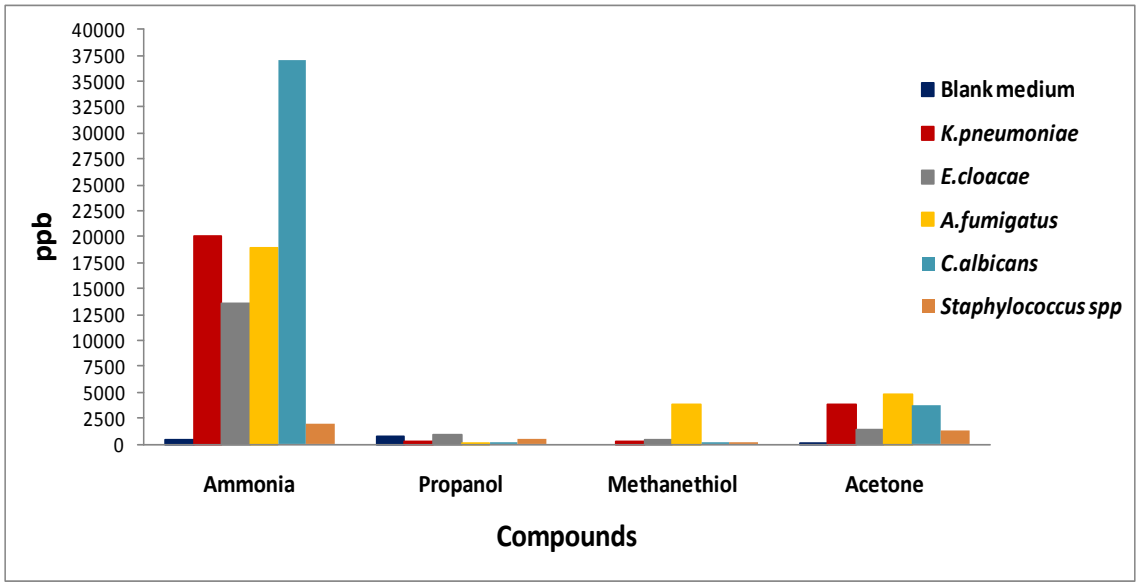



**Figure C.9:** Bar chart of some compounds found in distinctive amounts between blank medium and five VAP microorganisms by using SIFT-MS system after 24 hrs incubation at 37°C on NA. No replicates were analysed.

| Samples | LV | Scale | Overall | C1T | C2T | C3T | C4T |
|---|---|---|---|---|---|---|---|
| SS (All sensors) | 3 | MC | 95.83 | 100.0 | 87.5 | 100.0 | 100.0 |

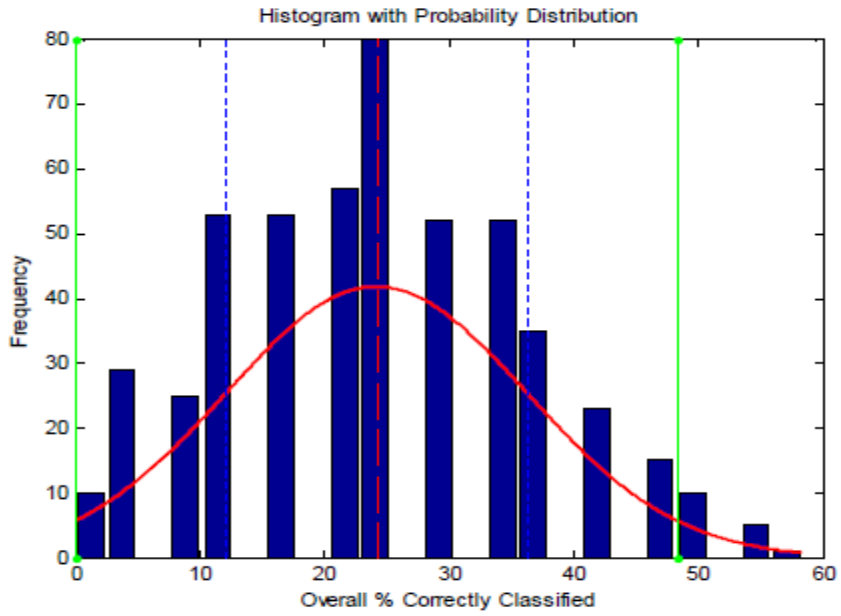



**Figure C.10:** Histogram for the resulting distribution for the *in vitro* model in saline solution+0.2% glucose-0.2% peptone at 37°C after 24 hrs incubation using PLS-DA method. (Key: SS: saline solution; LV: latent variable; MC: mean-centred scale).

# APPENDIX D PUBLICATIONS, ORAL PRESENTATIONS AND POSTERS

## D.1 Publications

1. **Planas, N**., Kendall, C., Magan, N. (2009). Early Discrimination Of Microorganisms Involved In Ventilator Associated Pneumonia Using Qualitative Volatile Fingerprints. *International Symposium on Olfaction and Electronic nose, Brescia 2009, Italy. Proceedings*.

2. **Camardo Leggieri, M**., **Planas Pont, N.**, Battilani, P., Magan, N. (2011). Detection and discrimination between ochratoxin producer and non-producer strains of *Penicillium nordicum* on a ham-based medium using an electronic nose. *Mycotoxin Research* **27**, 29-35.

3. **Humphreys, L**., Orme, R., Moore, P., Charaklias, N., Sahgal, N., **Planas Pont, N.**, Magan, N., Stone, N., Kendall, C.C. (2011). Electronic nose analysis of bronchoalveolar lavage fluid. *European Journal of Clinical Investigation* **41**, 52-58.

4. **Planas Pont, N**., Kendall, C., Magan, N. Analysis of volatile fingerprints for monitoring anti-fungal efficacy against the primary and opportunistic pathogen *Aspergillus fumigatus.* Being submitted to *Mycophatologia*.

5. **Planas Pont, N**., Kendall, C., Magan, N. Use of a similar and medically relevant lung fluid medium for discrimination between microorganisms involved in VAP and for building up a model for unknown samples. In preparation.

Student's contribution to papers 1, 4 and 5: to plan the experiments, prepare the samples and do the e-nose analyses, perform the statistical analyses and prepare a paper draft.

Student's contribution to paper 2: I assisted the first author to analyse the samples by the e-nose and obtain the results with statistical methods.

Student's contribution to paper 3: I analysed with the e-nose the latest clinical samples and I assisted the first author in providing any needed information related to the e-nose procedure.

## D.2 Oral presentations

1. Electronic Nose for early detection and discrimination of microorganisms involved in Ventilator-Associated Pneumonia (VAP). Presented at the *Postgraduate Students Conference*, September 2010, Cranfield Health, Cranfield University, UK.

## D.3 Posters

1. **Planas, N**., Sahgal, N., Kendal, C., Barr, H. and Magan, N. In vitro discrimination of microorganisms involved in ventilated associated pneumonia using the Electronic nose. *Multistrand Conference*, May 2008, Cranfield University, UK.

2. **Kendall, C**., Humphreys, ML., Charaklias, N., Orme, R., Moore, P., Sahgal, **Planas, N**, Magan, N., Ston, N. *IPEM annual Scientific meeting*, September 2008, Bath University, UK.

3. **Planas, N.**, Sahgal, N., Kendal, C., Barr, H. and Magan, N. Early discrimination of microorganisms involved in Ventilator Associated Pneumonia (VAP) using qualitative volatile fingerprints. *International Symposium on Olfaction and Electronic nose*, April 2009, Brescia University, Italy.

4. **Planas, N.**, Sahgal, N., Kendal, C., Barr, H. and Magan, N. Screening of drugs and discrimination of microorganisms involved in Ventilator Associated Pneumonia (VAP) using volatile fingerprints. *Postgraduate Students Conference*, September 2009, Cranfield Health, Cranfield University, UK.

5. **Planas, N.**, Sahgal, N., Kendal, C. and Magan, N. Volatiles fingerprints: use of a hybrid sensor array electronic nose to screen anti-fungal efficacy against *A.fumigatus* and *C.albicans*. *20$^{th}$ Anniversary World Congress on Biosensors*, May 2010, Glasgow, UK.

# Early Discrimination Of Microorganisms Involved In Ventilator Associated Pneumonia Using Qualitative Volatile Fingerprints

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**Abstract.** This study has examined the use of an electronic nose for the detection of volatile organic compounds produced by different microorganisms responsible for ventilator-associated pneumonia (VAP), an important disease among patients who require mechanical ventilation. Based on the analysis of the volatile organic compounds, electronic nose technology is being evaluated for the early detection and identification of many diseases. It has been shown that effective discrimination of two bacteria (*Enterobacter cloacae* and *Klebsiella pneumoniae*) and yeast (*Candida albicans*), could be obtained after 24 h and filamentous fungus (*Aspergillus fumigatus*) after 72h. Discrimination between blank samples and those with as initial concentration of $10^2$ CFU $ml^{-1}$ was shown with 24h incubation for bacteria and 48 h for fungi. Effective discrimination between all the species was achieved 72 h after incubation. Initial studies with mixtures of microorganisms involved in VAP suggest that complex interactions between species occur which influences the ability to differentiate dominant species using volatile production patterns. A nutrient agar base medium was found to be optimum for early discrimination between two microorganisms (*Klebsiella pneumoniae* and *Candida albicans*).

**Keywords**: Electronic nose, ventilator-associated pneumonia, volatile organic compound, volatile production patterns.

## INTRODUCTION

Today, one of the main risks for patients in hospitals is that of associated diseases, i.e., nosocomial infections. One of these infections is ventilator associated-pneumonia (VAP) which is defined as a pneumonia occurring in patients after more than 48 hours of mechanical ventilation via endotracheal or tracheotomy tube. This may affect 8 to 28% of all the intubated patients [1, 2] and the mortality rate may arise from 24 to 50% where high-risk pathogens are involved.

Gram-negative enteric rods, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the predominant microorganisms responsible for this infection. However, many others microorganisms can cause VAP including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Acinetobacter baumanii* depending on the onset of the illness. There are other microorganisms considered opportunistic organisms such as *Candida albicans* and *Aspergillus fumigatus* which can play a role. *Staphylococcus* sp. and *P.aeruginosa*, and others, belong to the group of microorganisms which potentially can be multi-drug resistant. Some of the criteria for diagnosing VAP are not very specific and can lead to an inaccurate diagnosis of the disease. For these reasons, the mortality and morbidity of VAP can be very high (24-50%).

Many infections are known to produce characteristic odours due to the release of volatile organic compounds (VOCs) from the microorganisms involved. The development of sensor arrays which can reflect changes in qualitative volatile fingerprints has provided promising potential tools for the rapid and early detection of microbial infections at the point of care [3].

The aim of the present study was to examine the potential for using such qualitative volatile fingerprints as biomarkers of VAP infections enabling rapid, point at care diagnosis and to facilitate more effective treatment. Thus this work describes initial studies on

the use of sensor arrays for the discrimination between five microorganisms and evaluation of the relative sensitivity for the detection of different concentrations of these microorganisms over 24-96 h periods. The media required for optimum detection and discrimination were also investigated.

## EXPERIMENTAL AND METHODS

### Species

*In vitro* studies were carried out with five microorganisms, two Gram negative bacteria (*Klebsiella pneumoniae* and *Enterobacter cloacae*), one Gram positive bacterium, (*Staphylococcus sp*), one yeast (*Candida albicans*) and one filamentous fungus (*Aspergillus fumigatus*). All of them, except *A.fumigatus*, were obtained from clinical isolates.

### Inocula and optimal media

In order to calculate the required concentrations of cells (CFU $ml^{-1}$), suspensions were made using calibration curves previously obtained. The optical densities were measured with a spectrophotometer (Camspec MS350). Initial experiments examined different media in order to find which one could give better results in terms of discrimination between microorganisms. Three kinds of media (Oxoid, UK) were tested: Nutrient Broth (NB), Brain Heart Infusion Broth (BHIB) and Tryptone Soy Broth (TSB) at 1% concentration were examined. *K.pneumoniae* and *C.albicans* ($10^4$ CFU $ml^{-1}$) were inoculated in 100 mL of sterilised broth. All the inoculated samples and blanks were incubated at 37°C in shaken flasks for 3 days. Subsequent experiments were performed using 1% Nutrient Broth in order to minimise interfering volatiles from the liquid media and at the same time permit the growth of the species.

### E nose system

Samples were tested using sensor arrays in the NST 3220 E nose from Applied Sensors (Sweden). This device consists of 12 MOS sensors, 10 MOSFET sensors and 1 humidity sensor. The headspace was analysed automatically from samples placed in the 12 position autosampler unit. Data were analysed with multivariate statistical methods such as principal components analysis (PCA) and cluster analysis (CA). In order to optimise the PCA results, replicates considered outliers were removed from the analysis if they were located far away from the mean group. The criteria for being considered an outlier was: samples with $\geq$ 25% response than the mean of the other replicates.

