CRANFIELD UNIVERSITY

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NOVEL FOOD APPLICATIONS OF ELECTRONIC NOSE TECHNOLOGY FOR DETECTION OF SPOILAGE FUNGI

INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

PhD THESIS

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Academic Year 2000-2001

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Novel Food Applications of Electronic Nose Technology for Detection of Spoilage Fungi

Supervisor: Prof. N. Magan

October 2001

This thesis is submitted for the degree of Doctor of Philosophy

ABSTRACT

This work investigated the potential use of the electronic nose (e-nose) for the rapid and early detection of fungal spoilage in intermediate moisture bakery products. Four xerophilic *Eurotium* spp., *Penicillium chrysogenum* and *Wallemia sebi* were grown on 2% wheat meal agar (WMA, 0.95 a_w) at 25°C. Discrimination between the fungal species and the control media based on their volatile patterns was possible after 48h, using the Bloodhound BH114 e-nose. Volatile patterns produced by four *Eurotium* spp. and the *Penicillium* sp. were also investigated on WMA at different water activities (0.95, 0.90 and 0.85). Enzyme assays using p-nitrophenyl substrates were used as an indicator of spore germination and growth, and compared with volatile patterns and growth rates. Only three of the seven enzymes assayed were found to change significantly i.e. N-acetyl-β-D-glucosaminidase, α-D-galactosidase and β-D-glucosidase. Earlier detection and differentiation of the control blanks and the fungal species were achieved after 48h incubation, using the e-nose when compared to enzyme assays.

In vitro study of volatile profiles from mycotoxigenic (*Aspergillus flavus*, *A. ochraceus* and *W. sebi*) and non-mycotoxigenic (*A. niger* and *P. chrysogenum*) fungi grown on WMA media demonstrated that differentiation of control blank media from the spoilage fungi was possible after 72h growth. Work on different mycotoxigenic and non-mycotoxigenic strains of *Fusarium moniliforme* and *F. proliferatum* showed that the e-nose could discriminate between the control blanks and the different strains after 48h growth. For all these studies the same three enzyme activities were found to be significant as in the previous work. Furthermore, the e-nose could detect fungal spoilage earlier than the enzyme assays.

Volatile patterns produced by two *Eurotium* spp. and *P. chrysogenum* colonising a bread analogue modified to 0.95 a_w at 25°C could be discriminated from the uninoculated bread after 40h incubation. This suggested that e-nose systems could be an earlier detection tool than enzyme assays. Furthermore, the same enzyme activities were found to be predominant as that observed for *in vitro* studies. *E.*

chevalieri and *P. chrysogenum* were used to determine the limit of detection on a bread analogue (0.95 a_w) using three inoculum concentrations (10^2, 10^4 and 10^6 spores ml^{-1}). The initial spore concentration of 10^6 spores ml^{-1} treatments and the uninoculated bread analogue could be discriminated after 24h incubation. In another study, the volatile patterns produced by two *Eurotium* spp. and *P. chrysogenum* in the presence of potassium sorbate were measured. The uninoculated bread substrate and those colonised by the spoilage fungi could be discriminated after 48h growth. The study was repeated using calcium propionate and differentiation could be achieved only after 24h incubation. In the final study, volatile patterns produced by two *Eurotium* spp. and *P. chrysogenum* growing on natural bread substrate could be differentiated along with the uninoculated bread after 24h growth prior to any visible signs of growth. Overall, this study suggests that for the first time e-nose systems could be used to detect qualitative changes in volatile patterns for early and rapid detection of activity of the spoilage moulds in bakery products.

ACKNOWLEDGEMENTS

I owe a great debt of gratitude to my supervisor, Prof. Naresh Magan, for giving me a fantastic opportunity, for his encouragement and guidance during the compilation of this thesis.

I wish to thank my parents and my family for their continual support financially and emotionally. Without their constant encouragement, I would not have completed this thesis. I would also like to thank my husband Rajiv for his encouragement and patience.

I am grateful to EPSRC and Campden and Chorleywood Food Research Association (CCFRA) for funding my research. I also acknowledge Bloodhound Sensors Ltd for their support.

Finally I would like to thank Dr. Phil Voysey at CCFRA and all my friends at Cranfield University especially the mycology group.

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ABBREVIATIONS AND NOTATIONS

AITC	-	allyl isothiocyanate
ANN	-	artificial neural network
ANOVA	-	analysis of variance
ATP	-	adenosine triphosphate
a_w	-	water activity
BCA	-	bicinchoninic acid
BOD	-	biochemiccal oxygen demand
BSA	-	bovine serum albumin
CA	-	cluster analysis
CCFRA	-	Campden and Chorleywood Food Research Association
CFUs	-	colony forming units
CoA	-	coenzyme A
CP	-	conducting polymer
Da	-	Dalton
DA	-	discriminant analysis
DG18	-	dichloran glycerol 18
DLC	-	discotic liquid crystal
DNA	-	deoxyribonucleic acid
ELISA	-	enzyme-linked immunosorbent assay
e-nose	-	electronic nose
EPS	-	extracellular polysaccharide
FTIR-PAS	-	fourier-transform infrared photoacoustic spectroscopy
GC	-	gas chromatography

HACCP	-	hazard analysis critical control point
HPLC	-	high-pressure liquid chromatography
I	-	current
L.S.D.	-	least significant difference
MAP	-	modified atmosphere packaging
m.c.	-	moisture content
MEA	-	malt extract agar
MOS	-	metal oxide semiconductor
MOSFET	-	metal oxide semiconductor field effect
MS	-	mass spectrometry
PARC	-	pattern recognition
PCA	-	principal component analysis
PCR	-	polymerase chain reaction
p.p.m.	-	parts per million
PVC	-	poly(vinyl chloride)
QCM	-	quartz crystal microbalance
r.p.m.	-	revolutions per minute
SAW	-	surface acoustic wave
SDE	-	steam distillation extraction
TCA	-	tricitric acid
TLC	-	thin layer chromatography
UV	-	ulttra-violet
V	-	voltage
var.	-	variety
WMA	-	wheat meal agar

Chapter 1

Introduction and Literature Review

1.1 INTRODUCTION

The decline in the use of preservatives and mould inhibitors as a result of legislation, especially in Europe, which limits the type and concentration of preservatives used, has paralleled an increase in the level of food spoilage by fungi. Its estimated that 5 to 10% of global food losses are due to moulds causing substantial economic losses in the food industry (Gourama & Bullerman, 1995).

From a microbiological point of view, foods with a high water activity (a_w) are susceptible to attack by spoilage fungi and have only a short shelf-life. Fungi are versatile even in environmentally stressed conditions, such as reduced water activity (a_w of 0.65 to 0.90) or low pH (< 3). Spoilage not only affects the quality but also the safety of food due to the production of mycotoxins, which are known to be carcinogenic and toxic. All these factors have underlined the need for methods, which are both rapid and sensitive for the early detection of the activity of spoilage moulds.

1.2 METHODS FOR DETECTING FUNGAL ACTIVITY

The main emphasis of many studies has been on the detection of fungal activity prior to visible moulding. A number of chemical and biochemical techniques are available for determining fungal activity (Magan, 1993a; Twiddy & Phillips, 1995; Gourama & Bullerman, 1995). These include biochemical markers characteristic of fungi, quantification of fungal enzymes, electrochemical methods and the presence of fungal volatiles (Magan & Evans, 2000, Table 1.1). However, some of these at best poorly correlate with fungal growth or biomass. The use of immunological techniques and

Table 1.1 Advantages and disadvantages of methods available for detection of fungi.

Method	Advantages	Disadvantages
Colony Forming Units (C.F.Us)	- Straight forward procedure - Sensitive method - Low levels of expertise required	- Tedious procedure - Long incubation periods (5-7 days) - Selective media required - Depends on fungal sporulation - Non-specific (all viable yeasts and moulds)
Ergosterol	- Sensitive method - Accurate measure of fungal growth	- Non-specific (total biomass) - Time consuming procedure - Intensive sample preparation - High levels of expertise required - Expensive equipment needed
Chitin		- Time consuming procedure - Intensive sample preparation - Non-specific (total biomass) - High levels of expertise required - Requires expensive equipment - Not very sensitive
Adenosine triphosphate (ATP)	Very sensitive method	- Time consuming procedure - Intensive sample preparation - High levels of expertise required - Non-specific (total biomass)
Enzymes	- Rapid and accurate procedure - Large number of samples analyzed	
Electrochemical	Rapid technique	- Medium and species specific - Lack specificity - Time consuming procedure
Enzyme-Linked Immunosorbent Assay (ELISA)	- Sensitive method - Large number of samples analyzed - Moderate ease of use - Method readily automated	- Expensive equipment and reagents required - High levels of expertise required - Non-specific reaction can give false positive or negative results - Specific to genus / species
Latex agglutination	- Simple and rapid method - Low levels of expertise required - Method readily automated	- Not as sensitive as ELISA - Non-specific reaction can give false positive or negative results

DNA probes have been applied to specific spoilage fungi enabling individual species to be differentiated. The main methods used are detailed in this section.

1.2.1 Colony forming units

Colony forming unit (CFUs) counts have been the traditional method used to quantify fungal contamination levels. The simplicity of this procedure is however, outweighed by its drawbacks. This approach is labour intensive, requires long periods of incubation (5-7 days) on selective media to obtain results, and measures only culturable fungi, thus providing a poor estimate of fungal biomass. CFU counts vary greatly depending on the degree of fungal sporulation (Schnurer, 1993; Schnurer *et al.*, 1999). Colonies, usually arising from spores, will result in an overestimation of heavily sporulating species (Jelen & Wasowicz, 1998). Thus it provides a better measure of sporulation rather than fungal biomass. Despite all its disadvantages, at present it is still the standard method used for determination of fungal contamination in the food industry.

1.2.2 Biochemical markers

Ergosterol: Ergosterol (ergosta-5-7, 22-trienol) is a major sterol component of the fungal cell membrane (Magan, 1993a) in *Ascomycetes* and *Deuteromycetes*, and is not found in significant concentrations in higher plants (Jelen & Wasowicz, 1998). Extraction of ergosterol involves refluxing the sample with methanol, saponification with strong alkali in ethanol and fractionation by high-pressure liquid chromatography (HPLC). Ergosterol is detected by ultraviolet absorption optimally at 282 nm, due to the presence of double bonds at positions 5 and 7. Recently a fluorodensitometric method has been developed for detection and quantification of ergosterol (Bailly *et al.*,

1999). This method is based on thermal treatment of thin layer chromatography (TLC) plates, which leads to the formation of a highly fluorescent ergosterol derivative.

Ergosterol content is influenced by substrate composition, growth phase of mycelium and water availability. Many studies have tried to determine the relationship between fungal growth and ergosterol production. Marfleet *et al.* (1991) showed a correlation between ergosterol levels and fungal biomass for three fungal species (*Eurotium amstelodami, Penicillium aurantiogriseum* and *Alternaria alternata*) over a range of a_w levels on wheat extract agar but not in liquid culture. Tothill *et al.* (1992) demonstrated a significant correlation between ergosterol content and total CFUs for naturally contaminated wheat at 0.95 a_w but not at 0.85 a_w. However, in wheat inoculated with the above individual species there was a statistically significant correlation between increase in ergosterol and total CFUs at both 0.95 a_w and 0.85 a_w. Interestingly, ergosterol content was found to increase prior to visible spoilage. Schurer and Jonsson (1992) and Magan (1993b) also obtained similar results. In contrast, Börjesson *et al.* (1990) found no correlation between ergosterol content and growth of *P. aurantiogriseum* on oats with 25% moisture content (= 0.95 a_w). Further studies by Börjesson *et al.* (1990; 1992) have shown a good correlation between ergosterol content and respiration of spoilage fungi but not with total CFUs.

Schnurer (1993) investigated the relationship between hyphal length, CFUs, and ergosterol level for three fungi (*Fusarium culmorum, Penicillium rugulosum* and *Rhizopus stolonifer*) with three different growth patterns. Hyphal length and ergosterol levels were found to increase for all three fungi on agar substrates. However, CFUs increased in parallel with hyphal length and ergosterol content only for the non-

sporulating *F. culmorum.*

Disadvantages of the assay include non-specificity, time-consuming procedures and do not provide any information on species composition (Schnurer *et al.*, 1999). Despite its limitations, the ergosterol assay is a sensitive and accurate measure of fungal growth.

Chitin: Chitin, a polymer of N-acetyl-D-glucosamine, is a major constituent of fungal cell wall. The assay consists of alkaline hydrolysis of chitin to glucosamine residue followed by deamination to aldehyde, which is measured colorimetrically. The disadvantage of the assay is that it is time-consuming and complex. Furthermore, chitin content does not increase proportionally with fungal growth. Chitin content can vary depending on the fungal species, culture age and growth conditions (Shahidi *et al.*, 1999). The assay lacks sensitivity and reproducibility so the results can be misleading (Magan, 1993a). For example, if the sample is contaminated with insect fragments as well as fungi then part of the chitin content can be as a result of insect exoskeletons. Again discrimination between viable and non-viable cells is not possible, giving an indication of past fungal activity.

Adenosine triphosphate (ATP): ATP has been suggested as a measure of microbial biomass. A study by Nieto-Fernandez *et al.* (1998) found a positive correlation between total ATP of the mycelium of *Phanerochaete chrysosporium* (white rot) and biomass. The assay relies on the bioluminescence technique, i.e., the oxidation of luciferin in the presence of luciferase and ATP (Jelen & Wasowicz, 1998). However, a good extraction technique is required to ensure that ATP is effectively released from the cells and that it remains stable while inactivating all enzyme activity. The drawback is that all living

plant and bacterial cells contain high levels of ATP and therefore, extraction of ATP

without homogenising the sample excludes the detection of internal fungi resulting in

an inaccurate total fungal biomass. This assay is still time-consuming and lacks

specificity (Gourama & Bullerman, 1995).

Respiratory activity of moulds: Measurements of carbon dioxide (CO_2), oxygen (O_2)

uptake, or heat production during aerobic respiration have been used to determine the

level of microbial activity. The level of respiration is dependent on the temperature and

moisture content (Lacey *et al.*, 1991). The equation used was:

$$C_6H_{12}O_6 \ + \ 6O_2 \ \longrightarrow \ 6CO_2 \ + \ 6H_2O \ +677.2cal$$
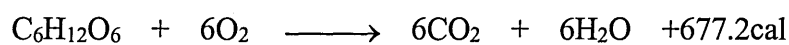
In such a method, inaccuracies may occur in measurements, and the assumption that

only carbohydrates are metabolised is incorrect as proteins and lipids may also be

metabolised and these have different respiratory quotients.

1.2.3   Enzymes

Studies on the fungal enzymes required for degradation of food substrates, have shown

considerable differences between fungal species in their ability to produce different

enzymes. Jain *et al.* (1991) utilized chromogenic 4-nitrophenyl substrates to assay

fungal enzymes in a microtitre plate assay. N-acetyl-β-D-glucosaminidase increased in

mouldy grain substrates at a range of a_w levels when compared to non-mouldy dry

grain. For *Eurotium amstelodami* there was also an increase in α-D-glucosaminidase.

Magan (1993b) demonstrated significant increases in some enzymes (N-acetyl-β-D-

glucosaminidase, α-D-galactosidase and β-D-glucosidase) prior to visible moulding.

Similarly, Marin *et al.* (1997; 1998) also found these three fungal enzymes to be predominantly produced by *Fusarium* spp. infecting maize. The advantages of this method include a rapid and accurate procedure, and large numbers of samples can be analyzed relatively quickly in a microtitre well format. No comparative studies have been carried out to evaluate fungal enzyme activities with other indicators of mould spoilage.

Spoilage fungi can also be detected by measuring the release of methanol from pectin by the action of the fungal enzyme pectinesterase, using gas liquid chromatography. This method has been applied to pure and mixed spore suspensions of *Aspergillus* and *Penicillium* species (Offem & Dart, 1983).