### Sampling method

Five ml of each mixture of media and bacterial inocula were placed in sterilised 25ml vials and sealed using screw caps and septa after 24, 48, 72 and 96 h. The samples were left for one hour at 37°C in the incubator in order to generate volatiles from the samples in the headspace. Sterile media without inoculum was used as a blank sample.

## RESULTS

### Separation of standards

Standards such as diluted alcohols (isopropanol, acetone) and distilled water were measured used periodically to evaluate drift in the hybrid sensor array responses over time. Overall, this was found to be <15% over the experimental period.

### Selection of optimal media for volatiles detection

NB was the only medium that achieved good discrimination between *K.pneumoniae* and *C.albicans* after 24 h incubation. TSB and BHIB only showed differentiation after 48 hrs incubation. The three different control media were classified in the same blank cluster (Figure 1).

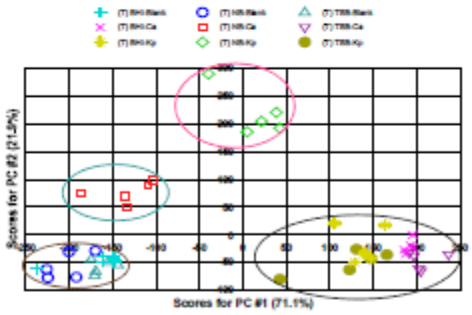


**FIGURE 1.** PCA plot showing the early discrimination between *C. albicans* (Ca) and *K. pneumoniae* (Kp) on 1% NB medium 24 h after incubation at 37°C. (Key: NB: Nutrient Broth; TSB: Tryptone Soy Broth; BHIB: Brain Heart Infusion Broth)

## Discrimination between species with similar initial concentration

The volatile fingerprints produced after 24 to 96 h using the same initial concentration ($10^4$ CFU $ml^{-1}$) for all of five of the microorganisms was examined. After 24 h it was possible to visualise at least three clusters of microorganisms: one group with the two Gram negative bacteria, another with *C.albicans* (yeast) and a third group formed by blank samples, *A.fumigatus* and the *Staphylococcus* sp. After 48 hours, the PCA showed clearer differences between blank samples and inoculated samples and after 72 hours, at least six clusters (blank and five species) were observed (Figure 2). After ninety six hours the discrimination was less clear.

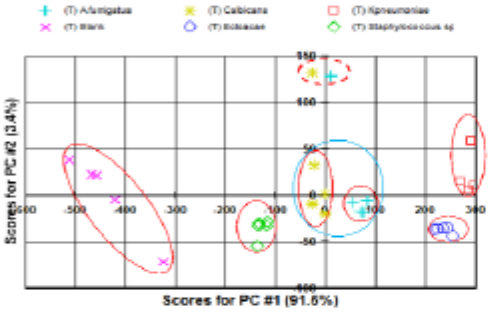


**FIGURE 2.** PCA score plot after 72 h at 37°C on 1% NB of five different microorganisms and blank samples. Dotted circle indicates two probable outliers

## Relative sensitivity of detection

Studies were carried out to examine sensitivity of the sensor array to discriminate between different initial concentrations $10^2$, $10^4$ and $10^6$ CFU $ml^{-1}$ of the same microorganism. The threshold of detection of the E-nose system was seen to vary with the microorganism. There was not discrimination between blank samples and the lowest initial concentration ($10^2$ CFU $ml^{-1}$) after 24 h in the samples inoculated with fungal species. For the Gram negative species there was clear discrimination between samples after 24 h.

Figure 3 shows the dendrogram generated from cluster analysis of the data for *Enterobacter cloacae* after 24 incubation with three well defined clusters: one with blank samples, another with the lowest concentration ($10^2$ CFU $ml^{-1}$) and a third cluster with the higher concentrations ($10^4$ and $10^6$ CFU $ml^{-1}$). After 48 hrs the discrimination between all the concentrations was less clear for Gram negative inoculations and in *C.albicans*. For *A.fumigatus* discrimination with the same initial concentration required 72 h of growth. With regards to the *Staphylococcus* strain there was not growth until 48h incubation. Due to that poor growth it was difficult to compare with the rest of bacteria and no conclusions can be made. A new type strain of *Staphylococcus* sp is now being used.

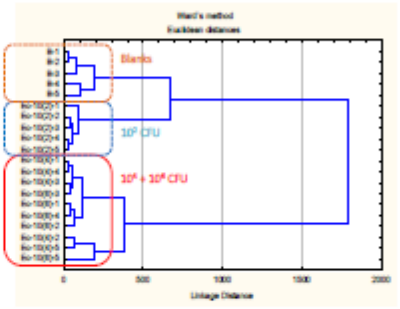


**FIGURE 3.** Dendrogram of three different concentrations of *E.cloacae* after 24 h incubation at 37°C on 1% NB. (Key: Ec: *Enterobacter cloacae*; 10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ CFU $ml^{-1}$)

## Mixture of microorganisms

Two experiments were performed by mixing different microorganisms: *C.albicans+E.cloacae* and *Staphylococcus* sp+*E.cloacae*; the latter experiment was performed twice. In all experiments blank samples were classified in a unique cluster as well as *C.albicans* and *Staphylococcus* sp samples alone. This did not happen with *E.cloacae* alone which was grouped with the rest of the samples in all cases and periods of incubation.

## DISCUSSION

The choice of growth media is important. This was demonstrated by the experiments with *K.pneumoniae* and *C.albicans* on three different media: Nutrient Broth (NB), Tryptone Soy Broth (TSB) and Brain Heart Infusion Broth (BHIB) at 1% concentration. Effective discrimination between the species was successful after 24 h incubation on NB. Depending on the group of microorganisms under investigation it is essential to first optimise the choice of media due to the nutritional variation between species. Furthermore, it is not always true that traditional solid media for

isolation of microbial species enhances volatile analysis. For example, Sahgal [4] found earlier discrimination (72 h incubation) between different dermatophytes using SBHIA (Sabouraud Brain Heart Infusion Agar) which is less commonly used in routine microbiology practice rather than SDA (Sabouraud Dextrose Agar).

Previous studies have suggested that the addition of specific enzymes to the liquid samples enhances volatile generation into the headspace and improves discrimination. This was successfully achieved with an enzymatic lipase-based cocktail for detection of *Mycobacterium tuberculosis* [5].

This study has shown that it is possible to discriminate between the main microorganisms which cause VAP in 24-48h with the same initial concentration. However, it was not until 72h when the maximum discrimination between blank samples and between all of the microorganisms was achieved. Due to the slow growth of *A.fumigatus* in comparison with the rest of the microorganisms, its cluster was grouped with the blanks after 24 h. Filamentous fungi grow slower than bacteria has been shown in previous studies with spoilage fungi [6]. With regards to bacteria and yeasts the discrimination between them was more rapidly achieved (24h after inoculation).

In terms of sensitivity the experiment performed by Magan *et al.* [7] studied the discrimination between three very close but high concentrations ($10^6$, $3.5x10^8$ and $8x10^8$ cells $ml^{-1}$) of *Pseudomonas aureofaciens* after 60 minutes incubation. The analysis showed that there was three-group formation: low concentration and milk control, medium and high concentration and the third the butanol control. These results are very closely paralleled in the current study although there were differences between the trials. In the present study it has been shown that the threshold of detection between blank and inoculated samples was achieved in different incubation times depending on the microorganism and initial concentration. As shown with *A.fumigatus* ($10^2$ CFU $ml^{-1}$) which required 72 h growth to discriminate inoculated from blank samples compared to only 24h growth with inoculation with a higher concentration ($10^6$ CFU $ml^{-1}$).

Few studies have been undertaken using mixtures of microorganism. However, one of these studies tested sputum samples from patients with a conducting polymer sensor array [5]. Samples consisted of three different bacteria (*Mycobacterium avium*, *M.tuberculosis* and *P.aeruginosa*) a mixture of these microorganisms and control sputum, all treated with lipase enzymes. They found clear discrimination between inoculated samples after 6 h at room temperature. The detection and discrimination of species in mixtures is a complex issue which is still being studied. We believe that with mixtures some may be more dominant than others (e.g. *E.cloacae*) which can be identified by their key volatile.

## CONCLUSIONS

The best discrimination between *K.pneumoniae* and *C.albicans* was achieved after 24h incubation with 1% Nutrient Broth. However, the optimisation of volatile production needs further improvement, for example evaluation of the use of enzymes to enhance volatile generation.

It was possible to differentiate between some bacteria and yeast species after 24-48 h incubation at a threshold of $\geq 10^2$ CFU $mL^{-1}$ as initial concentration. The discrimination between fungi required 72 h incubation. These findings show that potential exists for using this approach as a tool for the early detection of some bacterial infections in 24-48 hours with a relatively small amount of cells and using an easy and simple sampling method.

In mixtures of two different microorganisms one of them was usually seen to be predominant. This competitiveness of individuals in the mixture made interpretation of results more complex; however, the volatile pattern production could permit the identification of the predominant species and therefore guide selection of targeted therapy.

## ACKNOWLEDGMENTS

We are grateful to Cranfield Health and Gloucestershire Hospitals NHS Foundation Trust for financial support.

## REFERENCES

1. J. Chastre, J,Y, Fagon. *Am J Respir Crit Care Med.* **165**, 521-526 (2002).
2. M.H. Kollef. *Respiratory Care* **50**, 714-724.
3. A.P.F. Turner and N. Magan, *Nature Reviews: Microbiology* **2**, 161-166 (2004).
4. N. Sahgal. " Microbial and non-microbial volatile fingerprints: potential clinical applications of electronic nose for early diagnoses and detection of diseases", *Ph.D. Thesis,* Cranfield Health, Cranfield University 2008.
5. A.K. Pavlou, N. Magan, J. Jones, J. Brown, P. Klatser and A.P.F. Turner, *Biosensors and Bioelectronics* **20**, 538-544 (2004).
6. R. Needham. "Early detection and differentiation of microbial spoilage of bread using electronic nose technology", *Ph.D. Thesis,* Cranfield University 2004.
7. N. Magan, A. Pavlou and I. Chrysanthakis, *Sensors and Actuators B* **72**, 28-34 (2001).

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ORIGINAL PAPER

# Detection and discrimination between ochratoxin producer and non-producer strains of *Penicillium nordicum* on a ham-based medium using an electronic nose

**Marco Camardo Leggieri · Neus Planas Pont · Paola Battilani · Naresh Magan**

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**Abstract** The aim of this work was to evaluate the potential use of qualitative volatile patterns produced by *Penicillium nordicum* to discriminate between ochratoxin A (OTA) producers and non-producer strains on a ham-based medium. Experiments were carried out on a 3% ham medium at two water activities ($a_w$ ; 0.995, 0.95) inoculated with *P. nordicum* spores and incubated at 25°C for up to 14 days. Growing colonies were sampled after 1, 2, 3, 7 and 14 days, placed in 30-ml vials, sealed and the head space analysed using a hybrid sensor electronic nose device. The effect of environmental conditions on growth and OTA production was evaluated based on the qualitative response. However, after 7 days, it was possible to discriminate between strains grown at 0.995 $a_w$, and after 14 days, the OTA producer and non-producer strain and the controls could be discriminated at both $a_w$ levels. This study suggests that volatile patterns produced by *P. nordicum* strains may differ and be used to predict the presence of toxigenic contaminants in ham. This approach could be utilised in ham production as part of a quality assurance system for preventing OTA contamination.

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**Keywords** *Penicillium nordicum* · Ochratoxin A · Volatile patterns · Electronic nose · Dry cured ham · Early detection

## Introduction

Stored ham is commonly covered by mould whose type and extent depends on ecological conditions in the storehouse, especially the prevailing temperature and humidity conditions (Spotti et al. 1999). The microbial community is important as it determines the aroma and the organoleptic aspects and contributes to the characteristics of final products in different geographical regions (Leistner 1986); unfortunately, some component microorganisms can also cause deterioration or produce undesirable metabolites. Results from studies in various countries have shown that pork meat is the most important source of natural toxins, in particular ochratoxin A (OTA), when compared with other products of animal origin (Govaris et al. 2007). The kidney and liver are the main target organs for OTA, resulting in teratogenicity, carcinogenicity and mutagenicity (Govaris et al. 2007). It is also implicated in the aetiology of Balkan Endemic Nephropathy (EFSA 2006).

The presence of OTA in meat and meat products could be the result of either direct contamination with moulds or carry over from animals exposed to naturally contaminated feed (Gareis 1996). The presence of *Penicillium* as the dominant genus on dry-cured pork meat has been reported in Italy, with *P. nordicum* responsible for the production of OTA in proteinaceous food being observed sporadically (Spotti et al. 2001) or as an important component (26%) of the total population (Battilani et al. 2006). *Penicillium* has also been reported as dominant (88.3%) in Norway (Asefa et al. 2009), with *P. nalgiovense*, *P. solitum* and *P. commune* as the main species isolated. No further reports of *P. nordicum* on stored

pork meat are available, but possibly some misidentification of *P. verrucosum* could have occurred (Iacumin et al. 2008).

Mycotoxins and other non-volatile metabolites have traditionally been detected using high performance liquid chromatography coupled to diode array detection (HAPLC-DAD) and often also to mass spectrometry (LC-DAD-MS) (Smedsgaard 1997). A new validated HPLC-FLD method for OTA detection in dry cured meat and in blue cheese has recently been developed to minimise the matrix effect and to improve the analysis performance (Dall'Asta et al. 2007). These methods are destructive: they are applied at the end of the ham ripening period and can thus lead to a loss of commercial product.

There has been interest in using volatile production patterns to try and discriminate between spoilage fungi contaminating food products (Magan and Sahgal 2007; Cabañes et al. 2009). The main approach has been to use sensor arrays, based on different platforms such as conducting polymer, metal ion or metal oxide sensors. These sensors are often sensitive to specific groups of volatiles, e.g. alcohols, ketones, or terpenoids. Thus, volatiles passing over the sensor array change the resistance of the sensor based on adsorption patterns, which can be then normalised and compared between samples (Sahgal et al. 2007). The qualitative volatile patterns are then subjected to statistical analyses to compare treatments. Using this approach, and a conducting polymer sensor array, Keshri and Magan (2000) were able to discriminate fumonisin producer and non-producer strains of *Fusarium verticillioides* based on volatile fingerprints. Recently, Sahgal et al. (2007) and Cabañes et al. (2009) confirmed these results and demonstrated the potential for discriminating between toxigenic and non-toxigenic strains of the mycotoxigenic species, such as *P. verrucosum*, *Aspergillus flavus* and *A. carbonarius*, using metal ion- and metal oxide-based sensor arrays.

The objective of this study was to evaluate the effect of environmental conditions on growth and toxin production and the potential for discriminating between a toxigenic and non-toxigenic strain of *P. nordicum* using volatile production patterns on a ham-based medium for the first time.

## Materials and methods

### Strains

Two strains of *P. nordicum* were included in the study, an OTA and a non-OTA producer (Bogs et al. 2006). They are both held in the fungal collections of the Institute of Entomology and Plant Pathology, UCSC in Piacenza, Italy (MPVP P1669 and MPVP P1446, respectively) and the Max-Rubner Institute, Karlsruhe, Germany (BFE 838 and BFE 851, respectively).

### Inoculum preparation and inoculation

The strains were initially inoculated on Yeast Extract Agar [Peptone from casein (tryptone) 6.0 g; yeast extract 3.0 g; agar-agar 15.0 g; water 1,000 ml; Oxoid)] and incubated at 25°C for 7 days. The spores were collected using 10 ml of sterilised water and gently agitating the culture surface to remove conidia. The suspension was adjusted to $10^8$ conidia $ml^{-1}$ using a haemocytometer and used as an inoculum. A 0.2-ml aliquot was spread plated on the surface of each replicate Petri plate.

### Ham medium preparation

The ham medium used in this study was prepared with 30 g of lyophilised dry-cured pork (4% moisture, 11% NaCl, 67% total nitrogen, 18% fat), 15 g of technical agar and 1,000 ml water. The freeze-dried cured ham was prepared by FOOD DRYING (Fontanellato Parma, Italy). The medium was sterilised and the molten mixture poured into 9-cm Petri plates; the basic medium (0.995 water activity; $a_w$), was adjusted to different $a_w$ levels in the range 0.98–0.80 by substituting water with glycerol/water solutions (Dallyn and Fox 1980). The $a_w$ was measured with an Aqualab device (Decagon Instruments, USA) to confirm accuracy of the target treatments.

### Growth studies and ochratoxin A analyses

The *P. nordicum* OTA producer strain was centrally inoculated on ham medium adjusted to the different $a_w$ levels and incubated at 25°C. The diameter of the colonies was measured in two directions at right angles to each other after 2, 5, 7, 9, 12 and 14 days. Three plugs (diameter 0.46 cm, weight 0.18 g), were removed from the inner, middle and outer area of the fungal colony and placed in a vial containing 1 ml of methanol, extracted for 1 h and shaken for 15 min. Detection and quantitative determination of OTA from ham medium samples was made without a clean-up step, analysing the final solutions by a HPLC-FLD quantitative method. The HPLC analysis was performed with a C18 column (Waters XTerra®, 250× 2.1 mm, 3 µm) on an Agilent 1100 chromatographic system under isocratic conditions at room temperature, with an aqueous $NH_3/NH_4Cl$ (20 mmol $l^{-1}$, pH 9.8): $CH_3CN$ 85:15 v/v mobile phase; the flow rate was 0.2 ml $min^{-1}$ and the injected volume was 20 µl. The FLD detection was obtained by means of the Agilent 1100 Fluorescence Detector ($\lambda_{ex}$=380 nm, $\lambda_{em}$=440 nm). The result was measured in ng of OTA $g^{-1}$ of ham medium. The limit of detection (LOD) and quantification (LOQ) were 0.1 µg $Kg^{-1}$ and 0.3 µg $Kg^{-1}$ respectively (Dall'Asta et al. 2007).

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**Table 1** Effect of incubation time and water activity on growth extension of *P. nordicum* (OTA producer), on a 3% ham medium at 25°C

| | Diameter (mm) | |
|---|---|---|
| Incubation time (days) | | |
| 2 | 4.6 | f |
| 5 | 11.9 | e |
| 7 | 15.6 | d |
| 9 | 19.2 | c |
| 12 | 24.3 | b |
| 14 | 27.3 | a |
| Water activity | | |
| 0.98 | 30.1 | a |
| 0.95 | 32.1 | a |
| 0.93 | 20.4 | b |
| 0.90 | 12.5 | c |
| 0.85 | 3.0 | d |
| 0.80 | 3.0 | d |

Different letters define significant differences ($P$=0.05)

### Volatile production patterns of *P. nordicum* strains

Experiments were carried out on a basic ham medium (0.995 $a_w$) and that modified to 0.95 $a_w$ for the detection of volatile fingerprints; 0.2 ml of inoculum of both fungal strains was spread plated over the whole surface of 20 plates (4 replicates for each condition). Plates were incubated at 25°C and three random agar plugs (1 cm diameter) were taken from each replicate after 1, 2, 3, 7 and 14 days and placed in 30-ml head space vials, sealed with screw caps and septa. The samples were incubated at 25°C for 1 h to allow a build-up of volatiles in the head space and then analysed.

### Electronic nose system

An AppliedSensor 3320 E-nose (AppliedSensor Group, Sweden) was employed in this study. The core sensor technology of this machine is based on a hybrid array of 10 metal-oxide-silicon field-effect-transistor (MOSFET) sensors and 12 metal oxide sensors (MOS), and a capacitance-based relative humidity sensor. These sensors are provided as standard in this specific system. The MOSFET sensors were sensitive to: hydrogen, amines (MOSFET 101A); amines and esters (MOSFET 102A); aldhydes and alcohols (MOSFET 103A); hydrogen (MOSFET 104A); hydrogen and amines (MOSFET 105A); amines, aldehydes, esters, alcohols and ketones (MOSFET 101B); hydrogen, amines and alcohols (MOSFET 102B); amines, aromatics, aldehydes, esters, ketenes and alcohols (MOSFET 103B); and hydrogen (MOSFET 104B) and amines, aldehydes, esters and ketones (MOSFET 105B). The sensor array was heated to 140°C.

The 12 MOS sensors were specific for air contaminants such as: hydrogen and carbon monoxide (MOS 101); hydrocarbons (MOS 102); alcohols, organic solvents (MOS 104); hydrocarbons (MOS 110); methane (MOS 111); propane and butane (MOS 112); hydrogen (MOS 113); organic solvents (MOS 114); alcohol (MOS 115); Freon (MOS 116); ammonia (MOS 117); and organic solvents, alcohol and hydrogen (MOS 118).

The e-nose system used has a 12-vial auto-sampler system. The 30-ml volume vials were sealed and placed into the sample holders which were maintained at 37°C. The computerized programme used consisted of 30 s to obtain the base line, 30 s to take a sample, 200 s flushing, resulting in a total time of 4.20 min. There are two gas filters connected to the inlet including a drying column of silica gel which acts as moisture trap, and a hydrocarbon filter which is in two parts: one is a moisture filter and the other is an active carbon adsorbent. The individual sample holders for the vials are temperature controlled at 37°C to maintain stability of presentation of vials for head space analyses. Only when equilibration has been achieved does the system become operative. The air enters the system at a flow rate of 60 ml/min. It employs a robotic double needle system to draw sample headspace from the sampling vial, and flush it over the hybrid sensor array; the headspace is passively replaced with air through the 2nd injection needle. The divergence response of each sensor was used in all analyses.

### Data analysis

Data on fungal growth, OTA production and the sensor responses were analysed with the Univariate analysis of variance in SPSS (Statistical Package for Social Science, ver. 15.0, 2006; SPSS, Chicago, Il, USA) and the Tukey test was applied to separate means. NSTSenstool 3320 software (program provided in the AppliedSensor 3320 machine) was used to perform the principal component analysis (PCA) on the response signal (mean-centred data). Statistica 7 was used to analyse the data for Cluster Analysis (CA). Euclidean distance and Ward's method were applied, respectively, to establish similarities between samples and to aggregate distances.

**Table 2** Colony diameters and ochratoxin A production by colonies of the toxigenic *P. nordicum* strain inoculated on 3% ham medium adjusted at different water activity levels after 14 days incubation

| $a_w$ | Diameter (mm) | | Ochratoxin A (ng/g) | |
|---|---|---|---|---|
| 0.98 | 47.6 | a | 7,992.7 | a |
| 0.95 | 51.3 | a | 4,458.2 | b |
| 0.93 | 33.0 | b | 1,390.1 | c |
| 0.90 | 23.0 | b | 481.7 | c |
| 0.85 | 3.0 | c | 0.0 | c |
| 0.80 | 3.0 | c | 0.0 | c |

Different letters define significantly differences ($P$=0.05)

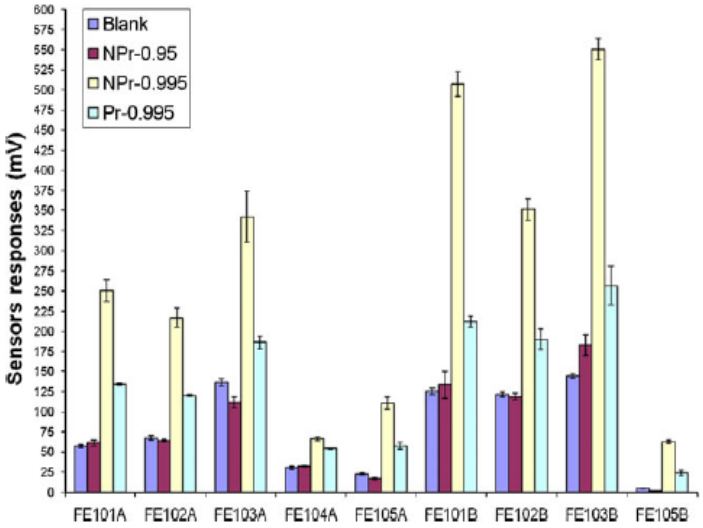



Fig. 1 Example of the mean response of the metal oxide sensors to volatiles produced by the toxigenic (MPVP1669, BFE 838) and non-toxigenic (MPVP1446, BFE 851) strains of *P. nordicum* after 7 days at 25°C on a 3% ham-based medium

## Results

### The effect of environmental factors on growth and OTA production

Growth of *P. nordicum* was slow but mycelial extension was visible after 2 days incubation. The colony diameter on the unmodified medium (0.995 $a_w$) was 30 mm (not included in data analysis). The mean diameter of colonies of the measured growth at all $a_w$ levels were about 27 mm after 14 days incubation (Table 1). The colonies were significantly larger at 0.98 and 0.95 $a_w$ when compared to lower $a_w$ treatments. The highest amount of OTA was produced after 14 days incubation at 0.98 $a_w$, followed by 0.95; the OTA amounts produced under water stress were significantly lower with no OTA produced at 0.85 and 0.80 $a_w$ (Table 2).