1.2.4 Electrochemical methods

Metabolites produced by growth of microorganisms in liquid media alter the medium's impedance (or capacitance) and conductance. Changes in these properties can be used as a measure of fungal growth (Magan, 1993a). A major problem with these techniques involves the selection of a suitable media, as ionic solutes and pH affect detection of electrical signals (Gourama & Bullerman, 1995). The media should be able to induce detectable and reproducible changes in either conductance or capacitance during fungal growth. Watson-Craik *et al.* (1989) demonstrated that conductance was both medium and species specific. Hence impedimetry and conductimetry are rapid methods provided well-defined conditions are used.

1.2.5 Fourier-Transform Infrared photoacoustic spectroscopy (FTIR-PAS)

FTIR-PAS, a sensitive probe of the surfaces of solid substrate, has been used to give a measure of the level of fungal infection. Recently FTIR-PAS has successfully been used in the detection of mycotoxigenic fungi in inoculated corn kernels (19-22% moisture content) (Gordon *et al.*, 1998). The FTIR-PAS spectra from *Aspergillus flavus* were shown to be significantly different from that of the control.

1.2.6 Immunoassays, Immunofluorescence and DNA probes

Antigens produced by fungal cell wall proteins can be detected by immunological methods. Some antigens are derived from components common to a wide range of fungi and hence are indicative of general fungal growth while others are genus- and species-specific. Girardin (1997) and more recently Li *et al.* (2000) have reviewed numerous immunological methods available that are rapid, specific, and sensitive, which could be carried out readily and automated.

Enzyme-Linked Immunosorbent Assay (ELISA): This assay involves absorption of specific antibodies onto microtitre plate wells (sensitizing the plate) and the addition of complementary fungal test antigens. The enzyme-linked antibody specific for test antigen then binds to the antigen forming a double antibody sandwich. Addition of the substrate produces a visible colour change, which is measured spectrophotometrically (Girardin, 1997).

Notermans *et al.* (1986) showed that the *Penicillium* antigen reacted with 43 of 45 *Penicillium* species tested. Antigen production correlated with mycelial weight and was

unaffected by culture condition, medium, temperature and a_w. The *Penicillium* antigen

also reacted with *Aspergillus flavus* and the levels of antigen correlated with aflatoxin

production. However, no correlation was observed between CFU and ELISA titre in

paprika samples contaminated with *A. flavus* (Kisko *et al.*, 1998). Thus ELISA assays

allow a number of samples to be tested simultaneously and automatically when

expensive equipment is available.

Latex agglutination: This approach incorporates the coating of latex beads with

antibodies sensitised to extracellular polysaccharide (EPS) and detection of visible

agglutination of the beads in the presence of EPS antigens (Twiddy & Phillips,

1995). Latex beads coated with antibodies from the extracellular polysaccharide

produced by *Penicillium digitatum*, specifically detected *Aspergillus* and *Penicillium*

species (Girardin, 1997).

A comparison of latex agglutination assay with ergosterol assay found both methods

comparable for *Penicillium* and *Aspergillus* species in pure culture. However, for

Fusarium species, the ergosterol assay was found to be more sensitive (Schwabe *et al.*,

1992). The latex agglutination assay has several advantages over the ELISA assay: it is

rapid with results being obtained in 10-20 minutes; does not require specific

equipment; is easy to carry out, but is not as sensitive as ELISA.

Fluorescent antibody techniques: Antibodies to specific fungi are linked directly or

indirectly to a fluorescent dye. Under a microscope with an UV light source, the

addition of the fluorescent antibody conjugate to the sample results in fluorescence, in

the presence of a specific fungal antigen. This method has been used for the detection

of mould in food and has been applied to the detection of *Penicillium aurantiogriseum* in barley (Warnock, 1971). Difficulties in this technique arise when working with large number of samples and when quantifying the level of mould present.

DNA probes: DNA sequences specific for moulds (i.e. internal transcript spacer of ribosomal DNA sequences) have been amplified using the Polymerase Chain Reaction (PCR), and the PCR primers have been used for specific detection of mould in food. The PCR method is sensitive and has been applied in the detection and quantification of *Eurotium amstelodami* and *Penicillium hordei* in grain (Roberts, 1992); *Fusarium culmorum* and *Fusarium graminearum* in cereals (Nicholson *et al.*, 1998); and *Alternaria alternata* in tomato products (Zur *et al.*, 1999).

Advantages of the method are specificity, sensitivity, reliability, and the ability to detect viable and non-viable cells. However, the limitations include inhibition of DNA amplification by compounds in food, breakdown of fungal DNA in food (Zur *et al.*, 1999), the requirement of expensive equipments and a high level of expertise.

1.3   FUNGAL VOLATILES

1.3.1   Why do fungi produce volatiles ?

Linton and Wright (1993) reviewed possible reasons for the production of fungal volatiles. Production of volatiles may be a way of removing inhibiting intermediates from the metabolism under unfavourable conditions. They are also thought to have an inhibitory effect on other fungi and may act as self-regulators of growth and development. For example, in *Geotrichum candidum*, volatile compounds have been shown to influence all stages of growth. Whereas, those from *Trichoderma viride* have been shown to inhibit the mycotoxigenic mould *Fusarium moniliforme*. Fungal volatiles also attract insects such as grain beetles and collembolans (Hedlund *et al.*, 1995). Volatiles produced by *Fusarium verticillioides* have been found to attract the sap beetle, *Carpophilus humeralis* (Bartelt & Wicklow, 1999), the advantage to the fungus being the dissemination of fungal spores.

1.3.2   Range of fungal volatiles

A range of fungal volatiles has been identified, which includes alcohols, esters, ketones, pyrazines and terpenes. Appendix A lists some of the volatile compounds produced by different fungal species on different grain substrates (Kaminski & Wasowicz, 1991).

Alcohols are by far the most commonly produced volatile compounds. For example, Börjesson *et al.* (1989) found alcohols to constitute 80% of the total volatile concentration (the exception being *E. amstelodami* where 50% were alcohols) and 90% of this alcohol was ethanol. Other studies (Sinha *et al.*, 1988; Tuma *et al.*, 1989)

revealed 3-methyl-1-butanol and 1-octen-3-ol to be predominant along with the ketone, 3-octanone. Since then, other alcohols have been identified such as 3-octanol (Nilsson *et al.*, 1996). Both geosmin and 2-methylisoborneol are commonly associated with off-odourous fungi.

The main esters found are acetates, which include ethyl acetate, isobutyl acetate and isopentyl acetate (Larsen & Frisvad, 1995a). They also identified aromatic esters such as 1-methoxy-3-methylbenzene. Besides esters, pyrazines such as methoxy pyrazines (Larsen & Frisvad, 1994), sulphurous compounds such as dimethyl disulphide (Börjesson *et al.*, 1993) and a number of mono- and sesquiterpenes are produced. Börjesson *et al.* (1989; 1990) identified heterocyclic compounds such as 3-methylfuran.

1.3.3   Production of fungal volatiles.

The metabolic pathway leading to the formation of volatiles gives important clues as to the relationship between various volatiles, non-volatiles and mycotoxins. Figure 1.1 illustrates the schematic pathway for the various volatiles (Pasanen *et al.*, 1996).

Ethanol is produced under anaerobic conditions. Alcohols are derived from amino acids (such as leucine, isoleucine, valine and phenylalanine) via the Ehrlich pathway (Jelen & Wasowicz, 1998). 1-octen-3-ol and other eight and ten carbon compounds are synthesized by oxidation of linoleic acid by lipooxygenase activity (acetate-fatty acids-alcohols pathway). Secondary alcohols, ketones and lactones are also produce via the fatty acid pathway. The breakdown of lipid through fungal lipase activity results in free fatty acids. These are oxidised to β-keto acids, which are subsequently decarboxylated to methyl ketones. The corresponding secondary alcohols are formed

Aromatic secondary
metabolites

Glucose

Nitrogen
compounds

Ethanol

Amino acids
(e.g. Leucine, Valine)

Pyruvate   Malonate

Aromatic amino acids
(Phenylalanine)

Alcohols

Acetate
(Acetyl-CoA)   Polyketides   Ochratoxins

TCA-cycle:
Oxalo-acetate

Methyl ketones

Threonine   Aspartate

Mevalonate   Fatty acids

Lactones
Esters   Alcohols
Aldehydes

Secondary
alcohols

Methionine

Geranyl pyrophosphate

Sulphur
compounds

Farnesyl pyrophosphate

Monoterpenes
2-methylisoborneol

Sesquiterpenes
Geosmin

Trichothecenes

Figure 1.1 Main metabolic pathways for production of some volatile and toxic metabolites of fungi (Pasanen *et al.*, 1996).

by the reduction of methyl ketones. Lactones are produced from γ-keto acids. Enzyme catalyzed reactions between alcohols and acyl-CoA compounds result in the formation of esters.

Pyrazines are thought to be synthesized by the condensation reaction between acetoin and ammonia (Jelen & Wasowicz, 1998). Dimethylsulphide is produced from methionine. The mevalonic acid pathway gives rise to a variety of terpenes such as geosmin and 2-methylisoborneol.

1.3.4 Relationship between mycotoxins and fungal volatiles.

A number of studies have investigated the relationship between mycotoxin and volatile production. Abramson *et al.* (1983) found that the total volatiles peaked at two and six weeks, declining thereafter for barley at 16 and 20% moisture content (m.c.). However, more volatiles were produced at 20% m.c. Ochratoxin A was also detected in barley (20% m.c.) initially at week 6, which then increased during weeks 14 to 62.

A later study by Pasanen *et al.* (1996) examined a mycotoxigenic strain of *Fusarium sporotrichioides* grown on straw, wheat and oat grains. A similar composition of volatiles was produced on both grain types. *F. sporotrichioides* produced equal amounts of terpenes and ketones, and to a minor extent alcohols. Interestingly, large amounts of trichothecenes were also synthesized. As seen from Figure 1.1, trichothecenes are synthesized via the terpenoid route, the same pathway for the production of terpenes. Furthermore, Pasanen *et al.* (1996) suggested that the production of a large amount of ketones was due to the high lipid content of grain. An earlier investigation by Börjesson *et al.* (1989) also indicated the relationship between

terpenoid compounds and trichothecene production by *F. culmorum*, suggesting terpenes as intermediates in their synthesis. Jelen *et al.* (1997a) observed trichodiene production by toxigenic strains of *Fusarium* spp. grown on wheat, the greatest amount being produced by *F. sambucinum*. As the concentration of trichodiene decreased, trichothecene content was observed to increase, suggesting trichodiene as an intermediate in trichothecene biosynthesis. Similar correlation between trichodiene and trichothecene production was also observed for *Fusarium* spp. on naturally contaminated grain spikelets (Jelen *et al.*, 1997b). They suggested that the sesquiterpene trichodiene could be used as a volatile marker for trichothecene biosynthesis.

Research into production of volatiles by non-mycotoxigenic and mycotoxigenic fungal strains has identified differences in their volatile profiles. Studies on aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* grown in liquid culture have shown a correlation between sesquiterpene production and initiation of aflatoxin biosynthesis (Zeringue *et al.*, 1993). The volatiles were absent during the first two days and the latter 8th and 10th days, and peaked in 3 day-old cultures. Aflatoxin concentration was observed to decrease with the decline in the amount of sesquiterpenes present. Furthermore, individual strains showed differences in their sesquiterpene volatile profiles. In contrast to these findings, Jelen *et al.* (1995) found volatile profiles of sesquiterpenes to be similar for all mycotoxigenic strains (although the concentrations were different) in a study of 25 strains of *Fusarium sambucinum* grown on wheat. Non-mycotoxigenic strains produced less volatile sesquiterpenes with less chemical diversity in comparison to mycotoxigenic strains. The different

observations by Zeringue *et al.* (1993) and Jelen *et al.* (1995) may be due to different substrates used in their studies. Pasanen *et al.* (1996) also demonstrated differences in volatile composition between toxigenic and non-toxigenic strains of *Penicillium verrucosum*. *P. verrucosum* that synthesized ochratoxin showed an accelerated production of ketones compared to that for a non-toxigenic strain, in which alcohols were predominant. Ochratoxins are derived from polyketides (see Figure 1.1) and phenylalanines. Similarities between the polyketides and fatty acid pathways may result in increased production of ketones in ochratoxin-producing strains.

Although many authors have examined specific groups of volatile compounds, no attempts have been made to study the use of overall volatile patterns to discriminate between mycotoxigenic and non-mycotoxigenic fungi, and correlate this with other indicators such as enzyme activities.

1.3.5 The relationship between volatiles and fungal growth.

During different fungal growth stages, the relative proportion of volatile compounds varies. However, the profile of volatile types remains the same (Börjesson *et al.*, 1989; 1990; 1992; 1993). Börjesson *et al.* cultured *E. amstelodami*, *A. flavus*, *Penicillium cyclopium* and *Fusarium culmorum* on moist wheat. The CO_2 content indicated the metabolic activity of the fungi. Prior to visible signs of growth, about two days after inoculation, not only did the CO_2 content increase, but also some volatile compounds were detected. These may be specifically associated with fungal growth. For example, 3-methyl-1-butanol was found to be predominant at the early stages of growth of *A. flavus* and *P. cyclopium*. The volatile compounds increased in concentration during

growth, with the exception of *F. culmorum* where 2,2,5-trimethylhexane was only exclusive to the first six days. 3-methylfuran was present during early stages of growth of some *Penicillium* and *Aspergillus* spp. With *P. roqueforti* the ratio of terpene production to CO_2 production markedly increased during late growth stages, which may be due to a shortage of growth factors. However, very few authors have attempted to study early detection of volatiles prior to visible signs of growth.

Some authors have examined changes in volatile production over a period of time. Zeppa *et al.* (1990) detected 11 and 16 volatile compounds from an isolate of *Trichoderma viride* after 3 and 12 days respectively, with an increase in concentration of derivatives of α-pyrone and a decrease in alcohols. Similarly, Bartelt and Wicklow (1999) also studied the changes in volatile pattern of *Fusarium verticillioides* inoculated on corn kernels over a period of 8 days. After 2 days incubation (visible fungal growth), 2-methyl-1-propanol, ethanol, 1-propanol, acetaldehyde and ethyl acetate were detected. This was followed by the additional presence of 3-methyl-1-butanol, 2-methyl-1-butanol and aromatic compounds after 5 days. Only ketones and aromatic compounds were detected after 8 days.

Production of volatiles may not be related to the amount of fungal biomass produced. For example, Dionigi and Ingram (1994) noted no correlation between rapid biomass production and an increased goesmin accumulation by *P. expansum*.

Volatile production may also be affected by sporulation. A number of studies have shown an increase in the volatile production during sporulation. For example, in *Penicillium discolor*, terpene production increased after 4 to 5 days when the fungus

begun to sporulate strongly (Nilsson *et al.*, 1996). Similarly, the appearance of geosmin from *P. expansum* (Mattheis & Roberts, 1992) and 2-methylisoborneol (Börjesson *et al.*, 1993) coincided with the onset of sporulation. A study by Larsen and Frisvad (1994) showed that strongly sporulating and less sporulating isolates of *Penicillium vulpinum* clustered separately in the dendrogram. During sporulation of *Fomitopsis pinicola*, Faldt *et al.* (1999) also noted an increase in 3-octanone, 1-octen-3-ol and some sesquiterpene hydrocarbons, suggesting fungal volatiles as attractants for insects, important in spore dispersal.

1.4   FACTORS AFFECTING FUNGAL VOLATILE PRODUCTION

Environmental conditions and substrate composition influence the production of volatile compounds, both qualitatively and quantitatively. Important environmental factors are a_w, pH, atmospheric composition, agitation, and temperature.

1.4.1   Atmospheric composition

The production of volatile compounds strongly correlates with carbon dioxide production (Börjesson *et al.*, 1990; 1992). Thus suggests that increased fungal metabolism in the absence of regular aeration leads to a depletion of oxygen. This anaerobic condition promotes alcohol production, especially ethanol (Börjesson *et al.*, 1989).