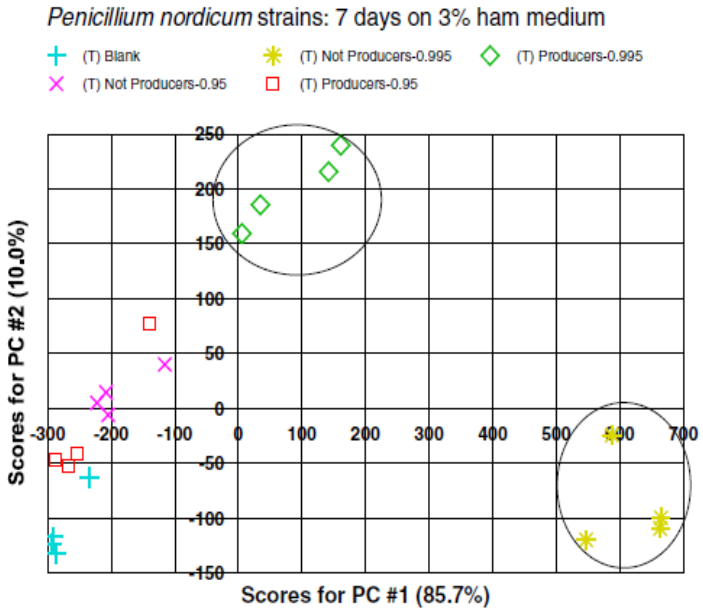



Fig. 2 The principal component analyses (PCA) plot of the data after 7 days incubation to show that there is some discrimination between the *P. nordicum* strains on a 3% ham-based medium based on the volatile production patterns

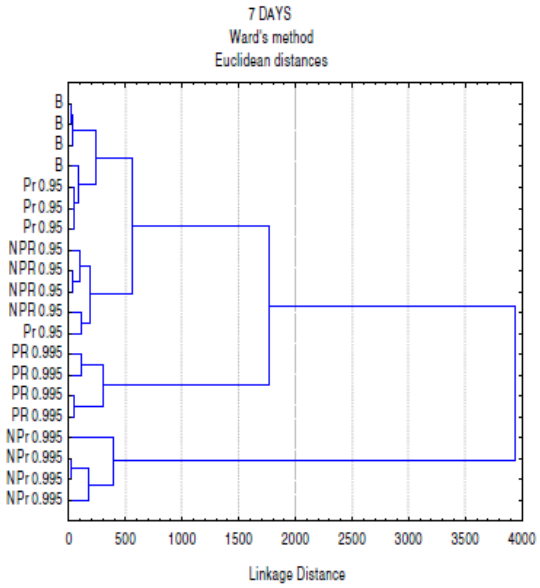



**Fig. 3** Cluster analyses after 7 days incubation of *P. nordicum* strains on a 3% ham-based medium. Key to treatments: *B* blank; *N PR 0.995* non-producer at 0.99 5$a_w$; *N PR 0.95* non-producer at 0.95 $a_w$; *PR 0.995* producer at 0.995 $a_w$; *PR 0.95* producer at 0.95 $a_w$

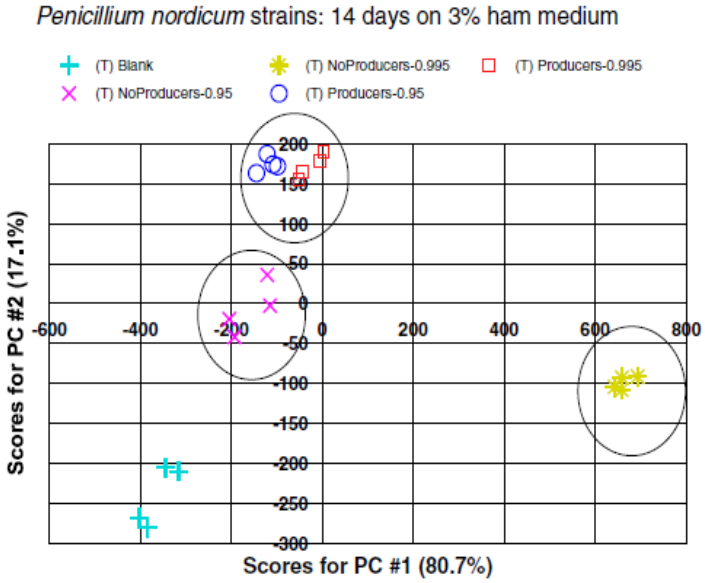
## Discrimination between *P. nordicum* strains based on volatile fingerprints

Figure 1 shows that there were differences in the relative response of the individual sensors in the array to the toxigenic and non-toxigenic strain of *P. nordicum* after 7 days incubation, suggesting differences in volatile production patterns. The PCA analyses after 1–3 days showed very little difference between treatments perhaps because of the very slow growth of *P. nordicum* (~5 mm colony diameter). However, after 7 days, *P. nordicum* strains were separated based on volatile production patterns, especially in the unmodified ham medium (0.995 $a_w$; Fig. 2). More than 90% of the variation was accounted for by PCA 1 and 2. This was confirmed by the cluster analyses which showed the spatial separation between treatments at 0.995 $a_w$, while at 0.95 $a_w$ this was less clear (Fig. 3). The results of the PCA after 14 days showed an effective separation between OTA and non-OTA *P. nordicum* strains at both $a_w$ levels examined (Fig. 4). However, use of the volatiles fingerprints produced by the OTA producer in the 0.995 and 0.95 $a_w$ treatments were not markedly different and thus could not be effectively differentiated. This was confirmed by CA (data not shown).

## Discussion

The application of the hybrid sensor array e-nose in detecting *P. nordicum* and OTA based on volatile patterns gave promising results. It was possible to separate the OTA producer strain with respect to the non-producer after 7–14 days incubation. The volatile fingerprints were also slightly different depending on $a_w$ of the medium after 14 days. This may be partially due to the biosynthetic pathways for OTA production being more active in the OTA-producing strain (MPVP1669) at 0.98 than 0.95 $a_w$. The use

**Fig. 4** The principal component analyses (PCA) plot of the data after 14 days incubation of *P. nordicum* strains showing the relative discrimination between treatments



of volatile patterns to separate the two strains after 7 days incubation was limited, and it was only possible with freely available water (0.995 $a_w$). The growth was slower at 0.95 $a_w$, and volatile production patterns may have been poorer resulting in the non-discrimination between the strains.

Previous work with *P. nordicum* grown on artificial media and incubated at 20°C for 7 days produced approx. 6 ppb of OTA (Battilani, unpublished data); the production of volatiles may be too low for effective discrimination using our approach without a concentration step to enhance the quantities of volatiles produced. As *P. nordicum* is a very slow growing fungal species, especially on the ham-based medium, this may be an important step to enhance volatile concentrations and perhaps also the overall fingerprint. Needham and Magan (2003) found some discrimination between toxigenic and non-toxigenic strains of *P. verrucosum* in relation to OTA production at 0.95 $a_w$ on wheat-based agar media after 2 days of incubation, although results for *A. flavus* were less clear.

Recent work on strains of the *Aspergillus* section *Nigri* species showed more promising results (Cabañes et al. 2009). Previous studies with food spoilage microorganisms have tried to take into account the volatile profiles produced and analysed by an e-nose combined with mass spectrometry (GC-MS). This showed a good correlation between volatiles identified during the development of the spoilage profile (Olsson 2000; Tognon et al. 2005; Bianchi et al. 2009). An interesting application of the e-nose approach was reported by Karlshoj et al. (2007); they developed a classification model for *P. expansum* spoilage of apples and a prediction model for patulin concentration usable for apple juice production. This was based on the correlation between e-nose response data to volatiles and HPLC quantification of patulin. The studies by Keshri and Magan (2000) certainly showed potential for discriminating between fumonisin producing and non-producing strains of *F. verticillioides* in vitro. Indeed, the volatile fingerprint approach has been shown to be faster than other more traditional approaches, and evidence of the potential for this approach has also been more recently reviewed by Sahgal et al. (2007).

The present study has shown that potential may exist for using volatile production patterns as an approach for better control of quality of stored meat. More detailed work is now required with *P. nordicum* and OTA production in cured pork meat products to evaluate whether the relationship between volatile production patterns and threshold levels of OTA can be correlated to enable rapid and real-time assessment of *P. nordicum* OTA and non-producers to define the potential risk to consumers more rapidly.

It may be possible to develop a miniaturised hand-held version containing a specific sensor array which can be optimised for use in the ham industry. This would require a small sample chamber or container which can be pressed directly against the ham surface to obtain volatile production pattern data without any destructive sampling. This type of data can be transmitted to a central control unit where background information exists, as obtained from the present study, to assist in validation of presence or absence of contamination by toxigenic strains of *P. nordicum*. This approach would enable a large amount of data to be rapidly collected and compared with more traditional analysis approaches. This could then be utilised as part of a quality assurance programme in this industry.

## References

Asefa DT, Gjerde RO, Sidhu MS, Langsrud S, Kure CF, Nesbakken T, Skaar I (2009) Moulds contaminants on Norwegian dry-cured meat products. Int J Food Microbiol 128:435–439

Battilani P, Pietri A, Giorni P, Formenti S, Bertuzzi T, Toscani T, Virgili R, Kozakiewicz Z (2006) *Penicillium* populations in dry-cured ham manufacturing plants. J Food Prot 70:975–980

Bianchi F, Cereri M, Mangia A, Mattarozzi M, Musci M, Concina I, Falasconi M, Gobbi E, Pardo M, Sberveglieri G (2009) Differentiation of the volatile profile of microbiologically contaminated canned tomatoes by dynamic headspace extraction followed by gas chromatography-mass spectrometry analysis. Talanta 77:962–970

Bogs C, Battilani P, Geisen R (2006) Development of a molecular detection and differentiation system for ochratoxin A producing *Penicillium* species and its application to analyse the occurrence of *Penicillium nordicum* in cured meats. Int J Food Microbiol 107:39–47

Cabañes FJ, Sahgal N, Bragulat MR, Magan N (2009) Early discrimination of fungal species responsible of ochratoxin A contamination of wine and other grape products using an electronic nose. Mycotoxin Res 25:187–192

Dall'Asta C, Galaverna G, De Dea LJ, Virgili R, Neviani E, Dossena A (2007) A new validated HPLC-FLD method for detecting ochratoxin A in dry-cured meat and in blue cheese. Mycotoxin Res 23:132–137

Dallyn H, Fox A (1980) Spoilage of material of reduced water activity by xerophilic fungi. In: G.H. Gould and J.E.L. Corry (eds) Society of Applied Bacteriology, Technical Series No. 15:129–139

EFSA (European Food Safety Authority) (2006) Opinion of The Scientific Panel on contaminants in the Food Chain of the EFSA on a request from the Commission related to ochratoxin A in food, EFSA J 365, 1-56. http://www.efsa.europa.eu. Accessed 15 May 2008

Gareis M (1996) Fate of ochratoxin A on processing of meat products. Food Addit Contam 13(suppl):35–37

Govaris A, Solomakos N, Pexara N (2007) Ochratoxin A in foods of animal origin. J Hell Vet Med Soc 58:313–320

Iacumin L, Chiesa L, Boscolo D, Manzano M, Cantoni C, Orlic S (2008) Moulds and ochratoxin A on surfaces of artisanal and industrial dry sausages. Food Microbiol 26:65–70

Karlshoj K, Nielsen Per V, Larsen T (2007) Prediction of *Penicillium expansum* spoilage and patulin used for apple juice production by electronic nose analysis. J Agric Food Chem 55:4289–4298

Keshri G, Magan N (2000) Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of *Fusarium* spp. using volatile production profiles and hydrolytic enzymes. J Appl Microbiol 89:825–833

Leistner L (1986) Mould-ripened foods. Fleischwirtschaft 66:1385–1388
Magan N, Sahgal N (2007) Electronic nose for quality and safety control. In: Nollet L, Toldra F (eds) Chapter 6, advances in food diagnostics. Blackwell, Iowa, USA, pp 119–129
Needham R, Magan N (2003) Detection and differentiation of toxigenic *Penicillium verrrucosum* strains on bakery products using an electronic nose. Aspects Appl Biol 68:217–222
Olsson J (2000) Modern methods in cereal grain mycology. PhD dissertation, Acta Univ Agric Sueciae – Agraria
Sahgal N, Needham R, Cabanes FJ, Magan N (2007) Potential for detection and discrimination between mycotoxigenic and non-toxigenic moulds using volatile production patterns: a review. Food Addit Contam 24:1164–1168
Smedsgaard J (1997) Analytical Abstracts Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr 760:264–270
Spotti E, Busolli C, Palmia F (1999) Sviluppo di colture fungine di importanza rilevante nell'industria dei prodotti carnei. Ind Conserve 74:23–34
Spotti E, Chiavaro E, Lepiani A, Colla F (2001) Contaminazione da muffe e da Ocratossina A in prosciutti stagionati e in fase di stagionatura. Ind Conserve 77:3–13
Tognon G, Campagnoli A, Pinotti L, Dell'Orto V, Cheli F (2005) Implementation of the electronic nose for the identification of mycotoxins in durum wheat (*Triticum durum*). Vet Res Commun 29:391–393

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ORIGINAL ARTICLE

# Electronic nose analysis of bronchoalveolar lavage fluid

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## ABSTRACT

**Background** Electronic nose (E-nose) technology has been successfully used to diagnose a number of microbial infections. We have investigated the potential use of an E-nose for the diagnosis of ventilator-associated pneumonia (VAP) by detecting micro-organisms in bronchoalveolar lavage (BAL) fluid in a prospective comparative study of E-nose analysis and microbiology.