Low oxygen/carbon dioxide ratios influence the fungal metabolism, which then affect volatile production (Larsen & Frisvad, 1995b). *Penicillium* species produced mainly alcohols, ketones and esters when grown *in vitro* in Petri dish cultures, and terpenes

when grown in a flow-through system (Nilsson *et al.*, 1996). During fungal growth, CO_2 is removed by an unknown rate of diffusion from the Petri dish, whereas, in a flow-through system the flow of CO_2 is controlled. In an earlier study by Dionigi and Ingram (1994), *Penicillium expansum* was observed to produce a higher concentration of geosmin in an atmospheric composition of 10% O_2:90% N_2 when compared to the control (20% O_2:80% N_2). However the isolate used was thought to be acclimatized to reduced oxygen concentrations.

1.4.2 Agitation

Sinha *et al.* (1988) and Tuma *et al.* (1989) studied volatile production associated with mouldy wheat in ventilated and non-ventilated storage bins at different moisture contents (m.c.). Ventilation, used to cool and dry grains, disrupts the development of microflora and volatile production. The dominant volatiles, namely 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone occurred in greater amounts in non-ventilated than ventilated bins. The increase in the level of fungal volatiles corresponded to heavier fungal infection and a reduction in seed germinability.

1.4.3 Water availability

High initial moisture content was found to accelerate the volatile production in non-ventilated bins (Sinha *et al.*, 1988; Tuma *et al.*, 1989). Bins, containing wheat of 18.2% and 25% (= 0.95 a_w) moisture contents, had higher levels of volatiles than that for 15.6% and 20% m.c. respectively. 3-methyl-1-butanol was common to all bins regardless of the initial moisture content and levels of ventilation. However, the highest levels of 3-methyl-1-butanol were found in bins with m.c.s of 25% and 18.2%. High

levels of 1-octen-3-ol and 3-octanone, present in the 18.2% m.c. non-ventilated bins, were associated with invasion by *Aspergillus versicolor*, *A. flavus* and *Penicillium* spp.. These fungi were absent in ventilated and non-ventilated bins containing drier grain (15.6% m.c.). Wasowicz & Kaminski (1988) also noted that the total amount of volatiles was more pronounced in wheat and maize samples with high moisture contents. The total amount of volatiles was observed to increase by 20 and 5 folds respectively in maize with 25% and 17% m.c. The contents of 3-octanone and 1-octen-3-ol also increased in 25% m.c. maize grain. All these studies have focused on the effect of water availability on volatile production from grain undergoing spoilage generally. Further investigations are required to study volatile production by spoilage fungi *in vitro* and *in situ* at different a_w levels.

Few studies have examined the effect of water stress on the production of volatiles by spoilage fungi. In investigations of 2-heptanone production by *Trichoderma viride* grown on agar media, volatile production was observed to increase with decreasing a_w values (Gervais *et al.*, 1988; Gervais, 1990). Water stress caused by lowering the a_w from 0.99 to 0.80 after 5h resulted in a reduction in 2-heptanone production. The subsequent return to 0.99 a_w after 10h, corresponded to an increase in volatile production.

1.4.4 Temperature

Temperature and seasonality are also associated with volatile production. Temperature in non-ventilated bins (m.c. 15.6%) ranged from –6.3 to 23.8 °C and the moisture content from 13.4 - 16.0% (Sinha *et al.*, 1988). The greatest amount of volatiles was

detected in the middle of the bins, during summer when the temperature of the grain was between 20.9 – 21.8 °C. Tuma *et al.* (1989) showed that 1-octen-3-ol production correlated with *Penicillium* spp. and temperature. Thus suggesting a possible temperature-effect on *Penicillium* spp., enabling them to produce 1-octen-3-ol. Furthermore, high levels of volatiles were associated with increased levels of microfloral activity during storage in autumn and again in spring as the temperature increased.

Other studies have examined volatile production at different temperatures. *Pencillium expansum* was found to produce higher concentration of geosmin at 40°C than at 20°C, again suggesting geosmin biosynthesis to be associated with temperature (Dionigi & Ingram, 1994). Similarly, Bartelt and Wicklow (1999) also showed the progression of the volatile profile of *Fusarium verticillioides* over a period of 8 days was retarded at 20°C and accelerated at 30°C, although the volatile patterns were qualitatively similar.

1.4.5   Substrates

The composition of volatiles produced can vary depending on the substrate used (Pasanen *et al.*, 1996), however the substrate type does not greatly influence the volatile production. Table 1.2 list volatiles produced by a variety of *Aspergillus, Penicillium, Fusarium* spp., and other fungi on different cereal substrates.

Börjesson *et al.* (1989; 1990; 1992) investigated volatile compounds produced by spoilage fungi grown on a variety of agar and grain substrates. Profiles of the volatiles were significantly different between species rather than between substrates. Even more surprisingly, was the difference between strains of the same fungus. With

Table 1.2 Fungal volatiles identified in inoculated cereal cultures by different authors[a] (from Abramson, 1991).

Fungal volatile	Cereal		
	Corn	Barley	Wheat
1-butanol			c
2-butanol			c, d
3-methyl-1-butanol	a	b	c, d
1-pentanol	a	b	
1-hexanol	a		c
2-octen-1-ol			c
1-octanol			c
3-octanol	a		c
1-octen-3-ol	a	b	
phenylethanol			c
2-ethyl-5-methylphenol		b	
hexanal	a		
2-(2-furyl)pentanal		b	
benzaldehyde			c
3-octanone	a		c
2-hydroxy-3-butanone	a		
nonanone			c
2-methylacetophenone		b	
butyl acetate			c
amyl acetate			c
octyl acetate			c
2-methylfuran			d
2-(1-pentyl)furan		b	
3-methylanisole		b	

[a]Key: a, Richard-Mollard *et al.* (1976); b, Wilkins & Scholl, (1989); c, Kaminski *et al.* (1987); d, Börjesson *et al.* (1989).

regard to substrates, *Penicillium aurantiogriseum* produced greater amounts of 8-carbon alcohols and 3-methyl-1-butanol and lower amount of terpenes on grains compared to those from cereal-based agar. They suggested that this may be explained by the slower growth of fungi on agar than on grain, which has a higher lipid content (see Figure 1.1). In addition, certain volatiles such as 1-octen-3-ol, were detected only on grain substrates and the dominant volatiles were the same regardless of the grain type. Alcohols were more pronounced on cereal-based agar media than those from mineral nutrient substrates (these have adjusted carbon/nitrogen ratio), where terpene production was higher as a result of possibly a shortage of certain nutrients. Similarly, *P. commune* produced a series of esters when grown on malt extract agar (MEA) whereas on dichloran glycerol 18 agar (DG 18) the fungus produced mono- and sesquiterpenes. *A. versicolor* produced 19 volatile metabolites on DG18 and only one on MEA. Kiviranta *et al.* (1998) also noted differences in volatile production from *Aspergillus fumigatus* and *Penicillium brevicompactum*. However, in the case of *P. brevicompactum*, esters were detected on DG18 and terpenes on MEA. Trace metals such as copper and zinc, are also important for volatile production (Larsen & Frisvad, 1995c).

Only one study to date by Harris *et al.* (1986) investigated the production of fungal volatiles on agar and wheat bread. *Aspergillus niger* and *Penicillium roqueforti* were noted to produce greater amounts of alcohols and ketones (such as 3-octan-ol and 1-octene-3-ol) on bread than on agar media. *P. roqueforti* was also found to produce large quantities of 2-methylisoborneol and damascenone on bread substrate. Much more extensive research is needed in the area of fungal volatile production on real natural

substrates with regards to spoilage fungi. Furthermore, studies are required to evaluate the effect of preservatives (in bread substrate) on production of volatiles by fungi.

Volatile production is also affected by the carbon source present in the culture media. For instance replacement of glucose with fructose in the media resulted in an increase in sesquiterpenes accumulation by *Lentinus lepideus* (Sprecher & Hanssen, 1982). Furthermore, calcium ions and potassium nitrate (as a source of nitrogen) were also found to increase production of terpenes. Jacobsen and Hinrichsen (1997) found that the volatile profiles of *Penicillium candidum*, *P. nalgiovense* and *Geotrichum candidum* grown on glucose, peptone and maize oil substrates were qualitatively similar and quantitatively different. However, the dominant volatiles were observed to be the same irrespective of the substrate. For example, 3-methyl-butanol was the dominant volatile for *G. candidum* and 1-octen-3-ol for *Penicillium* spp. Principal component analysis of the volatile compounds revealed *G. candidum* as one group and the *Penicillium* spp. as another, indicating that species rather than substrate to be the important factor in volatile production.

1.4.6 Method of collection

Differences in methods of collection of volatiles can affect the relative proportion of volatiles. For example, Tuma *et al.* (1989) found that inverting and shaking the flask containing *A. repens* inoculated wheat resulted in a 10-fold increase in 1-octen-3-ol and detection of 3-octanone, in comparison to that of an undisturbed flask. This disturbance releases the intergranular volatiles in the caked grain and increased the surface area for active transfer of volatiles. Furthermore, quantitative differences in the volatile profiles

found by other studies may be due to differences in isolation and concentration techniques utilized. As shown by Larsen and Frisvad (1995b), the qualitative profiles of volatiles were similar for diffusive sampling from headspace and purging and trapping of headspace. However, the quantitative profiles of volatiles were different, particularly at lower purge flow. On the other hand, steam distillation extraction (SDE) gave a completely different volatile profile for the same fungal species.

1.5   VOLATILES AS TAXONOMIC MARKERS

Studies on other fungal species revealed specific volatiles such as 3-methylfuran, to be produced in similar amounts regardless of the fungal species and grain substrates (Börjesson *et al.*, 1992). While other volatile compounds especially terpenes were both species- and strain-specific (Börjesson *et al.*, 1989; 1993; Larsen & Frisvad, 1995c; Nilsson *et al.*, 1996; Larsen, 1997). For example, thujopsene was produced by *Aspergillus* species but not by *Penicillium* species. *A. candidus* produced a monoterpene not found in the other five fungi examined, suggesting the use of terpenes for differentiating between fungi.

Some authors have suggested the use of volatile profiles, rather than individual volatile compounds to classify fungi at a species level, as the combination of volatiles is unique to each species (Larsen & Frisvad, 1994; Larsen & Frisvad, 1995c; Larsen, 1997; Wilkins *et al.*, 1997; Korpi *et al.*, 1998; Fischer *et al.*, 1998; 1999). Larsen (1997) classified *Penicillium* species within 2 days after inoculation and in a mixed culture of *P. roqueforti* and *P. commune* (in a ratio of 1000:1) identification was possible within 3 days. Larsen and Frisvad (1995a) reclassified some *Penicillium* described as varieties,

as separate taxa and vice versa. For example, only *P. roqueforti* var. *carneum* produced geosmin and cluster analysis of the volatile profiles showed separate clusters for *P. roqueforti* var. *carneum* and *P. roqueforti* var. *roqueforti*. However, all these studies used gas chromatography/mass spectrometry (GC-MS) to identify and quantify volatile compounds rather than newer faster techniques such as the electronic nose to detect and discriminate fungi based on their overall volatile profiles.

1.6   VOLATILES AS INDICATORS OF FUNGAL GROWTH

Production of volatile compounds with time may act as chemical markers for rapid characterization and detection of early fungal growth. Volatiles may be used as early indicators for detecting spoilage of stored grain (Sinha *et al.*, 1988; Tuma *et al.*, 1989; Kaminski & Wasowicz, 1991; Magan & Evans, 2000) and for quality control (Larsen & Frisvad, 1995c) or for microbial growth in buildings (Wilkins *et al.*, 1997; Korpi *et al.*, 1998). Very few studies have correlated fungal volatiles to other indicators of fungal spoilage such as CFUs and ergosterol (Borjesson *et al.*, 1990; 1992). Further research is required to compare fungal volatiles with other markers of fungal activity, such as enzymic activity, especially as an early indicator of spoilage prior to visible signs of growth.

1.7 THE ELECTRONIC NOSE

All previous studies have utilized gas chromatography (GC) or a combination of GC-mass spectrometry (GC-MS) to quantify and identify fungal volatile compounds. Modification of the method includes the use of porous polymer (e.g. Tenax GC) to trap and concentrate volatiles, which can then be released by thermal desorption (Stephan *et al.*, 2000). However, this method is still time-consuming and requires expensive equipment. Recent development and applications of electronic nose technology (Gardner & Bartlett, 1994; Bartlett *et al.*, 1997) has given rise to the possibility of a new tool for early detection of fungal growth based on volatile patterns.

The electronic nose to some extent mimics the natural olfactory system and hence the term 'Electronic Nose' (Shurmer, 1990; Pearce, 1997). Persaud and Dodd (1982) originally proposed the concept of an artificial nose system in 1982 at Warwick University in the UK (Gardner & Bartlett, 1994). The term 'electronic nose' appeared around the beginning of the 1990s and Gardner and Bartlett (1994) defined the electronic nose as 'an instrument, which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern-recognition system, capable of recognizing simple or complex odours'.

The electronic nose consists of three basic building blocks (Figure 1.2). The sensor array represents the sensors in the nose; the circuitry represents the conversion of the chemical reactions on the human sensors to electrical signals into the brain (Craven *et al.*, 1996). Finally the software represents the brain itself. The signal pattern from the sensors is firstly pre-treated and then further processed by pattern recognition (PARC) methods, such as principal component analysis (PCA), discriminant analysis (DA),

cluster analysis (CA) and artificial neural networks (ANN). The data from the sensor array is comparative rather than quantitative so a classification or "fingerprint" of the sample can be obtained. There is no identification of the components in the sample headspace.

Headspace: The volatiles normally exist as the headspace above the liquid or solid sample. There are two types of headspace, i.e. static (sensors are moved into the headspace of the sample) and dynamic (the volatiles are pumped to the sensors) headspace. The advantages of headspace analysis are that there is no sample preparation as it is a non-invasive method, and it is more representative for large quantity of samples (Reineccius, 1996). Volatile profiles are not changed by artefacts, which can be generated in the extraction/distillation method for collecting volatiles.

The sample is placed in a closed vessel and allowed to equilibrate so that the headspace becomes saturated with volatile compounds. The equilibration time depends on the volatility of different compounds, the temperature, the surface area of the sample and the volume of the headspace. The headspace is transferred into the chamber containing the sensor array using an automated sampling system (Craven *et al.*, 1996). After a time the headspace is removed from the chamber, replaced by clean air and the sensors return to their baseline values.
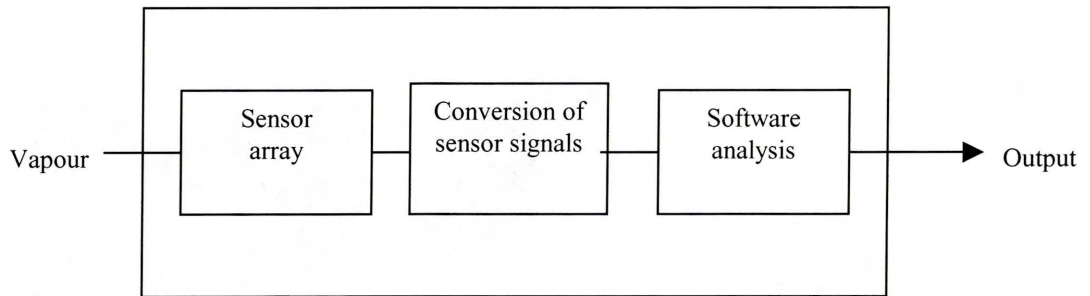
Figure 1.2 Principal components of the electronic nose (Hodgins & Simmonds, 1995).

Figure 1.3 Bloodhound BH114 electronic nose.

Sensors: A variety of sensors have been used in electronic nose technology ranging from metal oxide semiconductor to conducting organic polymer sensors (Schaller *et al.*, 1998; Strike *et al.*, 1999). The Bloodhound BH114 electronic nose uses an array of 14 conducting polymer sensors (Figure 1.3). Table 1.3 shows the commercially available electronic nose systems.