**Materials and methods** BAL samples were collected using a blind technique from 44 patients following a minimum of 72 h mechanical ventilation. Control samples were collected from six patients mechanically ventilated on the intensive care unit (ICU) immediately following elective surgery. Quantitative microbiological culture and E-nose headspace analysis of the BAL samples were undertaken. Multivariate analysis was applied to correlate E-nose response with microbiological growth.

**Results** E-nose fingerprints correctly classified 77% of the BAL samples, with and without microbiological growth from patients not on antibiotics. Inclusion of patients on antibiotics resulted in 68% correct classification. Seventy per cent of isolates, cultured in the laboratory from the clinical samples, were accurately discriminated into four clinically significant groups.

**Conclusions** E-nose technology can accurately discriminate between different microbial species in BAL samples from ventilated patients on ICU at risk of developing VAP with accuracy comparable with accepted microbiological techniques.

**Keywords** Electronic nose, mechanical, complications, ventilation, ventilator associated pneumonia.

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## Introduction

Ventilator associated pneumonia (VAP) is the most common health care associated infection in the intensive care unit (ICU), affecting up to 28% of patients receiving mechanical ventilation [1]. Patients with VAP have a longer duration of mechanical ventilation and hospital stay [2] and a 2–10 fold increased risk of death [1]. Subsequently, VAP has major cost implications, of up to $25,072 (2005 US Dollars) in hospital charges per patient [3].

Determining when a mechanically ventilated patient develops VAP remains difficult. A variety of techniques exist, using a combination of clinical symptoms and signs and the results of microbiological analysis. Microbiological culture of bronchoalveolar lavage (BAL) fluid is probably the best technique, but results are usually not available for 24–48 h after sample collection. A number of means of sample collection exist, including the aspiration of tracheal secretion, blind or formal BAL, or protected specimen brush; however, the preferred method of sample collection remains uncertain [4,5]. The Clinical Pulmonary Infection Score (CPIS) has been proposed as a way of combining the clinical parameters with microbiological findings into a more accurate clinical tool for the diagnosis of VAP [6], but some studies suggest that it is of limited use [7].

Electronic nose (E-nose) technology was developed in the early 1980s in an attempt to replicate the mammalian olfactory system's ability to detect volatile organic compounds (VOC's) [8]. VOC's are organic compounds that can easily become gases or vapours; E-noses can also detect other types of volatile compounds. VOC's produced by metabolic or microbiological

activity are in part excreted via the lungs and can be detected in exhaled breath. The potential of E-nose technology has been investigated for the diagnosis of a number of infections [9]. Studies have shown accurate diagnosis of urinary tract infection (UTI) [10] and mycobacterium tuberculosis infection [11]. E-noses are easily portable, suited to near patient testing and have the potential to give a diagnosis of infection with minutes or hours as opposed to days with conventional means. This in turn may allow earlier initiation of antibiotic therapy with the potential to improve patient outcome as well as avoiding inappropriate antibiotic use. In this preliminary study, we have investigated the use of E-nose technology to detect the presence of micro-organisms in BAL fluid from mechanically ventilated patients by comparing E-nose analysis of BAL samples with quantitative microbiological culture.

## Materials and methods

With local research ethics committee approval, we studied 50 patients receiving mechanical ventilation on the ICUs at Cheltenham General and Gloucestershire Royal Hospitals. If informed consent could not be obtained from the patient, assent from a relative was obtained and patient's consent was sought at the earliest possible opportunity. In cases where the patient subsequently refused consent, the relevant samples were destroyed and the data were not analysed. In accordance with the standard definition of VAP, sample collection did not commence until patients had been receiving a minimum of 72 h of mechanical ventilation [12]. Samples were collected three times per week until mechanical 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 h of admission to the ICU with no evidence of pulmonary problems. It is extremely difficult to find a suitable control group for critically ill, mechanically ventilated patients. For this reason, we have used a small number of controls to see if any obvious discrimination occurred with the E-nose. In a larger study, the issue of adequate controls will need to be carefully considered. For a statistical power of 80%, with 5% significance, a sample size of 100 samples was calculated assuming an incidence of microbiological growth of 25% within our patient population.

### Bronchoalveolar lavage collection

BAL was performed using a blind technique as previously described by Garrard [13]. 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 it could be advanced no further. Twenty millilitres 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 E-nose analysis. Quantitative microbiological culture was performed according to the standard hospital protocol.

### E-nose analysis

BAL samples collected from patients were stored at 4 °C and allowed to warm to room temperature prior to analysis. The samples were then pipetted into 25 mL glass vials and allowed to equilibrate at room temperature for 1 h. The headspace gas was then analysed using the E-nose (NST 3320; Applied Sensor, Linkoping, Sweden) comprising a hybrid metal oxide semiconductor sensor array of 24 individual sensors. The headspace gas is drawn over the sensor array via a sampling port which pierces a diaphragm on the vial containing the sample for analysis.

To remove patient related confounding factors and to construct a laboratory-based model to assess the ability of the E-nose to discriminate between microbiological species; bacterial slopes taken from the BAL samples were created. These were created from the portion of the BAL samples sent to the hospital microbiology laboratory for standard microbiology and culture. The purpose of this laboratory-based model was to validate the performance of the E-nose to discriminate between micro-organisms grown in culture media, without the effect of patient confounding factors. The micro-organisms used were all isolated from BAL samples from patients. These were then used to culture the organisms in question in a laboratory setting without the presence of the BAL fluid. Organisms isolated from the bacterial slopes were maintained on nutrient agar plates. One colony per species was inoculated in 10 mL sterile nutrient broth (LabM, Bury, Lancs, UK) and incubated for 4 h at 37 °C in a rotary shaker at 0.3 *g*. Thereafter 100 μL of each microbial suspension was transferred into a fresh 10 mL sterile nutrient broth and incubated for 18 h at 37 °C in a rotary shaker at 0.3 *g*. Subsequently, 5 mL from each suspension was transferred into 25 mL glass vials and left for 1 h 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 E-nose. These studies were repeated at least twice. The same manufacturer's nutrient broth was used to culture all the organisms used in this part of the study to avoid the potential for a different volatile fingerprint being generated by different

 

culture media. It is important to note that the clinical samples were the patient's BAL fluid which was not cultured in broth prior to headspace analysis. This maintains the aim of near patient testing with the E-nose.

### Data analysis

The response generated by the E-nose sensors in the form of normalized, 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. Multivariate analysis techniques were applied to the data. Principal component analysis (PCA) was used as a data reduction technique to explore the variance in the dataset and fed into linear discriminant analysis (LDA) to correlate E-nose response with the findings of culture of the lavage samples. LDA is a statistical technique used to classify samples into one of two or more groups based on a set of features which describe the samples. The samples are assigned to one of a number of predetermined groups based on observations made about the sample. Because of the sample size, leave-one-out cross-validation was used to evaluate the how the results would translate to an independent dataset. 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.

## Results

Ninety-six samples were obtained from 44 patients and six samples from six control subjects. There were 32 men and 18 women. Full details of the organisms isolated following microbiological growth of the lavage samples are shown in Table 1. In summary, there were eight Gram-positive, seven Gram-negative, nine fungal, 10 mixed growth (more than one species isolated) and 67 samples with no growth. Fourteen samples were excluded from the data analysis because of technical problems with the sample processing and analysis. These were problems with storage and transport as opposed to inherent problems with the E-nose itself. The mixed growth samples were excluded from the four group data analysis.

### E-nose discrimination of isolated microbiological species

Discrimination of the individual microbial species using E-nose signatures, measured from isolates grown from the clinical samples, was not attempted because of the small numbers of any single species. We therefore grouped the isolated microorganisms into four clinically significant groups (Gram-positive, Gram-negative, fungi and no microbiological growth), according to their classification, PCA fed LDA was shown to accurately classify 81/98 of the samples (83%), with sensitivity of 74–95% and specificity of 77–100%. Testing the performance with a leave-one-out cross-validation demonstrated correct classification of 70% of samples with sensitivity of 56–84% and specificity of 81–97%. Figure 1 shows the distribution of the E-nose response with samples coded according to the microbiological growth. Clustering of the E-nose data into the four groups is clearly seen.

**Table 1** 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

| 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 |

### E-nose discrimination of clinical samples

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, a two-group PC-fed LDA classification model was developed, to evaluate the discrimination of E-nose fingerprints from 88 samples; 58 with no microbiological growth and 30 with microbiological growth (Fig. 2). Sixty-eight per cent of samples were correctly classified by the leave-one-out cross-validated model, with sensitivity and specificity of 67–69%.

Twelve samples were collected from patients who had not received antimicrobial therapy within 48 h of sampling. Nine

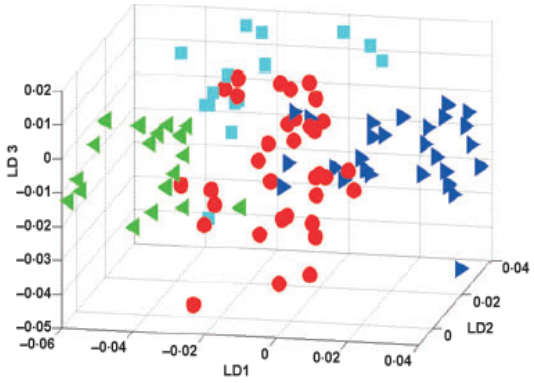


**Figure 1** Scatter plot of linear discriminant scores of the electronic nose response to micro-organisms isolated from clinical bronchoalveolar lavage samples. Each measurement is coded according to the microbiological classification into four clinically significant groups, Gram-positive (▶), Gram-negative (◀), Fungi (●) and no microbiological growth (■).

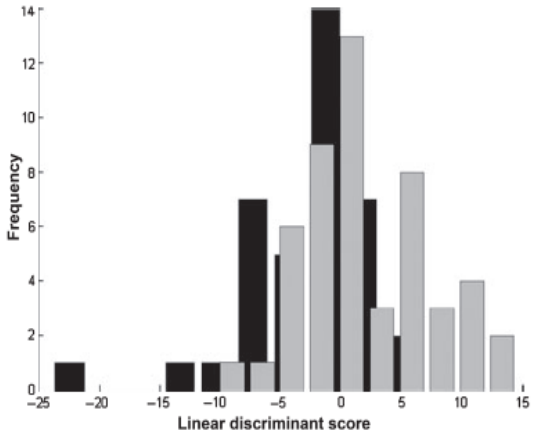


**Figure 2** Histogram of linear discriminant scores differentiating samples with microbiological growth (black) and samples without microbiological growth (grey). The E-nose fingerprints from samples with no microbiological growth are seen to cluster in two sub-groups and a significant number of them are misclassified (to the left of zero) overlapping samples with microbiological growth.

of these samples were culture negative and three grew organisms. A further two-group PC-fed LDA classification model was developed to evaluate the discrimination of E-nose fingerprints of these nine no growth samples, from patients not on antibiotics and 30 samples with microbiological growth

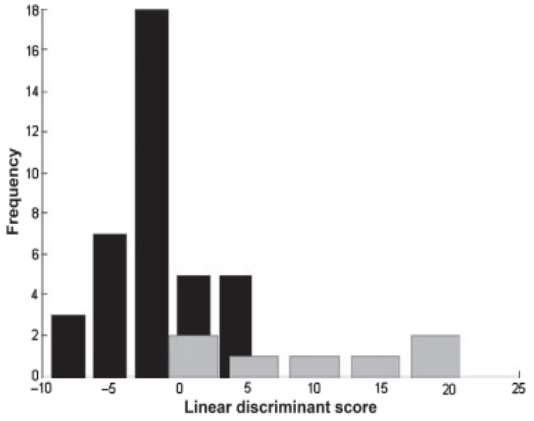


**Figure 3** Histogram of linear discriminant scores differentiating E-nose fingerprints measured from samples with microbiological growth (black) and samples without microbiological growth taken from patients not on antibiotics (grey).

(Fig. 3). Seventy-seven per cent of samples were correctly classified by the leave-one-out cross-validated model, with sensitivity and specificity of 56–83%.

A clinically significant four group classification (Gram-positive, Gram-negative, fungi and no microbiological growth) was then developed, to evaluate the discrimination of E-nose fingerprints from 29 samples; nine with no microbiological growth, from patients not on antibiotics, five samples with Gram-positive growth, seven samples with Gram-negative growth and eight with fungal growth. Samples exhibiting mixed microbiological growth were excluded from this analysis. Seventy-six per cent of samples were correctly classified by the model, with sensitivity of 60–100% and specificity of 81–100%.

## Discussion

This study demonstrates that E-nose technology has the ability to accurately discriminate between different groups of micro-organisms isolated from clinical BAL samples grown in nutrient broth, with 83% of samples being correctly classified in a clinically significant four group model (Gram-positive, Gram-negative, fungi and no microbiological growth). These findings suggest an E-nose may be able to detect the presence of and type of micro-organisms in BAL fluid, thereby potentially allowing for earlier initiation of targeted antibiotics in patients with clinically suspected VAP. We believe this is the first time that E-nose technology has been used to analyse BAL samples for the presence of micro-organisms.

 

There are various types of E-nose available commercially but they all share the same basic design. This consists of a sample delivery system, a sensor array over which the volatiles pass; and some form of data processing software which produces an output. This is an attempt to replicate the chemical sensor array found in the mammalian nose, signal processing in the olfactory bulb and pattern recognition performed by the brain.

The sample delivery system facilitates the generation of a headspace (containing the volatile compounds). The headspace is then injected into the detection system. An efficient sample delivery system is vital to ensure constant operating conditions. The sensor array (or detection system) reacts when in contact with volatile compounds. This reaction takes the form of a change in electrical properties. Each individual sensor reacts to all volatile compounds, but in a different way. The adsorption of the volatile compound onto the sensor causes a physical change; this is then recorded as a digital value. The data are then fed into the data processing software. As previously stated, we have used an E-nose with a hybrid MOS sensor array. Another type of E-nose with a different sensor array (for example, conducting polymer) may have produced different discrimination results. To ensure consistent results, we use substances such as acetone and ethanol at different concentrations that act as controls prior to analysing study samples.

We have demonstrated that E-nose analysis of BAL samples from ventilated patients has the ability to discriminate between different species (Fig. 1). This is in agreement with other published work on clinical infections. Pavlou showed that an E-nose could discriminate between different organisms causing UTIs [14] and different species of mycobacterium [11]. The only published studies investigating the diagnosis of VAP using an E-nose have compared E-nose analysis of breath samples with either computed tomography scans of the chest or a CPIS to diagnose VAP [15,16].

In the study comparing E-nose analysis of exhaled breath samples from mechanically ventilated patients with CT scanning of the chest, 25 sets of exhaled gas samples were taken from 25 patients. Each set consisted of five consecutive breath samples. The samples were taken within 48 h of CT scanning (average time 17·3 ± 14·5 h). Twenty-five CT scans were performed in 23 patients; two patients underwent two CT scans each. CT detected 12 cases of pneumonia and 13 pneumothoraces. No explanation for this high rate of pneumothorax is given. The authors do not state the time interval between the initiation of mechanical ventilation and CT scanning or whether any of the patients were receiving antimicrobial therapy prior to samples being taken. When using a prediction set different to the training set a diagnostic accuracy of 80% was achieved for E-nose breath analysis compared with chest CT. These results are promising and suggest that E-nose technology is able to discriminate between the breath of those with pneumonia and those without; but as with our study the sample size is small. The basis on which this discrimination occurs is unknown.

CPIS has been shown to have a moderate performance when compared with pathological diagnosis with a sensitivity of between 72% and 77% and a specificity of between 42% and 85% [17,18]. The inter-observer agreement in calculating CPIS has been shown to be poor [19]. Comparison of the E-nose fingerprints measured in this study with recorded CPIS will be reported elsewhere.

The instrument used to analyse breath in the above study was a Cyranose 320 (Cyrano Sciences, Pasedena, CA, USA). This is a hand held, portable device as opposed to the workbench based E-nose we used. Collecting breath samples for analysis with our system would have been difficult and prone to contamination. We were particularly concerned about the high levels of VOC's given off by the materials used in the construction of endotracheal tubes and breathing circuits. For these reasons, we elected not to attempt breath analysis using the E-nose.

The VOC's of interest detected from our BAL samples are likely to be alcohols, phenols and ketones. To gain more information on the nature of these VOC's and changes in the breath of mechanically ventilated patients, we have performed analysis of the breath of our study patients using Gas Chromatography-Mass Spectrometry. These results will be reported elsewhere.

In the clinical setting a simple two group model differentiating BAL samples with and without microbiological growth correctly classified 68% of samples in a leave-one-out cross-validated analysis. Crossover of a significant number of E-nose fingerprints from samples without demonstrated microbiological growth were seen to be overlapping the samples with microbiological growth, as classified by their E-nose signature. Indeed the no growth samples appear to cluster into two sub-groups, one of which overlies the growth samples (with negative discriminant scores). There are likely to be a number of reasons for this finding; however, the most obvious of these is the use of antimicrobial therapy prior to sampling. Eighty-nine of 102 (87%) of the samples were taken from patients who had been given antibiotics for at least 24 h prior to sampling. Other factors such as host response could also influence the results.

Deeper scrutiny of the misclassified samples in the clinical model (Fig. 2) indicated that most of these were in the 'no growth' group. These are samples that had no microbial growth during the culture period. Previous studies have shown that even 24 h of prior antimicrobial therapy can affect culture results [20]. Torres demonstrated that prior antibiotic use considerably decreased the sensitivity of BAL culture [5]. Montravers showed that 72 h of antimicrobial therapy could result in complete eradication of causative organisms [21]. As quantitative microbiological culture of BAL is used as the gold

standard for evaluation of the E-nose performance in this study, this false negative rate is of great concern. By this we believe that samples are correctly classified into a 'growth' group by the E-nose but microbiological culture has failed because of the use of antibiotics. This has led to the accuracy of the E-nose on the clinical samples appearing to be much lower than expected in comparison with the performance of the E-nose identification of species isolated in the laboratory.

The effect of antibiotics on the microbiological culture of the BAL samples was the motivation behind the development of the second two-group model shown in Fig. 3, as the use of antimicrobial therapy prior to sampling appears to affect the measured volatile signature. Samples collected from patients who had received antimicrobial therapy within 48 h of sampling were removed from the analysis. An improvement to 77% for the two group leave-one-out cross-validated classification was demonstrated, with little overlap between the two groups. However, removal of a large number of samples leads to a diminished statistical significance. Development of a four group classification, mirroring that constructed with the E-nose fingerprints measured from the species isolated in the laboratory, resulted in the correct classification of 76% of samples. This classification rate is comparable with current microbiological culture and sensitivities. Microbiological culture of BAL has been shown to have a sensitivity of between 19% and 83% and specificity of between 36% and 83% [22].

The accepted cutoff for the presence of infection as opposed to colonization in the use of quantitative BAL culture is usually $> 10^4$ CFU $mL^{-1}$. Five of our positive growth samples achieved $10^4$ CFU $mL^{-1}$ and 10 of the samples achieved $10^5$ CFU $mL^{-1}$. All other positive samples achieved $10^3$ CFU $mL^{-1}$. There appears to be no difference in the E-nose's ability to discriminate based on the level of growth. Our positive growth samples occurred in patients who had been receiving prior antimicrobial therapy and in one control patient who had not. Souweine suggest that in this situation the diagnostic threshold for infection should be reduced to $10^3$ CFU $mL^{-1}$ for BAL [20]. At present, we are unable to differentiate between colonization and infection. However, we have considered the presence of any growth in the BAL samples to have been significant, as the identification of any growth with surveillance sampling allows earlier identification of likely infecting organism in suspected VAP and earlier initiation of appropriate antibiotics.

The clinical signs and symptoms of VAP can be vague and unreliable. Clinical manifestations in combination with other diagnostic modalities are the usual method of diagnosing VAP. Chest radiography may be sensitive but typically is non-specific. Quantitative cultures obtained by various methods including BAL can be equivocal in the diagnosis of VAP. They can also take up to 48 h to yield results. Lung histology has been considered to be the gold standard but this also has inherent problems. Corley showed the prevalence of pneumonia in open post-mortem lung biopsies determined by each of four pathologists varied from 18% to 38% [23]. Various biomarkers including CRP, Procalcitonin and soluble triggering receptor (sTREM) in BAL fluid have all been evaluated as novel ways of diagnosing VAP [24]. We have shown that E-nose technology has the potential to offer rapid, near patient analysis of BAL samples with an accuracy level comparable with that of accepted microbiological techniques. However, larger multi-centre studies are required to validate these preliminary results. The biggest advantage of E-nose diagnosis is its ability to give results within minutes or hours compared with days for microbiological methods. It has been shown that delayed treatment with appropriate antibiotics increases the risk of death from VAP by up to seven fold [25]. It is also important to establish the absence of infection rapidly as this decreases antibiotic usage, with associated side effects, and the development of resistance. The ability to institute early, targeted antimicrobial therapy has been shown to improve patient outcome and reduce rates of antimicrobial resistance and side effects. [1,26].

In conclusion, we have demonstrated that E-nose technology can be used to provide rapid, near patient analysis of BAL fluid to identify the presence of micro-organisms. The accuracy is comparable with that of current microbiological techniques and a result can be obtained in minutes or hours. This may permit more rapid diagnosis of VAP, thereby allowing earlier institution of antibiotic therapy where necessary, which has been shown to improve patient outcome. Prevention of the use of antibiotics in equivocal cases where patients may receive unnecessary antibiotic therapy pending a negative culture result could also be achieved. The ability to recognize the presence of Gram-positive, Gram-negative or fungal infection would enable the use of more targeted therapy prior to culture results, leading to reduced incidence of antibiotic resistant organisms. Further work is required to refine our models towards a target of accurate identification of the infecting organism.

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**References**

1 Chastre J, Fagon JY. Ventilator-associated pneumonia. *Am J Respir Crit Care Med* 2002;**165**:867–903.
2 Rello J, Ollendorf DA, Oster G, Vera-Llonch M, Bellm L, Redman R *et al*. Epidemiology and outcomes of ventilator-associated pneumonia in a large US database. *Chest* 2002;**122**:2115–21.
3 Anderson DJ, Kirkland KB, Kaye KS, Thacker PA 2nd, Kanafani ZA, Auten G *et al*. Underresourced hospital infection control and prevention programs: penny wise, pound foolish? *Infect Control Hosp Epidemiol* 2007;**28**:767–73.
4 Bartlett JG, Alexander J, Mayhew J, Sullivan-Sigler N, Gorbach SL. Should fiberoptic bronchoscopy aspirates be cultured? *Am Rev Respir Dis* 1976;**114**:73–8.
5 Torres A, El-Ebiary M. Bronchoscopic BAL in the diagnosis of ventilator-associated pneumonia. *Chest* 2000;**117**:198S–202S.
6 Fartoukh M, Maitre B, Honore S, Cerf C, Zahar JR, Brun-Buisson C. Diagnosing pneumonia during mechanical ventilation: the clinical pulmonary infection score revisited. *Am J Respir Crit Care Med* 2003;**168**:173–9.
7 Lauzier F, Ruest A, Cook D, Dodek P, Albert M, Shorr AF *et al*. The value of pretest probability and modified clinical pulmonary infection score to diagnose ventilator-associated pneumonia. *J Crit Care* 2008;**23**:50–7.
8 Persaud K, Dodd G. Analysis of discrimination mechanisms in the mammalian olfactory system using a model nose. *Nature* 1982;**299**:352–5.
9 Turner AP, Magan N. Electronic noses and disease diagnostics. *Nat Rev Microbiol* 2004;**2**:161–6.
10 Aathithan S, Plant JC, Chaudry AN, French GL. Diagnosis of bacteriuria by detection of volatile organic compounds in urine using an automated headspace analyzer with multiple conducting polymer sensors. *J Clin Microbiol* 2001;**39**:2590–3.
11 Pavlou AK, Magan N, Jones JM, Brown J, Klatser P, Turner AP. Detection of Mycobacterium tuberculosis (TB) *in vitro* and *in situ* using an electronic nose in combination with a neural network system. *Biosens Bioelectron* 2004;**20**:538–44.
12 Kollef MH. What is ventilator-associated pneumonia and why is it important? *Respir Care* 2005;**50**:714–21; discussion 21–4.
13 A'Court CH, Garrard CS, Crook D, Bowler I, Conlon C, Peto T *et al*. Microbiological lung surveillance in mechanically ventilated patients, using non-directed bronchial lavage and quantitative culture. *Q J Med* 1993;**86**:635–48.
14 Pavlou AK, Magan N, McNulty C, Jones J, Sharp D, Brown J *et al*. Use of an electronic nose system for diagnoses of urinary tract infections. *Biosens Bioelectron* 2002;**17**:893–9.
15 Hockstein NG, Thaler ER, Lin Y, Lee DD, Hanson CW. Correlation of pneumonia score with electronic nose signature: A prospective study. *Ann Otol Rhinol Laryngol* 2005;**114**:504–8.
16 Hockstein NG, Thaler ER, Torigian D, Miller WT Jr, Deffenderfer O, Hanson CW. Diagnosis of pneumonia with an electronic nose: correlation of vapor signature with chest computed tomography scan findings. *Laryngoscope* 2004;**114**:1701–5.
17 Fabregas N, Ewig S, Torres A, El-Ebiary M, Ramirez J, de La Bellacasa JP *et al*. Clinical diagnosis of ventilator associated pneumonia revisited: comparative validation using immediate post-mortem lung biopsies. *Thorax* 1999;**54**:867–73.
18 Papazian L, Thomas P, Garbe L, Guignon I, Thirion X, Charrel J *et al*. Bronchoscopic or blind sampling techniques for the diagnosis of ventilator-associated pneumonia. *Am J Respir Crit Care Med* 1995;**152**:1982–91.
19 Schurink CA, Van Nieuwenhoven CA, Jacobs JA, Rozenberg-Arska M, Joore HC, Buskens E *et al*. Clinical pulmonary infection score for ventilator-associated pneumonia: accuracy and inter-observer variability. *Intensive Care Med* 2004;**30**:217–24.
20 Souweine B, Veber B, Bedos JP, Gachot B, Dombret MC, Regnier B *et al*. Diagnostic accuracy of protected specimen brush and bronchoalveolar lavage in nosocomial pneumonia: impact of previous antimicrobial treatments. *Crit Care Med* 1998;**26**:236–44.
21 Montravers P, Fagon JY, Chastre J, Lecso M, Dombret MC, Trouillet JL *et al*. Follow-up protected specimen brushes to assess treatment in nosocomial pneumonia. *Am Rev Respir Dis* 1993;**147**:38–44.
22 Rea-Neto A, Youssef NC, Tuche F, Brunkhorst F, Ranieri VM, Reinhart K *et al*. Diagnosis of ventilator-associated pneumonia: a systematic review of the literature. *Crit Care* 2008;**12**:R56.
23 Corley DE, Kirtland SH, Winterbauer RH, Hammar SP, Dail DH, Bauermeister DE *et al*. Reproducibility of the histologic diagnosis of pneumonia among a panel of four pathologists: analysis of a gold standard. *Chest* 1997;**112**:458–65.
24 Chastre J, Luyt CE, Trouillet JL, Combes A. New diagnostic and prognostic markers of ventilator-associated pneumonia. *Curr Opin Crit Care* 2006;**12**:446–51.
25 Iregui M, Kollef MH. Prevention of ventilator-associated pneumonia: selecting interventions that make a difference. *Chest* 2002;**121**:679–81.
26 Niederman MS, Craven DE, Bonten MJ, Chastre J, Craig WA, Fagon J-Y *et al*. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *Am J Respir Crit Care Med* 2005;**171**:388–416.