The advantage of conducting polymers is their ability to respond rapidly and reversibly at room temperature. They are non-specific but can be highly sensitive (0.1 – 100 ppm) (Keller, 1999). Therefore, they can respond to a number of different compounds at low levels. The main drawbacks are the pronounced drift of the sensor responses and their extreme sensitivity to moisture due to the low operating temperature (< 50°C).

When a voltage (V) is applied across the electrodes, a current (I) passes through the polymer (Figure 1.4). The adsorption of molecules onto the polymer causes a change in the conductance of the polymer (usually an increase). A dynamic equilibrium develops as molecules are constantly being adsorbed and desorbed at the sensor surface.
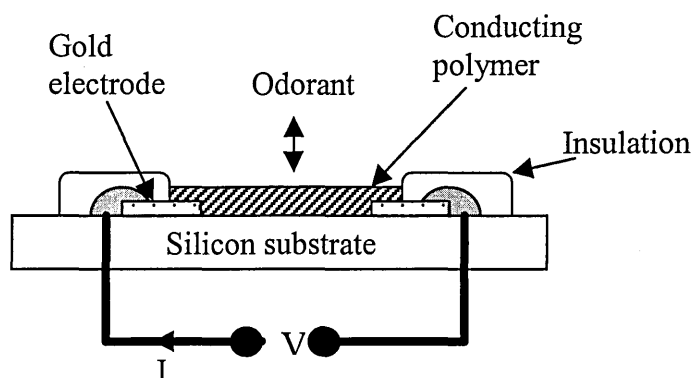
Figure 1.4 Schematic diagram of the conducting polymer sensor (Dodd *et al.*, 1991).

Table 1.3 Commercial availability of electronic noses.

Country	Company	Products	Technology
Germany	Airsense	PEN	10 x MOS
France	Alpha MOS	FOX 2000, FOX 3000, FOX 4000, FOX 5000	MOS, CP, QCM
U. K.	Bloodhound Sensors	BH114	14 x CP, DLC
USA	Cyrano Sciences	Cyranose 320	32 x composite polymer
Iceland	Element	FreshSense	MOS
Finland	Environics Industry Oy	MGD-1	IMCELL
USA	Estcal	4100 Vapor detector	6-15 x SAW
Germany	HKR Sensorsysteme	QMB6/HS40XL, HS40/MS	QCM, MS
Germany	Hewlett Packard	HP4440A	MS
Germany	Lennartz Electronic	MOSES II	8 x QCM, 8 x MOS (SnO_2)
U. K.	Marconi Applied Technologies	e-Nose 5000	CP, MOS, QCM
USA	Microsensor Systems	VaporLab	SAW
Germany	MoTech	VOCmeter, VOCcheck	QCM, MOS
Sweden	Nordic Sensor Technologies	NST 3210, NST 3220, NST 3220A	MOSFET, MOS
Belgium	OligoSense	Under development	CO
U. K.	Osmetech plc	MultiSampler-SP	CP
Germany	RST Rostock	Sam	QCM, SAW, MOS
Switzerland	Smart Nose	Smart Nose-300	MS

Source website http://nose.uia.ac.be/review/.
Key: Metal oxide semiconductors, MOS; Conducting Polymers, CP; Surface acoustic wave, SAW; Quartz crystal microbalance, QCM; Mass spectrometry, MS; Metal oxide semiconductor field effect, MOSFET; Discotic liquid crystal, DLC.

The sensors respond to molecules ranging in molecular weight from 30 – 300 Da (Reineccius, 1996). They give a strong response to molecules, such as alcohols, ketones, fatty acids and esters, and reduced response to fully oxidized species, such as CO_2, NO_2, and H_2O. They are also sensitive to molecules containing sulphur and amine groups.

Limitations and advantages: The major limitation is the strong sensitivity to humidity. Other issues regarding limitation includes stability, drift, ageing and calibration of the sensor array (Strike *et al.*, 1999). Despite these shortcomings, the potential advantages of the electronic nose as an analytical tool are numerous and include rapid and reproducible results, simple to use and no sample preparation. Once a protocol has been established the system does not require highly trained operators, and automation and on-line analysis may be possible.

1.8   PATTERN RECOGNITION TECHNIQUES

The sensor array response data requires processing and interpretation using pattern recognition (PARC) techniques. The first step of the PARC technique is the pre-processing of the sensor signals. This involves normalisation of the sensor response data, which effectively sets the sensor response values so that they all lie in the range of 0, to +1. This gives equal weighting to each sensor compensating for the differences in the magnitudes of the signals and reduces the effect of sample variation (Pearce, 1997). A range of PARC techniques can be then applied to the pre-processed data.

Principal component analysis (PCA): Principal component analysis is a linear pattern recognition technique used to summarise the pattern of correlation among observed variables (e.g. sensor responses) without compromising or losing data and information (Gardner, 1991). PCA reduces a large number of observed variables to a smaller number of factors (namely principal components), which are projected onto a plane. The technique is unsupervised which means that the different classes or groups are not defined and the technique finds any hidden relationship between samples. PCA is carried out for a particular set of sensor parameter such as divergence, for all 14 sensors. The first axis or principal component accounts for the largest degree of variation in the data. The second principal component, perpendicular to principal component 1, is drawn so that it accounts for the remaining variation of the data. Principal component 3 is drawn perpendicular to principal components 1 and 2, and so on. Principal components that account for at least 90% of the total variation are used (usually the first two principal components are sufficient) for the PCA plot, which shows how the samples relate to each other. A loading plot shows the degree of influence each variable in the data set has on each principal component, thereby revealing the variables, which contributes most of the information.

Discriminant analysis (DA): The selected parameter sensor data from the PCA are used in discriminant analysis. Linear discriminant analysis involves finding one or more linear combinations of the original variables that maximise the between-group differences relative to within–group differences. In this supervised technique the different classes are defined in the analysis.

Cluster analysis (CA): The between-group differences or the Mahalanobis's squared distance between groups from the discriminant analysis are used for cluster analysis to construct the dendrograms (Gardner, 1991). The greater the Mahalanobis's squared distance between groups, the greater the dissimilarity between groups.

1.9   APPLICATIONS OF ELECTRONIC NOSE TECHNOLOGY

At present electronic noses have been developed and applied primarily in three areas: medicine, environment and food industry. Table 1.4 summarizes some of the different applications of electronic noses. Electronic noses have been applied in other areas such as military defence (Newman, 1991) in the detection of chemical warfare gases (such as nerve and mustard gases). Other industry where the electronic nose has been applied includes tobacco; flavour and fragrances; packaging; petrochemicals; pharmaceuticals; water; power and healthcare.

Environmental analysis: Environmental applications include testing of ground water for odours, monitoring of toxic emissions such as dioxins (Staples, 1998), air quality and odour emission from treatment plants (Keller, 1999). A study by Stuetz *et al.* (1999) showed the application of an electronic nose system in monitoring odour emission and biochemical oxygen demand (BOD) in wastewater treatment works.

Table 1.4 Applications of electronic nose technology in the environmental, medical and food industries.

Applications	References
Classification of pathogenic bacteria	Gibson *et al.*, 1997; Gardner *et al.*, 1998; Pavlou *et al.*, 2000
Diagnosis of diabetes	Wang *et al.*, 1997
Evaluation of breath alcohol	Paulsson & Winquist, 1999
Detection of ketosis in dairy cattle from breath samples	Elliott-Martin *et al.*, 1997; Gardner *et al.*, 1999
Monitoring of packaging quality	Holmberg *et al.*, 1995; Forsgen *et al.*, 1999; Culter, 1999
On-line quality monitoring of bioprocess (fermentation)	Namdev *et al.*, 1998; Liden *et al.*, 1998; Bachinger *et al.*, 2000
Evaluation of odour emissions at waste treatment plants	Brockreis & Jager, 1999; Stuetz *et al.*, 1999
Monitoring agricultural malodour emissions	Persaud *et al.*, 1996a; Persaud *et al.*, 1996b; Persaud *et al.*, 1996c; Misselbrook *et al.*, 1997
Classification of gas emission from automotive leather	Kalman *et al.*, 2000
Fish freshness	Di Natale *et al.*, 1997; Olafsdottir *et al.*, 1997
Meat freshness	Winquist *et al.*, 1993; Funazaki *et al.*, 1995; Eklov *et al.*, 1998; Blixt & Borch, 1999
Classification of vegetable oils	Martin *et al.*, 1999
Italian vinegar classification	Anklam *et al.*, 1998
Cheese maturity	Schaller *et al.*, 1999
Tomato maturity and quality	Maul *et al.*, 1998; Sinesio *et al.*, 2000
Classification of strawberry varieties	Hirschfelder *et al.*, 1998
Fruit ripeness	Llobet *et al.*, 1999; Hines *et al.*, 1999
Evaluation of mycological quality of cereals	Stetter *et al.*, 1993; Borjesson *et al.*, 1996; Jonsson *et al.*, 1997; Borjesson & Olsson, 1998; Evans *et al.*, 1999; Evans *et al.*, 2000
Detection of off-odour in sugar	Kaipainen *et al.*, 1997
Classification of beer and off-taints	Pearce *et al.*, 1993; Tomlinson *et al.*, 1995
Authentication of wine	Di Natale *et al.*, 1996; Di Natale & D'Amico, 1998
Differentiation of coffee brands and roasting	Gardner *et al.*, 1992

Medical: There have been advances in the application of electronic nose technology in the field of medicine as a rapid diagnostic tool for diabetes (Wang *et al.*, 1997) and detection of pathogenic bacteria (Gibson *et al.*, 1997; Gardner *et al.*, 1998) such as *Helicobacter pylori* (Pavlou *et al.*, 2000). Applications also include analyses of biological samples such as urine from patients affected by kidney disease (Di Natale *et al.*, 1999) and differentiation of cerebrospinal fluid from serum (Thaler *et al.*, 2000) which can prove useful in clinical patient management.

Food and food processing: The majority of the current literature concentrates on usage of electronic nose technology in the food, and drinks industry, with an emphasis on rapid on-line, and on-site quality control. Applications range from classification and recognition of alcoholic and non-alcoholic beverages, to a large variety of foods.

The electronic nose has been applied to test the freshness of fish and meat. Classification as a function of the storage time can be correlated with freshness of food products. Di Natale *et al.* (1997) observed good classification of codfish during the first 3 days of storage. Studies on evaluating the maturity of cheeses and fruit ripeness are promising. Llobet *et al.* (1999) reported the use of the electronic nose in determining banana ripeness. The application area of quality control of raw materials includes sugar (Kaipainen *et al.*, 1997) and cereals. Electronic noses have the advantage of being able to discriminate between acceptable and unacceptable products that human sensory panels cannot assess for safety reasons (e.g. carcinogens, toxins and solvents). Although the electronic nose would not replace sensory panels and analytical techniques such as GC/MS, the technology is more likely to complement them.

Applications in the beverage industry include detection of taints in beer and authenticity of wine vintage. Pearce *et al.* (1993) showed discrimination of artificially tainted and untainted lager samples. Gardner *et al.* (1992) studied the differentiation of different coffee blends and roasts using the electronic nose. Better discrimination was achieved for different roasts than blends.

All the studies concerning microbial spoilage use the presence of bacterial and yeast volatiles. To date there is relatively little or no literature on detection of fungal volatiles using the electronic nose as all studies (Stetter *et al.*, 1993; Börjesson *et al.*, 1996; Jonsson *et al.*, 1997; Evans *et al.*, 2000) have focused on grain quality and classification in general. Evans *et al.* (2000) studied real-time analysis of volatiles produced grain of differing quality. Studies are required to evaluate electronic nose technology as a tool for early detection of spoilage fungi based on their volatile patterns. Jonsson *et al.* (1997) used the electronic nose to differentiate between various grain qualities. Similarly, Börjesson *et al.* (1996) showed a classification rate of 90% when two categories were used: good and bad grain quality. Jonsson *et al.* (1997) also showed that some relationship existed between volatiles and quantitative measures of fungal growth as the measured ergosterol and fungal CFU values correlated with artificial neural network predicted values of mouldiness.

1.10 OBJECTIVES

This study is the first attempt at using electronic nose technology for early detection and differentiation of spoilage moulds in food products. The main objectives of the project were, firstly, to determine the relationship between *in vitro* growth patterns of spoilage fungi under different environmental conditions and detection of volatiles using the electronic nose, and secondly, to compare mould activity using volatile patterns with other indicators of spoilage *in vitro*, and in model and real food substrates. The following studies were performed to address these objectives.

- Early detection and differentiation of fungal species *in vitro* using the electronic nose based on their volatile patterns prior to visible signs of growth.

- Correlation of growth rates with volatile patterns of spoilage fungi at different a_w levels.

- *In vitro* studies to examine the effect of a_w on volatile profiles produced by xerophilic fungi using the electronic nose, and to correlate this with quantitative enzyme analysis using p-nitrophenyl substrates.

- Differentiation of mycotoxigenic and non-mycotoxigenic fungi using their volatile patterns to examine any relationship between volatile and mycotoxin-production and make comparison with hydrolytic enzyme activities.

- A comparative study of volatile profiles and hydrolytic enzymes produced by non-toxic and mycotoxigenic strains of *Fusarium* spp. as an early indicator of fungal growth.

- Development of a bread analogue for use as a model food substrate.

- Examine the potential of early detection of volatile patterns produced by spoilage fungi colonizing bread analogue using the electronic nose and comparison with other indicators of mould spoilage.

- Study the effect of different inoculum concentrations on volatile patterns and correlate with fungal populations on bread analogues to determine the threshold limit of detection.

- Consider the effect of preservatives at different concentration in bread analogues on fungal volatile patterns and correlate with enzyme activity and fungal populations.

- Detection and correlation of volatile patterns produced by fungi on real bread substrate with other indicators of spoilage.

The flowchart in Figure 1.5, schematically outlines the different phases of work presented in this thesis.

Phase I: *In vitro* studies

Detection of spoilage fungi on wheatmeal agar (2%) using an electronic nose:

✓   Volatile patterns

Early detection and differentiation of fungal growth on wheatmeal agar at different water activities:

✓   Volatile patterns

✓   Specific enzyme activities

✓   Growth rate at different water activities

Phase II: Mycotoxigenic/non-mycotoxigenic fungi

Differentiation of mycotoxigenic fungi using electronic nose and correlation with enzyme activities:

✓   Volatile patterns

✓   Specific enzyme activities

Early detection and differentiation of mycotoxigenic and non-mycotoxigenic isolates of *Fusarium* spp.:

✓   Volatile profiles

✓   Specific enzyme activities

Phase III: Bread analogues

Early detection of fungal growth on bread analogue slices (modified to 0.95 a_w):

✓   Fungal volatile profiles

✓   Specific enzyme activities

✓   Fungal populations (CFUs)

Bread analogues inoculated with different inoculum concentrations of spoilage fungi to determine the minimum limit of detection using an electronic nose:

✓   Fungal volatile patterns

✓   Fungal populations

Phase IV: Preservatives

Effect of different concentrations of sorbate on fungal volatile patterns and comparison with other indicators of mould spoilage:

✓   Volatile patterns

✓   Specific enzyme activities

✓   Fungal populations

Effect of different concentrations of propionate on fungal volatile patterns and comparison with other indicators of spoilage:

✓   Volatile patterns

✓   Specific enzyme activities

✓   Fungal populations

Phase V: Real substrate

Detection of volatile patterns of spoilage fungi grown on bread equilibrated to 0.95a_w:

✓   Volatile patterns

✓   Specific enzyme activities and fungal populations

Figure 1.5 A flowchart outlining the different phases of work presented in this thesis.

Chapter 2

Materials and Methods

2.1 MATERIALS

Substrates: The cereal grains used were wheat, oats, maize, soya, sorgum, barley, linseed and oilseed rape. Campden & Chorleywood Food Research Association (CCFRA, Chipping Campden, Glos, U. K.) supplied the white wheat flour.

Fungal isolates and maintenance: The following fungal cultures were used (Plate 2.1): *Aspergillus flavus* Link; *A. ochraceus* Wilhelm; *A. niger* van Tieghem; *Wallemia sebi* (Fr.) von Arx; *Penicillium chrysogenum* Thom; *Eurotium amstelodami* Mangin; *E. rubrum* Mangin; *E. herbariorum* (wiggers) Link; *E. chevalieri* Mangin; *Fusarium moniliforme* Sheldon (isolates 6N, 25N, 150N, and 48N) and *Fusarium proliferatum* Nirenberg (isolates 20N, 112N, 58N, and 55N). With the exception of *P. chrysogenum*, all the fungal cultures are held in the culture collection of Food Technology Department, University of Lleida, Spain. *P. chrysogenum* was isolated from mouldy brown bread and identified. The fungal cultures were regularly maintained on 0.95 a_w 2% malt extract agar (MEA; modified using glycerol; Merck Ltd., U.K.; pH 5.8) media at 25°C.
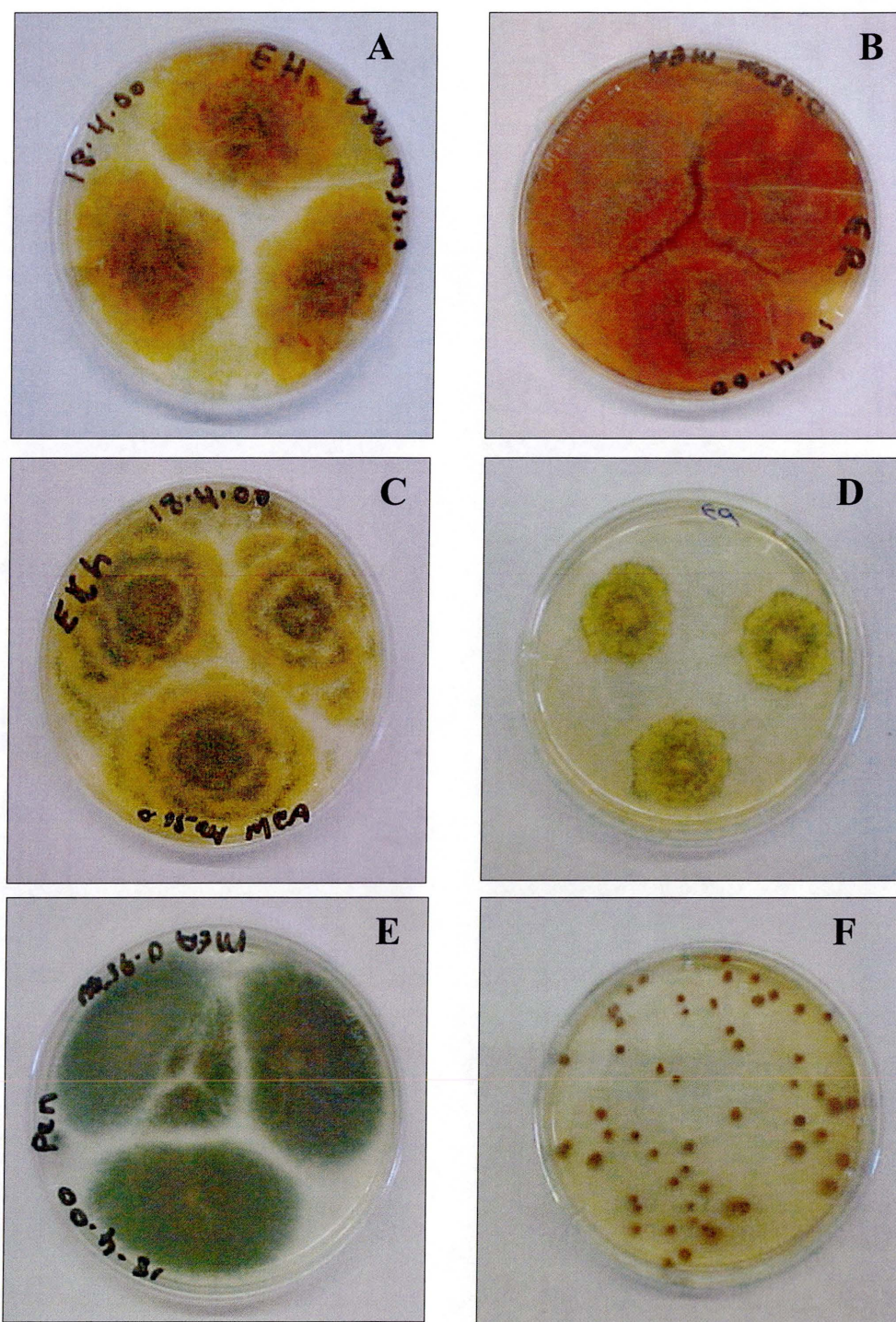
Plate 2.1 Six spoilage fungi grown on 2% malt extract agar modified to $0.95a_w$ and incubated at 25°C: (A) *Eurotium herbariorum*; (B) *E. rubrum*; (C) *E. chevalieri*; (D) *E. amstelodami*; (E) *Penicillium chrysogenum* and (F) *Wallemia sebi*.

2.2   MEDIA AND INOCULATION OF FUNGI FOR *IN VITRO* STUDIES

Wheatmeal (2%) agar (2%; WMA; Lab M No. 2) was prepared by the addition of homogenised dry wheat grain to agar and distilled water. The a_w of the medium was modified with the non-ionic solute glycerol (BDH, U.K.) (Table 2.1). The pH and a_w of the media were determined using a Microcomputer pH meter (HI 8424, Hanna Instruments Ltd, Leighton Buzzard, Bedfordshire. U.K.) and an Aqua Lab (Lab Cell, Basingstoke, Hants U.K.). The pH of the media was in the range of 5.9-6.0 irrespective of the a_w value.

Table 2.1. Amount of glycerol added to obtain the relevant water activity for wheatmeal agar media.

Water activity	g glycerol/100ml distilled water
0.95	23.0
0.90	41.3
0.85	65.0

Using a sterile loop, spores from the fungal culture were collected in a bottle containing sterile distilled water, agar and 0.01% tween 80. The bottles were vigorously shaken and the spore concentration (spores ml^{-1}) of each species measured, using a haemocytometer (Thoma, BDH, U.K.).

The WMA plates (90mm diameter) were inoculated with the spore suspension (0.2ml) by spread plating to obtain an even covering on the surface of the agar, using a surface sterilised bent Pasteur pipette. Plates were enclosed in polyethylene bags

and incubated at 25°C. Uninoculated agar media plates served as controls at each

sampling time.

2.2.1 Differentiation of fungi based on their fungal volatile profiles

WMA media (0.95 a_w) were inoculated with the following (approx. 10^6 spores ml^{-1}):

E. amstelodami; *E. chevalieri*; *E. rubrum*; *E. herbariorum*; *W. sebi* and *P.*

chrysogenum. The plates were incubated at 25°C for up to 72h. Four replicates of

each treatment were sampled after 24, 48, and 72h with the electronic nose as

described in Section 2.8.

2.2.2 Comparative study of hydrolytic enzyme activity, fungal volatile profile and growth rate at different a_w levels

The usage of electronic nose technology to differentiate between control and the

fungi at different a_w levels was studied. Wheatmeal agar media modified to three

different a_ws (0.85, 0.90 and 0.95 a_w) were inoculated with conidia of the following

fungi (approx. 10^6 spores ml^{-1}): *E. amstelodami*; *E. chevalieri*; *E. rubrum*; *E.*

herbariorum and *P. chrysogenum*. Twenty replicates per species and per a_w were

incubated at 25°C.

For 0.95 a_w, four replicate plates were sampled using the electronic nose after 48, 72,

and 96h and for 0.90 a_w the plates were sampled after 48, 72, 96, 120 and 144h

growth. For lower a_w levels of 0.85 the volatile measurements were made after 3, 5,

7, 11 and 14 days due to slower growth of the fungal species.

Discs of agar were removed from each treatment plate for enzyme extraction and enzyme activity determination as described in Section 2.5. The growth rate for each fungal species was determined at different a_w levels (Section 2.7).

2.2.3 Detection of mycotoxigenic and non-mycotoxigenic fungi based on enzyme activity and volatile profiles

The potential for discriminating between mycotoxigenic and non-mycotoxigenic species based on volatile profiles was determined. The toxigenic fungi used were *A. flavus*; *A. ochraceus* and *W. sebi*. For comparison *P. chrysogenum* and *A. niger* were used. WMA media (0.95 a_w) plates were inoculated with each of the above five fungi (approx. 10^6 spores ml^{-1}) and incubated at 25°C up to 120h. Five replicates of each fungal species were sampled destructively with the electronic nose after 72 and 120h incubation. Sub-samples of each replicate were taken for subsequent enzyme assays.

2.2.4 Study of different mycotoxigenic and non-mycotoxigenic strains of *Fusarium* spp. using an electronic nose and quantitative enzyme assays

The possibility of using volatile profiles to differentiate between strains of *Fusarium* spp. was investigated. Four strains of *F. moniliforme* (6N, non-producer; 25N, fumonisin producer; 150N, zearalenone and fumonisin producer; and 48N, trichothecene producer) and of *F. proliferatum* (20N, non-producer; 112N, fumonisin producer; 58N, trichothecene producer; and 55N, trichothecene and fumonisin producer) were used in this study. The mycotoxin profiles of the strains have been previously reported (Sala 1993; Sala *et al.* 1994).

WMA media (0.95a$_w$) were inoculated with spore suspension (approx. 10^6 spores ml^{-1}) of each strain and species (16 replicates per treatment) and incubated at 25°C for up to 96h. Volatile profiles of four replicates per strain were destructively sampled using the electronic nose after 48, 72 and 96h. Sub-samples from the replicates were used for enzyme analyses (Section 2.5).

2.3   PREPARATION AND INOCULATION OF BREAD ANALOGUES

Bread analogue: Bread dough was prepared by mixing together white wheat flour (200g); margarine (10g); salt (1g); yeast (1g) and sugar (1g) with water (126ml)/glycerol (14g) mixtures to obtain 0.95 a$_w$ (Patterson & Damoglou, 1986), placed in a baking tin lined with grease-proof paper, sealed with autoclave tape and sterilised for 15 minutes. When cooled, the bread analogue was sliced (approx. size and weight 2 x 2cm; 5g) with a sterile knife and placed in sterile Petri dishes (55mm diameter; BDH, U.K.) in the flow bench. Water activity and pH of the bread analogue were determined respectively with an Aqua Lab and pH meter.

(a) Bread analogue with 0.15% potassium sorbate: Bread analogue (0.95 a$_w$; pH 5.78) was prepared as described with the addition of 0.15% (total dry weight basis) potassium sorbate (0.32g; Sigma Chemical Co. U.K.).

(b) Bread analogue with 0.3% potassium sorbate: Bread analogue (0.95 a$_w$; pH 5.90) was prepared as described with the addition of 0.3% (total dry weight basis) potassium sorbate (0.64g).

(c) Bread analogue with 0.15% calcium propionate: Bread analogue (0.95 a_w; pH 5.77) was prepared as described with the addition of 0.15% (total dry weight basis) calcium propionate (0.32g; Aldrich Chemical Co. Inc., U.K.).

(d) Bread analogue with 0.3% calcium propionate: Bread analogue (0.95 a_w; pH 5.71) was prepared as described with the addition of 0.3% (total dry weight basis) calcium propionate (0.64g).

Inoculation of bread analogues: Spore suspensions of the fungi were prepared in glass Universal bottles (containing 0.95 a_w water/glycerol solution and 0.01% tween 80) and the concentrations checked using a haemocytometer, as described in Section 2.2. Aliquots (100μl) of the spore suspension were pipetted randomly onto each slice of the bread analogue contained in the Petri plate (Fustier *et al.*, 1998). Uninoculated bread analogue slices served as a control for each sampling time. Petri dishes containing the slices of bread analogue were placed in a humidity chamber (a surface-sterilised box containing 300ml of glycerol/water solution modified to 0.95 a_w) and incubated at 25°C.

2.3.1 Detection of spoilage fungi on bread analogues using their volatiles patterns, enzyme activity and CFUs

Slices of bread analogue (0.95 a_w; pH 5.75; no preservative) were inoculated with the following species as spore inoculum (approx. 10^6 spores ml^{-1}): *E. amstelodami*, *E. chevalieri* and *P. chrysogenum*. Uninoculated bread slices were used as controls at each sampling time. Twenty four replicates per treatment were incubated at 25°C for up to 72h. Samples were taken for an initial CFU count at time = 0 hr. Volatile

profiles of six replicates were taken using the electronic nose after 26, 40, 48 and 72h. Sub-samples of each replicate were taken for subsequent population assays (CFUs, 1g) and enzyme assays (2g) (Sections 2.5 and 2.6).

2.3.2 Effect of inoculum concentration on fungal volatile patterns and CFUs on bread analogues

Three spore concentrations (approx. 10^2, 10^4, 10^6 spores ml^{-1}) of *E. amstelodami* and *P. chrysogenum* were used in this investigation. Bread analogue slices (0.95 a$_w$; no preservative) were inoculated with the different spore concentrations of each spoilage species. Uninoculated bread analogues were used as a control for each sampling time. Twelve replicates per treatment were incubated at 25°C for up to 72h. Samples were taken for initial CFU count at time = 0 hr. After 24, 48 and 72h the volatile patterns of four replicates were measured using the electronic nose and samples of each replicate (1g) were taken for CFUs determination.

2.3.3 Effect of preservatives on fungal volatile profiles, enzymic activity and CFUs

(a) Potassium Sorbate: Bread analogues (0.95 a$_w$) containing (a) no preservatives; (b) 0.15% potassium sorbate and (c) 0.3% potassium sorbate were used in this study. Slices of bread analogues were inoculated with conidia (approx. 10^6 spores ml^{-1}) of *E. amstelodami*; *E. chevalieri* and *P. chrysogenum*. Uninoculated bread analogues were used as controls for each sampling time. Sixteen replicates per treatment were incubated at 25°C for up to 96h. Samples were taken for an initial CFU count at time = 0 hr. Four replicates per fungal species were destructively sampled with the

electronic nose after 24, 48, 72, and 96h incubation. Sub-samples from replicates were used for population assays (CFUs, 1g) and enzyme assays (2g).

(b) Calcium Propionate: The above study was repeated using calcium propionate instead of potassium sorbate. Bread analogues (0.95 a_w) contained (a) no preservatives; (b) 0.15% calcium propionate and (c) 0.3% calcium propionate. Slices of bread analogues were inoculated with conidia (approx. 10^6 spores ml^{-1}) of *E. amstelodami*; *E. chevalieri* and *P. chrysogenum*. Uninoculated bread analogues were used as controls for each sampling time. Sixteen replicates per treatment were incubated at 25°C for up to 72h. Samples were taken for an initial CFU count at time = 0 hr. Four replicates per fungal species were destructively sampled with the electronic nose after 24, 48 and 72h incubation. Sub-samples from replicates were used for CFUs determination (1g) and enzyme assay (2g).

2.4 STUDIES OF SPOILAGE FUNGI ON NATURAL BREAD SUBSTRATE

Natural bread substrate: White wheat flour (460g); sugar (13.48g); salt (6.50g); dried yeast (8.03g); margarine (13.70g) and distilled water (230ml) were placed in a home bakery kit (Prima) and set on the normal mode setting (see Appendix B). After 2h 40 mins the bread was removed from the bakery in the flow bench and sliced (crusts removed). The bread slices (approx. size and weight 2 x 2cm; 5g) were placed in sterile Petri plates and were allowed to equilibrate to 0.95 a_w for 24h in a humidity chamber. The a_w (0.951 a_w) and pH (pH 5.53) of the slices were measured. The bread

slices were surface sterilised by spraying the bread surface with IPA and allowed to dry.