**Analysis of volatile fingerprints for monitoring anti-fungal efficacy against the primary and opportunistic pathogen *Aspergillus fumigatus***

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**Key words:** Hybrid sensor array, volatile fingerprints, anti-fungals, lung infections, volatile compounds.

## Abstract

The aims of this study were to use of qualitative volatile fingerprints obtained using a hybrid sensor array system (12 metal oxide semiconductor sensors, 10 metal oxide semiconductor field effect transistor sensors) to screen anti-fungals for controlling the important lung infecting fungus, *Aspergillus fumigatus*, especially in immune-compromised patients. SIFT-MS was also used to try and identify key volatiles produced by *A.fumigatus*. Initial studies were carried out to identify the $ED_{50}$ and $ED_{90}$ (effective dose) for inhibiting growth of *A.fumigatus* using three anti-fungal compounds, benomyl, tebuconazole and fluconazole. Subsequent studies involved inoculation of malt extract agar plates with spores of *A.fumigatus* (25, 37$^{o}$C) over periods of 24-72 h to examine the head space volatile fingerprints generated from the

sample treatments using the hybrid sensor array system to compare controls and $ED_{50}/ED_{90}$ concentrations. The sensor responses for divergence for treatments were used to build a model. This showed discrimination between treatments after 48 h incubation when benomyl and tebuconazole were used against *A.fumigatus* at 37°C, and after 72 h at 25$^{o}$C using Principal Components Analysis and Cluster Analysis. Studies using SIFT-MS showed that methyl pentadiene, ethanol, isoprene and methanol were key biomarker volatiles produced by *A.fumigatus* in the presence of anti-fungal compounds. This may also be a good approach for the development of rapid screening of anti-microbial compounds and potentially useful for the monitoring the possible build up of resistance to specific drug types. Volatile fingerprints produced by patient samples could also be used to evaluate whether lung infections are caused by bacteria or specific fungi to facilitate early diagnosis and enable the right drug treatment to be prescribed.

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# In vitro discrimination of microorganisms involved in ventilated associated pneumonia using the Electronic nose

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## Introduction

·There has been interest in using breath analyses as a rapid diagnostic measurement of ventilator associated pneumonia (VAP).
·VAP is caused by a range of individual or mixtures of micro-organisms.
·We have thus initiated a research project to examine the potential for using volatile fingerprints as biomarkers of specific microbial infections to facilitate more effective treatment.

**CULTURES**: Three different bacteria (*Enterobacter cloacae, Klebsiella pneumoniae* and *Staphylococcus sp)* and two fungi (*Aspergillus fumigatus and Candida albicans)* were cultured in conical flasks and analysed in the Enose.

Nutrient Broth 1%

Every 24hrs (96 hrs)

Specific vials for E-nose

Headspace analysis

E-nose-AS Nordic 3320

## Results

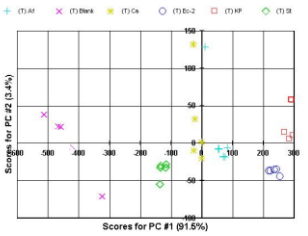


**Fig 1**: PCA Scores plot after 72 hours discriminating between five species with the same concentration ( $10^4$ CFU/ml)

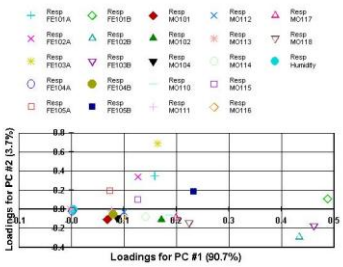


**Fig 2**: PCA Loadings plot indicating the contribution of sensors in separating samples along PC1 and PC2

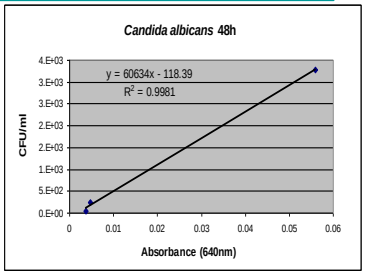


Fig 3: Calibration curve for *Calbicans* after 48hrs growth against absorbance at 640nm

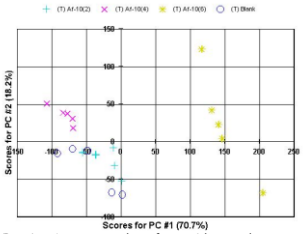


**Fig 4**: PCA Scores plot after 24 hours showing the relative discrimination between three concentrations of *A. fumigatus*. (1% Nutrient Broth medium)

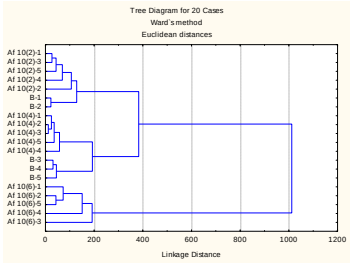


**Fig 5**: Hierarchical Cluster Analysis of data from Fig 4 showing the cluster of $10^6$ CFUs distinctly separated from the others.

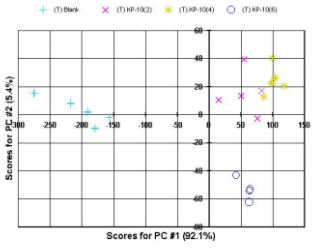


**Fig 6**: PCA Scores plot after 72 hours showing the relative discrimination between three concentrations of *K. pneumoniae* (1% Nutrient Broth medium)

## Conclusions

·Using PCA analyses it was possible to obtain good differentiation between the species after 72 hrs.
·The lowest concentration ($10^2$) is difficult to discriminate either from the blank (24hrs) or from $10^4$ (72hrs).
·Further studies are necessary in order to optimise a medium for rapid discrimination between different species causing VAP.
·Work is now in progress to identify the sensitivity of detection of each species and to examine the effect of different drug treatments in vitro on the species responsible for VAP.

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# Electronic nose analysis of bronchoalveolar lavage fluid for the diagnosis of ventilator associated pneumonia

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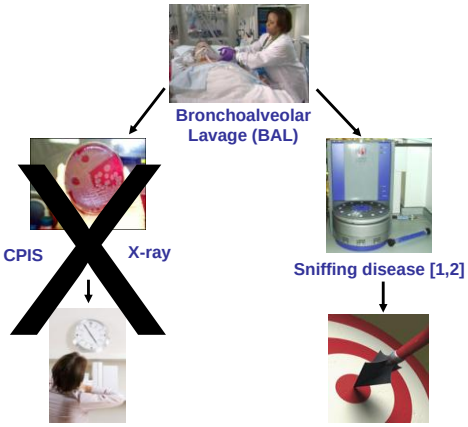
## 1. Introduction

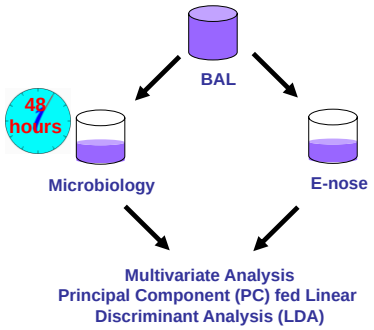
Diagnosis of ventilator associated pneumonia (VAP) is a significant problem on ITU




**Hypothesis:** E-nose technology can detect micro-organisms in BAL fluid, enabling targeted antibiotics to be administered earlier, improving patient outcome.

## 2. Materials and Methods

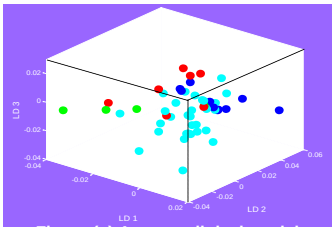
- Patients ventilated > 72 hours.
- Clinical pneumonia infection score (CPIS) recorded (modified from [3]).
- Blind bronchoalveolar lavage (BAL) with 20ml 0.9% NaCl.




## 3. Results

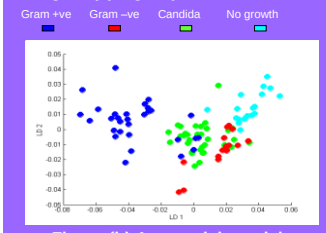
- Microbiological growth identified in 40% (21/52) samples collected from 27 patients.
- E-nose correctly classified 81% (42/52) of samples in a growth versus no growth model.

## 3. Results (cont.)



63% (33/52) samples correctly classified in a 4 group clinical model

**Figure (a) 4 group clinical model**




Micro-organisms grown from BAL samples were individually cultured for a 4 group lab model

E-nose analysis correctly identified 83% (81/98) of samples

**Figure (b) 4 group lab model**

### Samples with no microbiological growth

- 39% (12/31) samples with no microbiological growth were classified as infected by the E-nose.
- No correlation between CPIS and microbiological growth.
- CPIS correlates with E-nose prediction in 68% (21/31) samples with no microbiological growth.
- Could the microbiological culture have failed due to antibiotic use? (~90% of patients on antibiotics).
- Testing E-nose response of lab cultures to antibiotic therapy.
- Need more rigorous gold standard to test E-nose against.

## 4. Conclusions

- E-nose analysis provides a rapid and accurate method for the detection of micro-organisms in BAL fluid.
- Potential to identify infecting organism.
- Effect of antibiotics is under further investigation.
- Potential for point-of-care diagnosis facilitating earlier initiation of targeted antibiotic therapy, thereby improving patient outcome, reducing hospital stay and resistance to broad spectrum antibiotics.