The bread was inoculated with single cultures of *E. chevalieri, E amstelodami* and *P. chrysogenum* (approx. 10^6 spores ml^{-1}). Aliquots (100μl) of the spore suspension were pipetted randomly onto each slice of bread (Fustier *et al.*, 1998). Uninoculated bread slices served as control blanks at each sampling time. The Petri dishes containing the slices of bread were placed in the humidity chamber (twelve replicates per treatment) and incubated at 25°C for up to 72h. Samples were taken for an initial CFU count at time = 0 hr. Volatile profiles of four replicates were measured using the electronic nose after 24, 48 and 72h, and sub-samples were taken for CFUs determination (1g) and enzyme assays (2g).

2.5   QUANTIFICATION OF ENZYME PRODUCTION

2.5.1   Extraction of enzymes

For *in vitro* studies, three discs of agar (approx. weight 0.95g) were removed from the treatment plates using a cork borer (20mm diameter) and for bread studies 2g of bread were used. The agar discs/bread samples were placed in 4ml potassium phosphate extraction buffer (10mM; pH 7.2; BDH, U.K.). The bottles were shaken on a wrist action shaker (KS250 basic, IKA Labortechnik) for 1h at 4°C. The washings were decanted into 1ml plastic eppendorf tubes and centrifuged in a bench microfuge (13,000 r.p.m; Beckman Lite) for 15 minutes. The supernatant was removed and stored in aliquots at -20°C for total and specific enzyme activity determinations (Marin *et al.*, 1998).

2.5.2 Total enzyme activity determination

Seven hydrolytic enzyme activities were assayed using p-nitrophenyl substrates (Sigma Chemical Co., U.K.). Table 2.2 shows the substrate concentration and buffer used for each enzyme assay. Enzyme extract (40μl), substrate solution (40μl) and the appropriate acetate buffer (20μl; BDH, U.K.) were pipetted into the wells of the microtitre plate (Bibby Sterilin Ltd, Staffordshire, U.K.) and incubated at 37°C for 1h along with the appropriate controls. The control included substrate solution (40μl) and the appropriate buffer (20μl).

The reaction was stopped by the addition of 5μl 1M sodium carbonate solution (Sigma Chemical Co., U.K.) and left for 3 minutes. Enzyme extracts (40μl) were added to the control wells. The enzyme activity was measured, using a MRX multiscan plate reader (Dynex technologies Ltd., Billinghurst, West Sussex, U.K.; Revelation software version 4.21), by the increase in optical density at 405nm caused by the liberation of p-nitrophenol by enzymatic hydrolysis of the substrate. Total enzyme activity was calculated from the calibration curve of absorbance at 405nnm against p-nitrophenol concentration and expressed as μmol 4-nitrophenol released min^{-1} (see Appendix C).

Table 2.2 Summary of the enzyme assay, their substrates, concentrations, buffer and pH used.

Enzyme	Substrate	Substrate Concentration (mM)	Acetate Buffer/pH
β-D-fucosidase	4-nitrophenyl-β-D-fucopyranoside	2.0	25mM/5.0
α-D-galactosidase	4-nitrophenyl-α-D-galactopyranoside	4.0	25mM/5.0
β-D-glucosidase	4-nitrophenyl-β-D-glucopyranoside	2.0	25mM/5.0
α-D-mannosidase	4-nitrophenyl-α-D-mannopyranoside	4.0	25mM/5.0
β-D-xylosidase	4-nitrophenyl-β-D-xylopyranoside	2.0	25mM/5.0
N-acetyl-α-D-glucosaminidase	p-nitrophenyl-N-acetyl-α-D-glucosaminide	2.0	25mM/4.2
N-acetyl-β-D glucosaminidase	p-nitrophenyl-N-acetyl-β-D-glucosaminide	2.0	25mM/4.2

Calibration curves: Standard p-nitrophenol solutions (Spectrophotometric grade, Sigma Chemical Co. U.K.) of known concentrations were prepared using 25mM acetate buffer (pH 5.0 and pH 4.2). p-Nitrophenol solution (40µl), and the appropriate buffer (60µl) were pipetted into the wells of the microtitre plate and incubated at 37°C for 1h. Sodium carbonate solution 1M (5µl) was added and left for 3 minutes after which, absorbance at 405nm was measured. Calibration curves of absorbance at 405nm against p-nitrophenol concentration were plotted.

2.5.3 Specific enzyme activity determination

Protein concentration determination was carried out using a Bicinchoninic acid protein assay kit (BCA-1 kit; Sigma Chemical Co., U.K.). This kit consisted of bicinchoninic acid solution, copper (II) sulphate pentahydrate 4% solution and albumin standard (containing bovine serum albumin (BSA) at a concentration of 1.0 mgml^{-1}). Protein reduces alkaline Cu (II) to Cu (I), which forms a purple complex with bicinchoninic acid (a highly specific chromogenic reagent). The resultant absorbance at 550nm is directly proportional to the protein concentration.

The working reagent was obtained by the addition of 1 part copper (II) sulphate solution to 50 parts bicinchoninic acid solution. The reagent is stable for one day provided it is stored in a closed container at room temperature. Aliquots (10μl) of each standard or enzyme extracts were placed in the appropriate microtitre plate wells. Potassium phosphate extraction buffer 10mM pH 7.2 (10μl) was pipetted into the blank wells. The working reagent (200μl) was added to each well. After shaking, the plates were incubated at 37°C for 30 minutes. The plates were allowed to cool to room temperature (3 minutes) before measuring the absorbance at 550nm using the MRX multiscan plate reader. The protein concentrations in the enzyme extracts were obtained from the calibration curve of absorbance at 550nm against BSA concentration. These values were used to calculate the specific activity of the enzymes in nmol p-nitrophenol released min^{-1} μg^{-1} protein (see Appendix C).

2.5.4   Treatment of data

Analysis of variance (ANOVA) (Microsoft Excel add-in Analysis Toolpak) was used to compare the specific enzyme activity of treatments for each enzyme and at each sampling time.

2.6   COLONY FORMING UNITS

A bread sub-sample (1g) was placed in 10ml glycerol/water solution + 0.01% tween 80 (modified to 0.95 a_w), votexed and subsequently serially diluted to obtain the appropriate dilutions. Spore suspension (200μl) of each dilution was spread plated onto malt extract agar (2%) plates (modified to 0.95 a_w using glycerol) using a surface sterilised bent Pasteur pipette. Two replicates were set up for each treatment at each dilution and the plates were incubated at 25°C for up to 5 days. The number of colonies per plate was counted and expressed as CFU g^{-1} fresh weight (see Appendix D).

2.7   GROWTH RATE DETERMINATION

Different a_w levels: Wheatmeal agar media were modified to three different a_w levels (0.95, 0.90 and 0.85 a_w). Spore suspensions of the following were made: *E. amstelodami*; *E. chevalieri*; *E. rubrum*; *E. herbariorum* and *P. chrysogenum*. Instead of spread plating, the plates were point inoculated with spore suspension in the centre of each plate. Ten replicates per fungal species per a_w were incubated at 25°C. Growth rate measurements were determined by measuring the diameter of the colonies in two directions at right angles to each other, using a ruler.

2.8   SAMPLING OF VOLATILES

2.8.1   Electronic nose

Instrumentation: The Bloodhound BH114 electronic nose system (Bloodhound Sensors Ltd., Leeds, U.K.), controlled using computer software (Bloodhound data capture software version v1.0/0.14), which uses an array of 14 different conducting polymer sensors was used in this study. The array was predicted to give a good response over the range of volatiles that may be encountered (see Appendix E). The electronic nose was operated in the same constant temperature room (25°C) as the samples. Figure 2.1 illustrates the experimental set up used. Between each headspace sampling, the system utilises two calibration reference points: a sensor baseline, set by purified air headspace passed through activated charcoal filter unit and a control sample derived reference point (calibrant), using the headspace above deionised water. This corrects for sensor drift by subtracting the response to the calibrant from the actual response and confirms complete purging of the sensor between each headspace sampling.
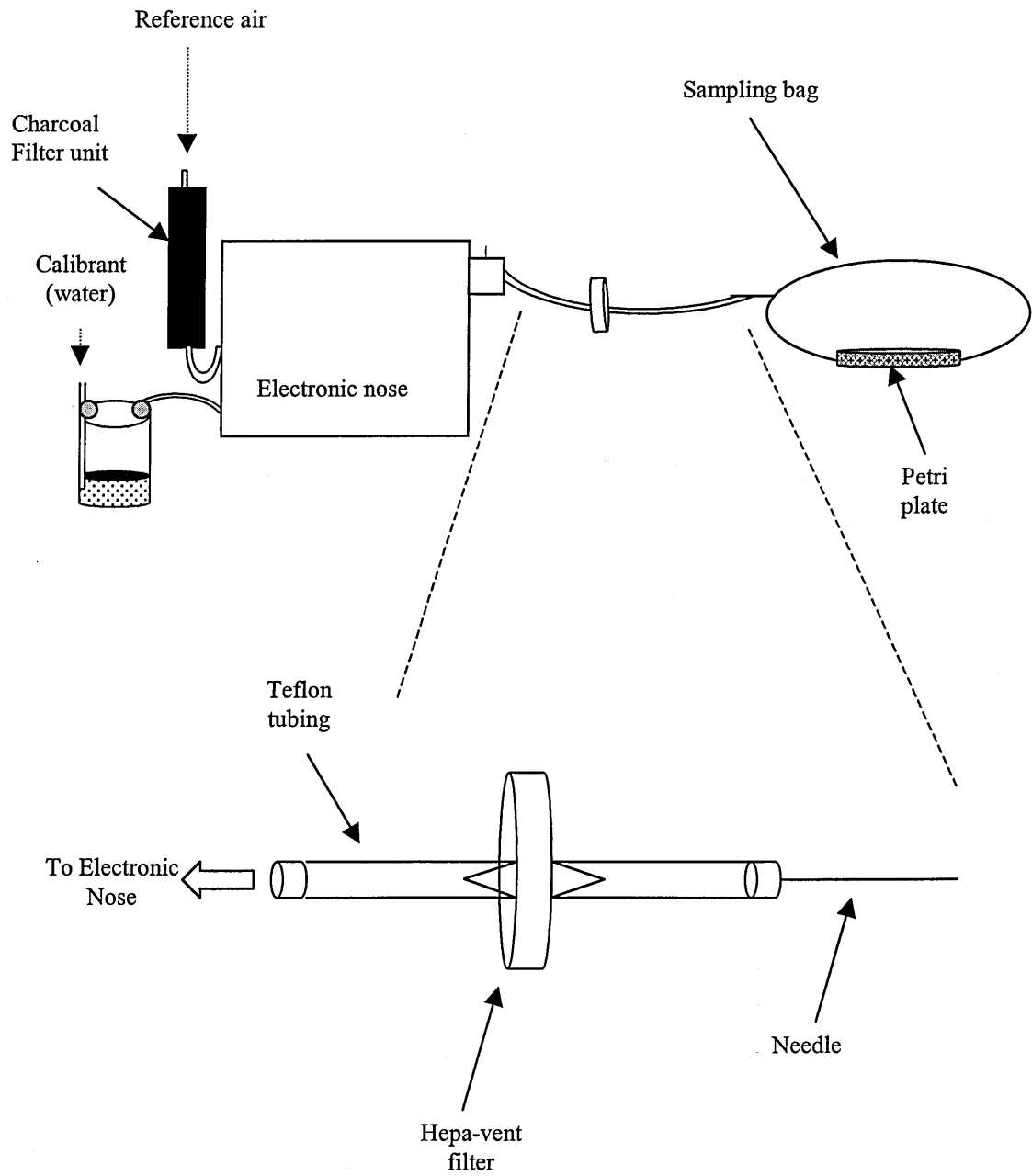
Figure 2.1 A schematic diagram of the experimental set-up of the electronic nose and the sampling device used.

Settings: The system used the differential sample mode and the sampling profile settings were as shown in Table 2.3. This mode of sampling reduces the effect of water from aqueous samples and helps to reduce sensor drift.

Table 2.3 Bloodhound BH114 electronic nose profile settings.

Settings	Time (Seconds)
Time delay	5
Injection	9
Desorption	5
Baseline Length	5
Hold Time	0

The sample headspace is introduced into the sensor array for 9 seconds (injection step). After this time the sensor array was purged for 5 seconds (desorption step) and the baseline monitored for a further 5 seconds. Between disconnecting the scanned sample and attaching the next, the pump was operated for 5 seconds in a faster mode to flush the sensors. A time delay of 5 seconds is used between completion of one headspace sampling and commencement of the calibration routine for the next.

2.8.2   Collection and sampling of fungal volatiles

Single Petri plate cultures or bread sample slices were placed in sampling bags (500ml capacity, size 152 x 229mm; BDH, U.K.), with the lid carefully removed, and filled with 300ml of filter-sterilised air and sealed. The bags were allowed to equilibrate at 25°C for 1h (for *in vitro* studies) or 2h (for bread analogue studies). The headspace from each bag was subsequently sampled in a 25°C constant

temperature room using the electronic nose system via a needle/filter apparatus (Figure 2.1).

The hepa-vent filter ($0.3\mu m$, Whatman international Ltd., Kent, U.K.) prevented fungal spores from entering the system and damaging the sensor array. The Teflon tube joints and connections were made airtight with parafilm.

Each study performed was of a randomised block design. Each block consisted of six to eight samples, one replicate of each sample type. Within each block, the sample order was randomised. Random sampling of headspace in a constant temperature room ensures effects such as drift of the sensor response, did not influence the discrimination.

Reference sample (10ml) of 2% butan-2-ol (Sigma Chemical Co., U.K.) was prepared. The lid of the Universal bottle had an air inlet and outlet. To the inlet was fixed a length of teflon tubing which passed into the butanol sample. As a result, when air was drawn through the vessel during sampling, the material was sparged to aid sampling. Reference butanol headspace was taken after every five actual headspace samples (i.e. fungal cultures or the control blanks) at 25°C.

2.8.3 Data analysis and treatment

The principle of conducting polymer sensors is that the adsorption of volatiles by a sensor causes a change in that sensor's electrical conductance. Therefore, the data recorded for each sample consisted of the response curves (change in conductance) of each of the 14 sensors over time. Figure 2.2 illustrates a typical sensor response curve. The raw data is processed and normalised (which removes baseline drift) by the Bloodhound software (version v1.0/0.14) before being exported into Microsoft Excel. Five parameters are measured from each of the 14 response curves (Ridgway *et al.*, 1999).
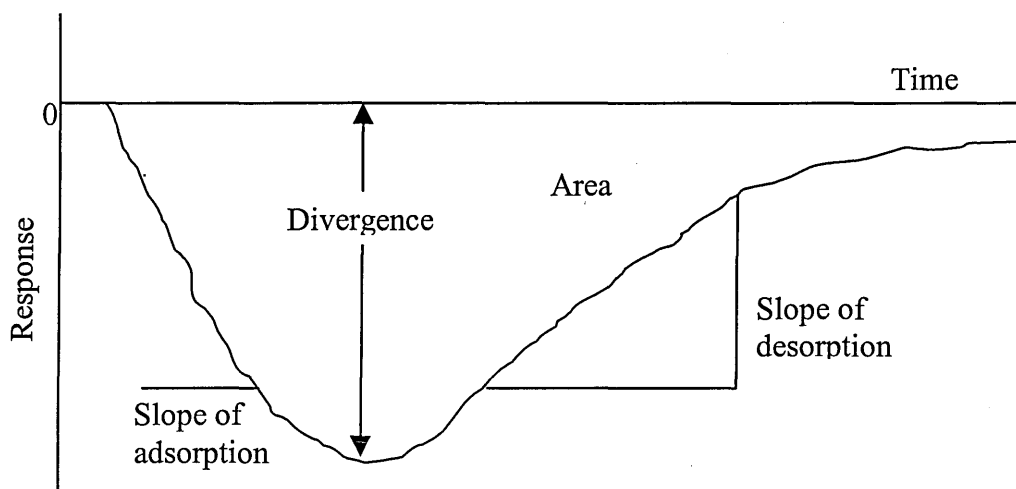
Figure 2.2 A typical sensor response curve showing the measured parameters.

♦ Divergence (div) – The maximum deflection from baseline.

♦ Absorption (abs) - Rate of increase in response on injecting sample headspace.

♦ Desorption (des) - Rate of decrease in response on purging.

♦ Ratio - Ratio of absorbance to desorbance.

♦ Area - Area under the response curve.

Normalised divergence and adsorption data for the 14 sensors were analysed using the program XLSTAT (Microsoft Excel add-in, version 3.4). Principal component analysis (PCA), discriminant analysis (DA) and cluster analysis (CA) techniques were applied to differentiate and classify the fungal species. Principal component analysis was carried out initially using all divergence and adsorption data. The sensor responses giving the most significant correlations were used in the PCA. For cluster analysis, the Mahalanobis's squared distances between groups (from the discriminant analysis) were used to construct the dendrograms.

Chapter 3

Results

3.1   DEVELOPMENT OF THE SAMPLING METHOD

3.1.1   Development of the system for collection of volatiles

The fungal cultures were placed in bags and filled with clean filtered air and sealed. The headspace inside the bag was allowed to equilibrate before being sampled by the electronic nose. The bag system had the main advantage of being disposable and economical, so there was no chance of cross-contamination between headspace samples. Furthermore, there was no density effect in the headspace as the air-filled bags were reasonably spherical. Some diffusion from the polythene bags may be possible but this would be negligible.

The bag system was further refined to ensure consistent and comparable sampling. The sample preparation included using a fixed volume of clean filtered air in each bag, which gave a constant headspace volume. The samples were analysed in set batches to ensure consistent equilibration time.

3.1.2   Electronic nose sampling

The Bloodhound electronic nose was left switched on in the morning to allow the sensor array to stabilize before the volatile measurements were made over the period of the afternoon. The headspace was sampled by the electronic nose via Teflon tubing of fixed length, attached to a filter and a needle. The filter prevented any fungal spores from entering the array but did not affect the volatile input. Care was taken to ensure that there was no carryover effect of previous headspace sample and the sensor array returned to the baseline. An interval of two minutes was allowed between each headspace sampling. Headspace of butan-2-ol solution (2%) was

sampled at regular intervals during each study to again ensure no carryover effect. Appendix F shows an example of the differentiation of butan-2-ol from the blank control and fungal species. Random sampling of the fungal cultures and the blank controls ascertained that effects such as sensor drift did not influence the differentiation.

Initially the sensor array used in this project consisted of 14 different types of sensors (See Appendix E). After the initial *in vitro* (see Section 3.2) study the array response significantly deteriorated. Bloodhound Sensors Ltd. replaced the array. The new array consisted of 14 sensors, however, six of the seven sensors were duplicates. To avoid redundancy and a reduction in discrimination, the duplicate sensors were not used in data analysis.

For each study, uninoculated agar plates or bread analogues were used as controls. The sensor array gave some responses to the control. However, this was regarded as a low background level, provided it was taken into consideration for each study.

3.2 DETECTION OF FUNGAL VOLATILE PROFILES AND DISCRIMINATION BETWEEN SPECIES

Initial *in vitro* studies showed that it was difficult to separate out the different species after only 24h growth on 2% wheatmeal agar media (using a spore lawn inoculum). However, after 48h, good reproducibility was obtained between replicates of the same species as shown for *P. chrysogenum* (Figure 3.1). Sensor drift was small over the sampling period.

Normalised data were used in principal component analysis in order to try to differentiate between fungal species and the milled wheat agar controls. This showed that good separation was possible for all six fungal species and the control (Figure 3.2). Figure 3.2B (an expanded scale of A) shows the discrimination of the four *Eurotium* species. These related *Eurotium* spp. clusters are closer together than that for *Wallemia sebi* and *P. chrysogenum*.

From the cluster analyses, it can be seen that four fungal species could be differentiated after 48h, prior to any visible growth (Figure 3.3), while *E. amstelodami* and *E. herbariorum* could not be discriminated.
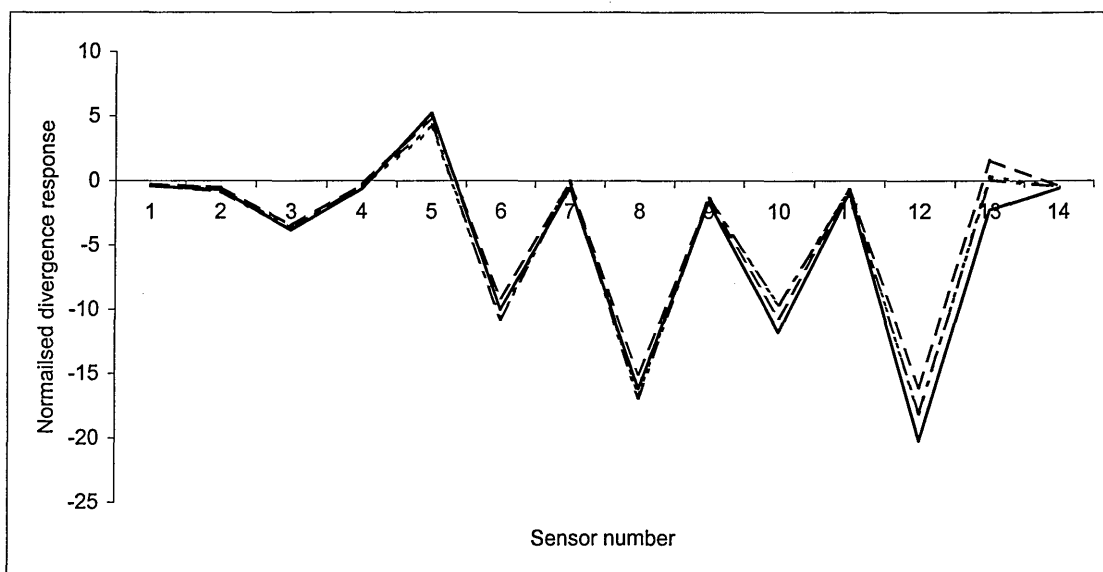
Figure 3.1 Normalised divergence responses of 14 sensors to volatiles produced by four replicates of *P. chrysogenum*.
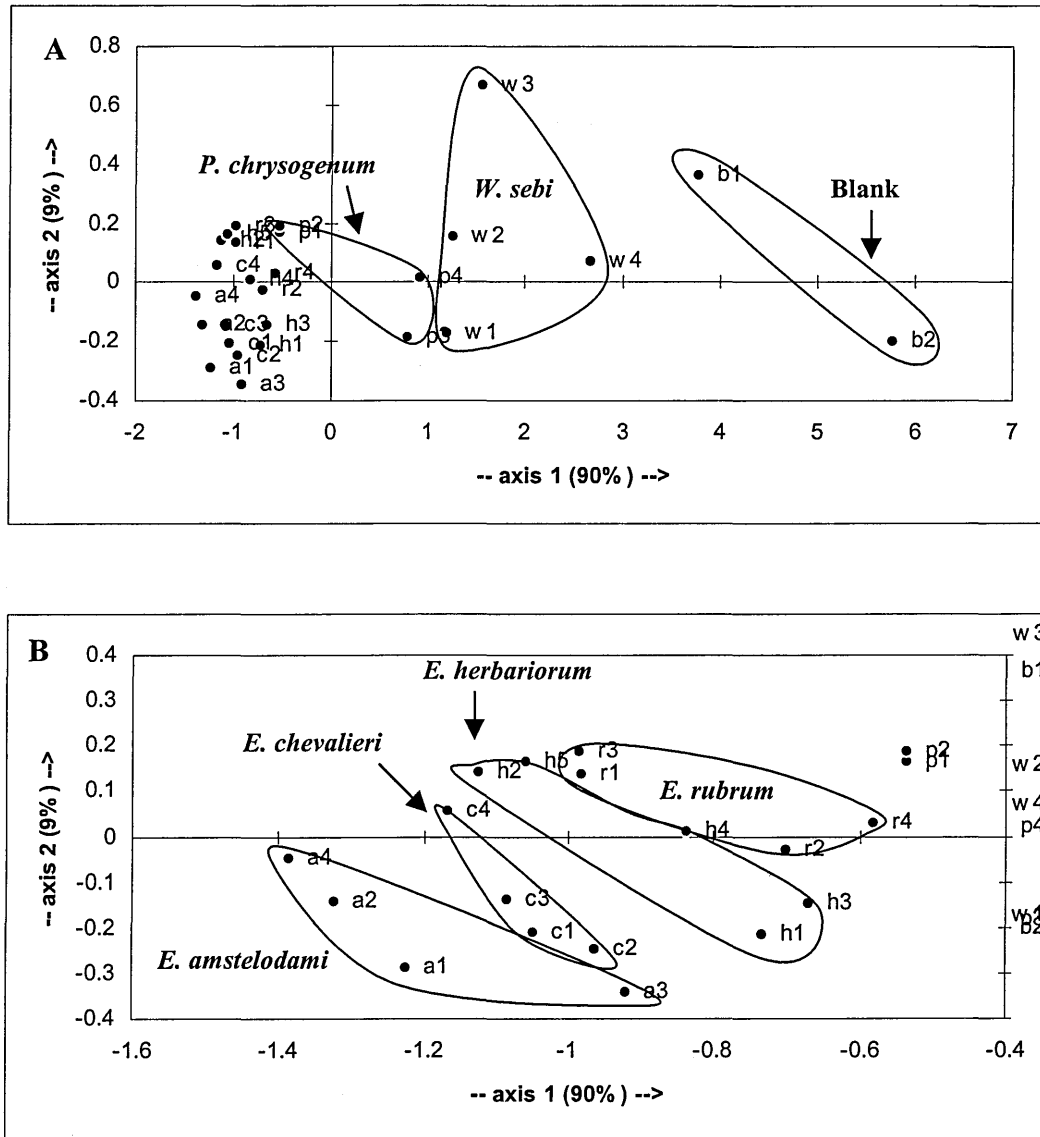
Figure 3.2 Principal component analysis of the response of the sensor array to volatiles from six fungi after 48h incubation. B is the same as A but is an expanded scale. Key to treatments: a, *Eurotium amstelodami*; c, *E. chevalieri*; r, *E. rubrum*; h, *E. herbariorum*; w, *Wallemia sebi*; p, *Penicillium chrysogenum* and b, control blank agar plates.
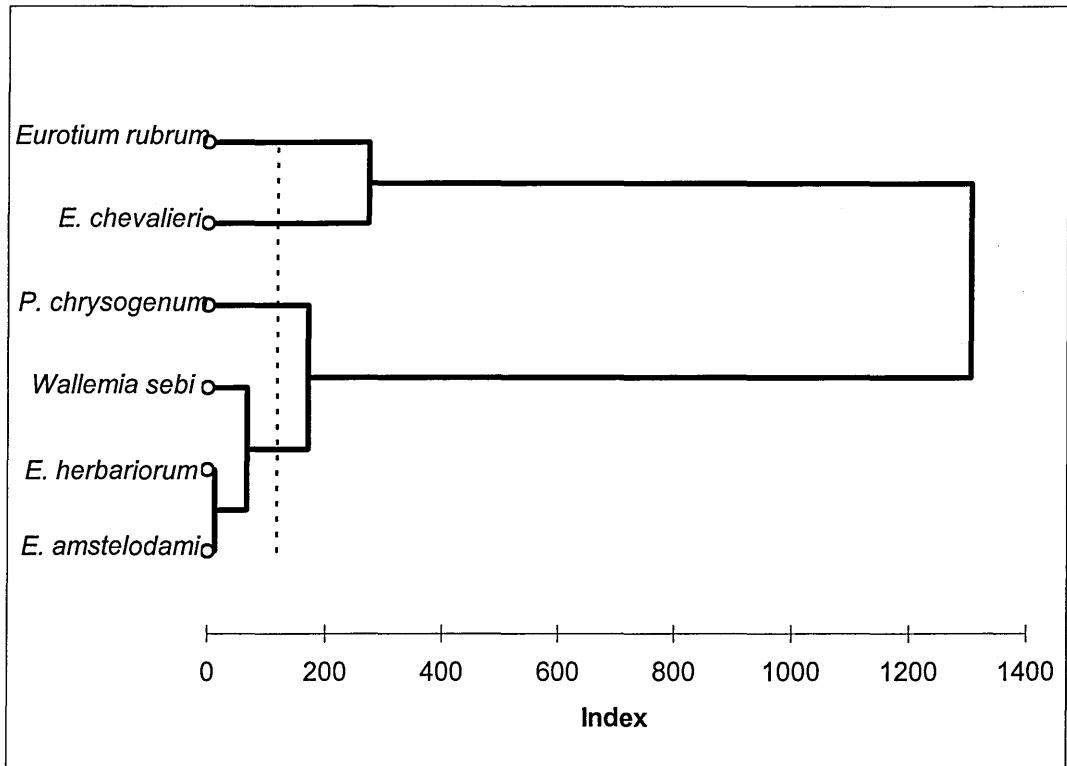
Figure 3.3 Cluster analysis showing classification of six fungi based on the response of the sensor array to their volatiles after 48h incubation.

3.3   *IN VITRO* STUDY OF SPOILAGE FUNGAL VOLATILE PATTERNS, ENZYME ACTIVITIES AND GROWTH RATES AT DIFFERENT WATER ACTIVITIES

3.3.1   Fungal volatile profiles

0.95 a_w: Figure 3.4 shows normalised divergence sensor response to volatiles produced by four replicates of *E. chevalieri*, grown as a spore lawn on milled wheatmeal agar modified to 0.95 a_w. The sensor array was found to show good reproducibility to the fungal volatiles.