## 5. References

[1] Persaud K, Dodd G. Analysis of discrimination mechanisms in the mammalian olfactory system using a model nose. Nature, 1982: 299(5881): 352-355
[2] Turner, A.P.F. and Magan, N. Electronic noses and disease diagnostics. Nature Microbiology Reviews, 2004: 2: 161-166
[3] Pugin J, Auckenthaler R, Mili N, Janssesns JP, Lew PD, Suter PM. Diagnosis of ventilator-associated pneumonia by bacteriologic analysis of bronchoscopic and nonbronchoscopic "blind" bronchoalveolar lavage fluid. Am. Rev. Respir. Dis., 1991: 143 (5): 1121-9

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NHS National Institute for Health Research

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## Early discrimination of microorganisms involved in Ventilator Associated Pneumonia (VAP) using qualitative volatile fingerprints

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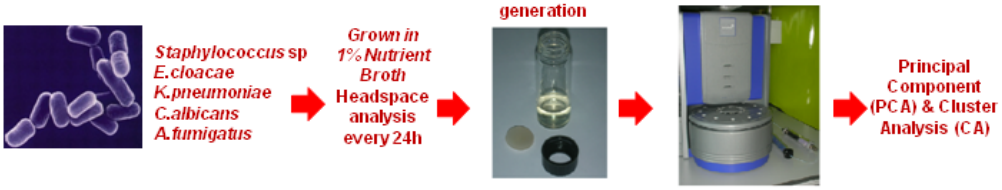
## Introduction

- VAP is one of the most common nosocomial infections, occurs >48h of ventilation. High rates of mortality (24-50%).
- Rapid, early detection of causative species would enable targeted antibiotic therapy and improved patient outcomes.
- A wide range of microorganisms may cause VAP: *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *E.coli*, *Acinetobacter baumanii*, *Staphylococcus* sp, *Streptococcus pneumoniae*, etc.
- **Objective: To investigate the capacity of a hybrid sensor array to discriminate diverse microorganisms involved in VAP by analysing volatile fingerprints.**

## Methods/Materials




## Results

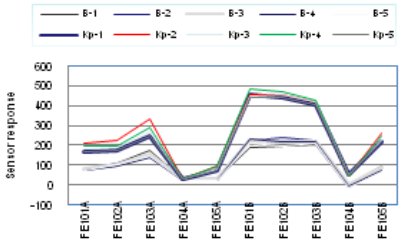



**Figure 1.** Measured response of MOSFET sensors for blank samples (B) and *K.pneumoniae* (Kp) after 24h incubation at 37° C on 1% NB (Nutrient Broth)

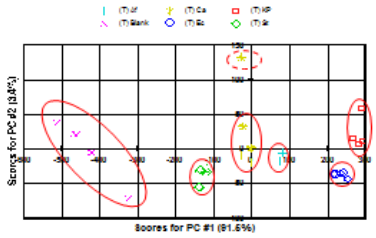



**Figure 2.** PCA score plot of E-nose response after 72h incubation at 37° C on 1% NB. Five replicates of each species. (Dotted circle shows two possible outliers.)

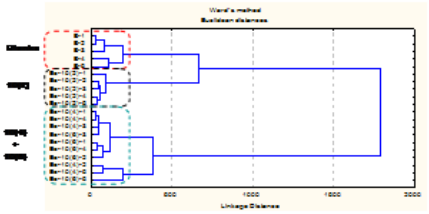


**Figure 3.** Dendrogram showing differentiation of E-nose response, using cluster analysis, to different initial concentrations of *E.cloacae* after 24h incubation at 37° C on 1% NB. (Key:10(2), 10(4) and 10(6): $10^2$, $10^4$ and $10^6$ CFU $ml^{-1}$)

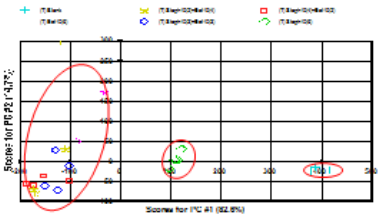



**Figure 4.** PCA score plot of E-nose response to mixtures of varying initial concentrations of *Staphylococcus* sp+*E.cloacae* after 48h incubation at 37° C on 1% NB

## Conclusions

- 5 microorganisms were well differentiated with initial concentration of $10^4$ CFU ml-1 after 72h incubation on 1% NB at 37° C.
- Discrimination from blank samples (same media without inocula) was achieved at $10^2$ CFU ml-1 after 24h incubation for bacteria, 48h for the yeast and 72h for the filamentous fungus.
- In mixtures of two microorganisms, usually one of them is predominant, which affects the measured volatiles.
- **Work in progress:** a) to assess the model developed with pure culture data for the validation of unknown samples; b) to evaluate the effect of drugs on volatile production patterns; c) to analyse clinical samples and to compare with gold standard microbiological methods; d) to identify the volatiles using GC-MS+SIFT-MS techniques and correlate with E-nose results.

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Acknowledgement: We are grateful to Cranfield Health and Gloucestershire Hospitals NHS Foundation Trust for financial support

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# Screening of drugs and discrimination of microorganisms involved in Ventilator Associated Pneumonia (VAP) using volatile fingerprints

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## Introduction

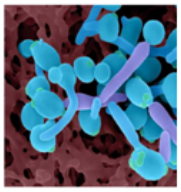
- VAP is one of the most common infections in intubated patients after 48 h of mechanical ventilation. A wide range of microorganisms may cause VAP: bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*; and also fungi such as *Aspergillus fumigatus* or *Candida albicans* in immuno-compromised patients.
- Rapid detection of causative pathogens and the screening of drug efficacy would enable improved patient outcomes.
- **Objectives: (a) to investigate the capacity of a hybrid sensor array Electronic nose for discriminating diverse microorganisms involved in VAP and (b) screening of some drugs using volatile fingerprints.**

## Methods/Materials

**Species used in these studies:** *A.fumigatus*, *C.albicans*, *K.pneumoniae*, *E.cloacae* and *S.aureus*

**Growth conditions:** 1) Nutrient Broth 1% and 2) SS+0.2%G-P for simulating the same conditions as bronchoalveolar lavage samples at 37°C

**Hybrid sensor array:** E-nose 3320 Applied Sensor



## Results

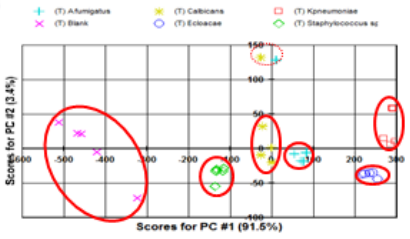



**Figure 1.** PCA score plot showing discrimination between microorganisms using the volatile fingerprints after 72h incubation at 37°C in 1% NB (Dotted circle shows two possible outliers)

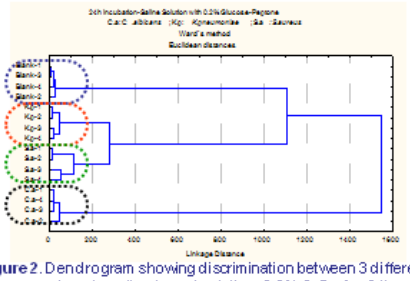



**Figure 2.** Dendrogram showing discrimination between 3 different microorganisms in saline-based solution+0.2% G-P after 24h of incubation at 37°C

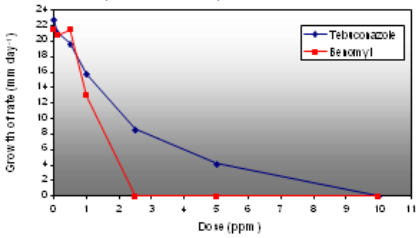



**Figure 3.** Dose response curves showing efficacy of two fungicides against *A.fumigatus* at 37°C in MEA

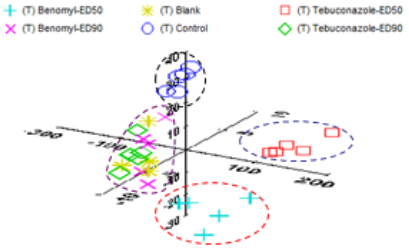



**Figure 4.** 3D PCA score plot after 48h incubation at 37°C in MEA showing discrimination between $ED_{50}$, $ED_{90}$ and control treatments for both fungicides against *A.fumigatus*

## Conclusions

- 5 microorganisms were differentiated with an initial concentration of $10^4$ CFU ml$^{-1}$ after 72h incubation in 1% NB at 37°C.
- 3 microorganisms (*S.aureus*, *K.pneumoniae* and *C.albicans*) were differentiated with initial concentration of $10^2$ CFU ml$^{-1}$ after 24h incubation at 37°C using a basic medium (Saline Solution+0.2% Glucose–Peptone).
- The volatile fingerprints produced in $ED_{50}$ and $ED_{90}$ treatments of Tebuconazole and Benomyl could be used to differentiate growth of *A.fumigatus* cultures.
- Work in progress: (a) to assess the model developed with pure culture data for the validation of unknown patient samples; (b) to analyse clinical samples and to compare with the gold standard microbiological methods; (c) to identify the key volatile biomarkers using GC-MS+SIFT-MS techniques and correlate these with the E-nose results.

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Acknowledgements: We are grateful to Cranfield Health and Gloucestershire Hospitals NHS Foundation Trust for financial support.

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## Volatiles fingerprints: use of a hybrid sensor array electronic nose to screen antifungal efficacy against *A.fumigatus* and *C.albicans*

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## Introduction

- *Aspergillus fumigatus* and *Candida albicans* are fungi which may play a role in many important diseases mainly in immuno-compromised patients.
- Rapid detection of causative pathogens and the screening of drug efficacy would enable improved patient outcomes.
- **Objectives: evaluate the efficacy of some anti-fungal agents using E-nose measurement of volatile fingerprints**

## Methods/Materials

**Species used in these studies: *A.fumigatus and C.albicans***
**Growth conditions: 1) Nutrient Broth 1% for *C.albicans* at 25C**
**2) Malt Extract Agar (MEA) for *A.fumigatus* at 25C and 37C**
**Hybrid sensor array: E-nose 3320 Applied Sensor Group (Sweden) with 23 sensors: 12 MOS, 10 MOSFET and 1 RH sensor**
**Antifungal agents: Benomyl and tebuconazole. $ED_{50}$/$ED_{90}$ concentrations were calculated**

## Results

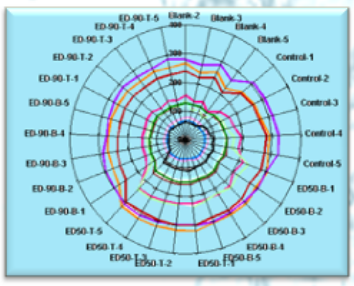

**Figure 1.** Radar plot with 12 MOS sensors (coloured lines) after 48h incubation of *A.fumigatus* in MEA amended with tebuconazole and benomyl $ED_{50}$/$ED_{90}$ concentrations, control and blank samples at 37C

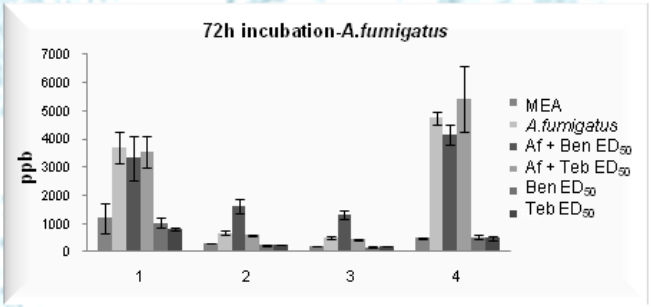


**Figure 2.** Comparison of volatiles produced by control/ blank samples with fungal treatments ($ED_{50}$ concentrations) found in SIFT-MS analysis after 72h incubation. 1: Dimethyl disulphide; 2: Isoprene; 3: Methanol; 4: Acetone

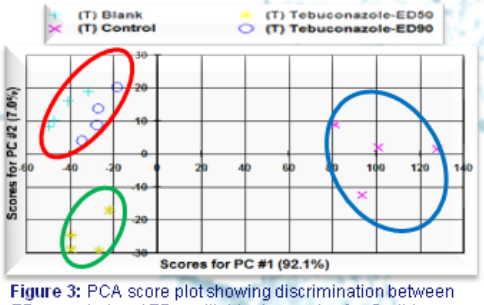


**Figure 3:** PCA score plot showing discrimination between $ED_{50}$, control and $ED_{90}$ with blank samples for *C.albicans* in NB1% at 25C after 48h incubation

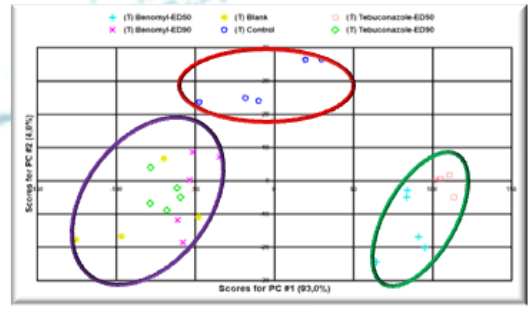

**Figure 4:** PCA score plot showing 3 clusters: control samples; $ED_{90}$ with blank samples and $ED_{50}$ *A.fumigatus* on MEA at 37C after 48h incubation

## Conclusions

- E-nose has shown a potential for screening the efficacy of anti-fungals agents for better effectiveness against these medically important fungi such as *A.fumigatus* and *C.albicans*.
- It might be possible to build a model to test unknown samples by using the data as a training set
- Volatile fingerprints could be used to identify when, how and which strains are developing resistance

Acknowledgements: We are grateful to Cranfield Health and Gloucestershire Hospitals NHS Foundation Trust for financial support.

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