Principal component analyses of the sensor response data were performed and 99, 97, 98% of the information were used at 48, 72 and 96h incubation, respectively. Figure 3.5 shows the analysis after 48h growth. Good differentiation was observed between the control and the five fungal species prior to any visible sign of fungal growth. However, the control blank and the *E. amstelodami* clusters were close to one another. The dendrogram (Figure 3.6) obtained from the cluster analyses demonstrated three separate groups: *E. herbariorum* alone in one group, *E. rubrum* and *Penicillium chrysogenum* in a second, and the remainder in a third group. The separation improved after 72h incubation for *P. chrysogenum* and the control (Figure 3.7). The cluster analyses (Figure 3.8) showed that it was possible to separate the control from the spoilage fungi. Furthermore, *P. chrysogenum*, *E. herbariorum* and *E. amstelodami* (Figure 3.8B) could be discriminated if the control blank was excluded from the analyses. *E. rubrum* and *E. chevalieri* could not be separated in the analysis.
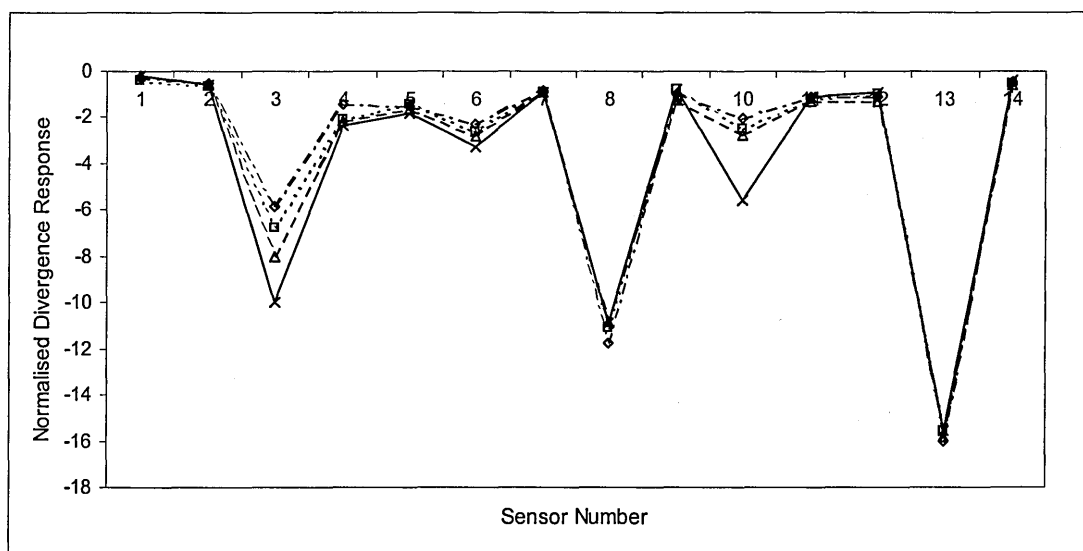
Figure 3.4 Normalised divergence response of sensor array to volatiles produced by four replicates of *E. chevalieri* after 48h growth on 2% wheatmeal agar at 0.95 a_w.
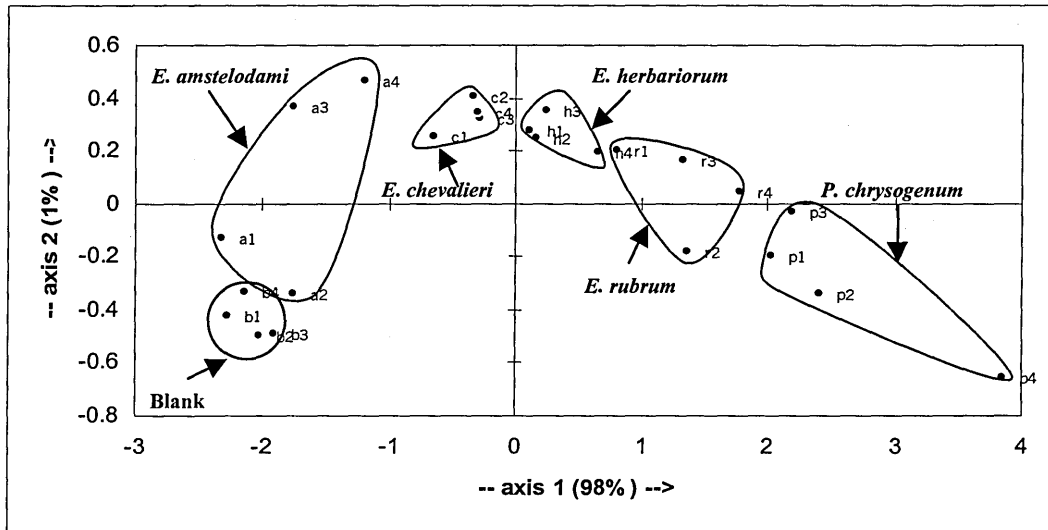
Figure 3.5 Principal component analysis of volatiles produced by fungi and control blank after 48h growth at 0.95 a_w. Data on axes 1 and 2 are 99%. Key to treatments: b, control blank agar plates; a, *Eurotium. amstelodami*; c, *E. chevalieri*; h, *E. herbariorum*; r, *E. rubrum* and p, *Penicillium chrysogenum*.
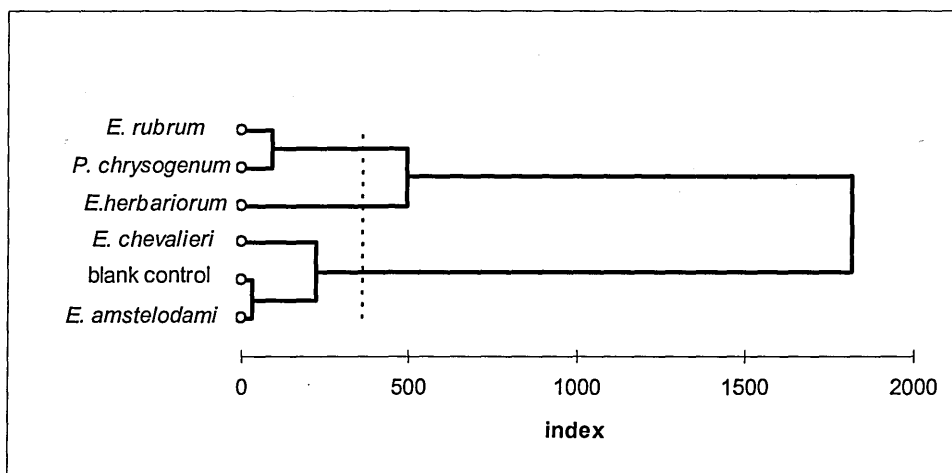
Figure 3.6 Cluster analysis of sensor responses to volatiles produced by blank control and five spoilage fungi on 2% WMA after 48h incubation at 0.95 a_w.
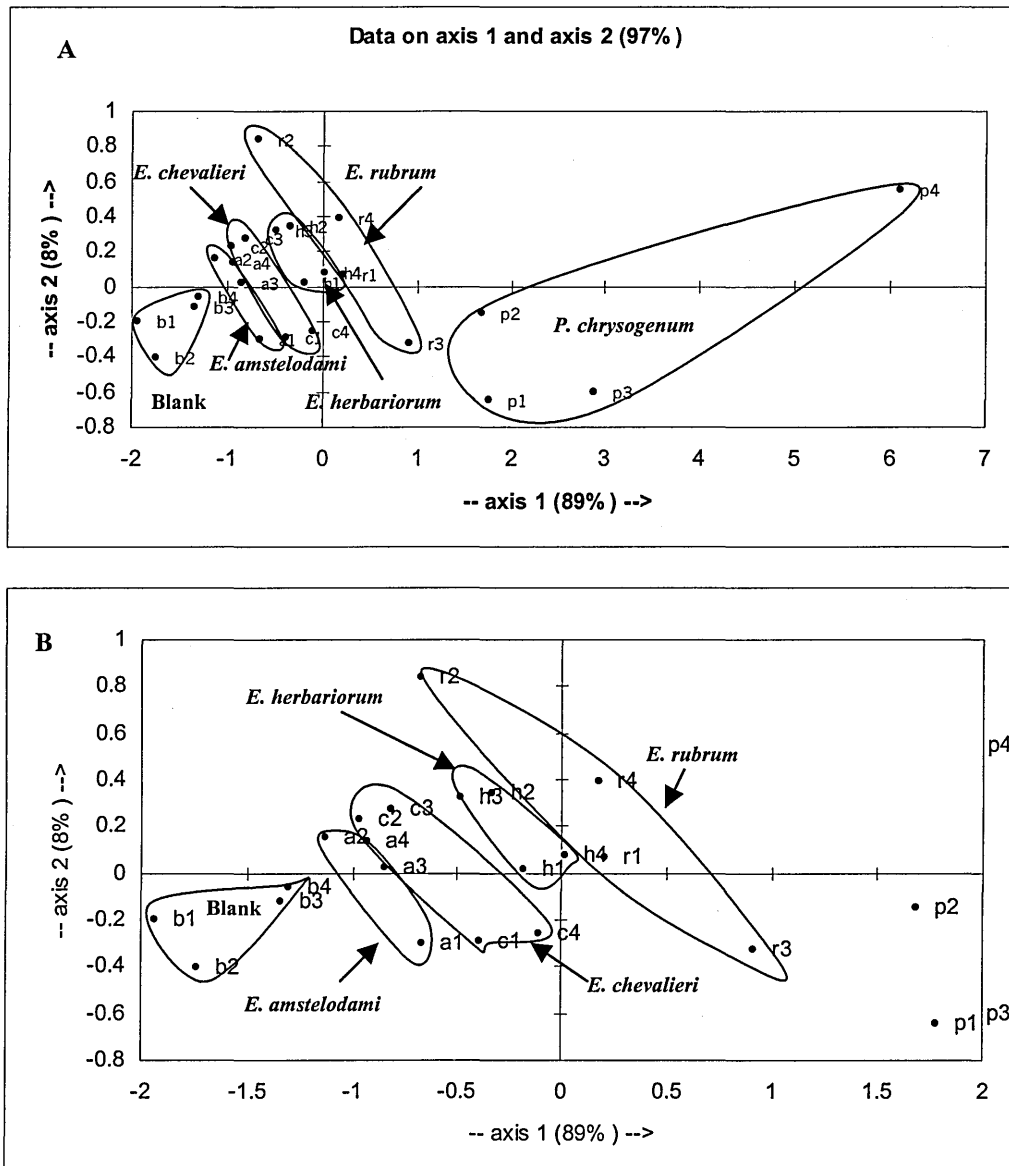
Figure 3.7 PCA of volatile profiles of fungi and control blank after 72h growth at 0.95 a_w. B is an enlarged scale of A. Key to treatments: b, blank control; a, *Eurotium amstelodami*; c, *E. chevalieri*; h, *E. herbariorum*; r, *E. rubrum*; and p, *Penicillium chrysogenum*.
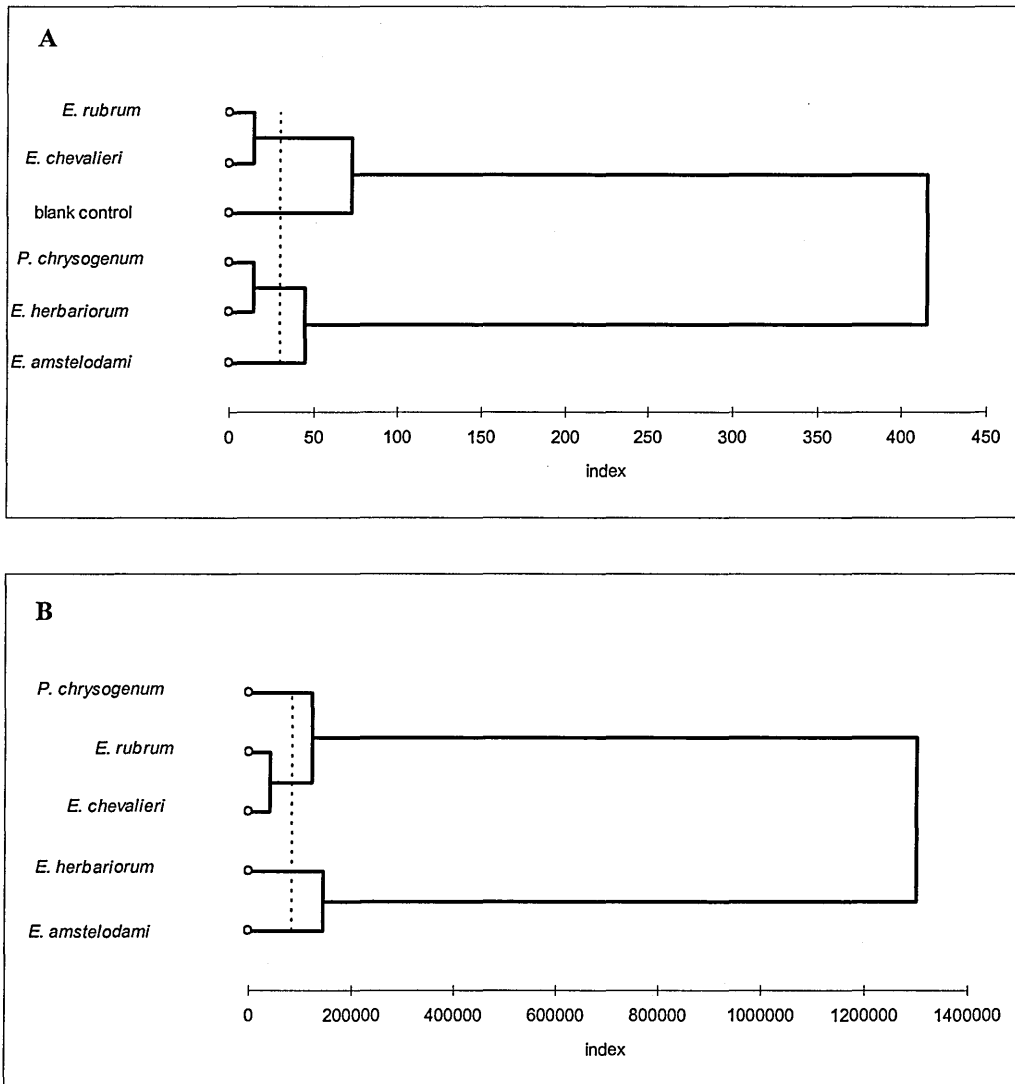
Figure 3.8 Cluster analysis showing separation of control blank and three fungal species after 72h growth at 0.95 a$_w$. B is cluster analysis excluding control blank.
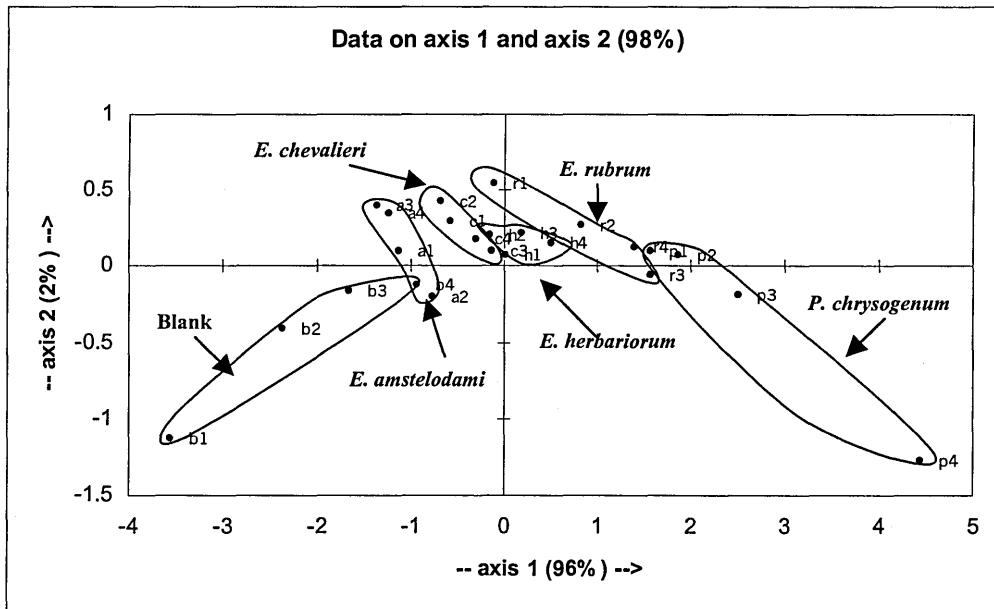
Figure 3.9 PCA showing differentiation of fungi and control after 96h incubation at 0.95 a_w. Key to treatments: b, blank control; a, *Eurotium amstelodami*; c, *E. chevalieri*; h, *E. herbariorum*; r, *E. rubrum*; and p, *Penicillium chrysogenum*.

After 96h growth, the control blank and the five fungal species could be still separated however, drift was also observed in the PCA (Figure 3.9).

0.90 a_w: Good reproducibility of the sensor array was achieved to volatiles produced by *E. chevalieri* after 48h incubation (Figure 3.10). The PCA analyses (Figure 3.11A) shows good separation was possible for the control and the xerophilic fungi at 0.90 a_w after 48h, prior to microscopic sign of growth. Some sensor drift was observed for sensor data at 120h incubation, however it was still possible to discriminate between the control blank and the species at visible growth (Figure 3.11B). After 144h growth, all the species along with the control blank could be differentiated as shown in Figure 3.11C. From the cluster analyses (Figure 3.12A) it can be seen that the control blank is separate from the fungal species. Again excluding the control blank from the analyses (Figure 3.12B) resulted in differentiation *of E. rubrum* and *E. herbariorum* after 48h and, *E. chevalieri* (Figure 3.12C) after 72h when signs of microscopic growth were observed.

0.85 a_w: At lower a_w, the sensor array was not as reproducible as seen in Figure 3.13. The principal component analyses of the sensor response data after 3, 5 and 7 days growth are shown in Figure 3.14. Figure 3.14A illustrates four separate clusters and the overlapping of *E. amstelodami* and *E. herbariorum*. It was possible to differentiate the control blank from the PCA, however, the measurements taken after 5 and 7 days show the replicates within the clusters to be spread out.
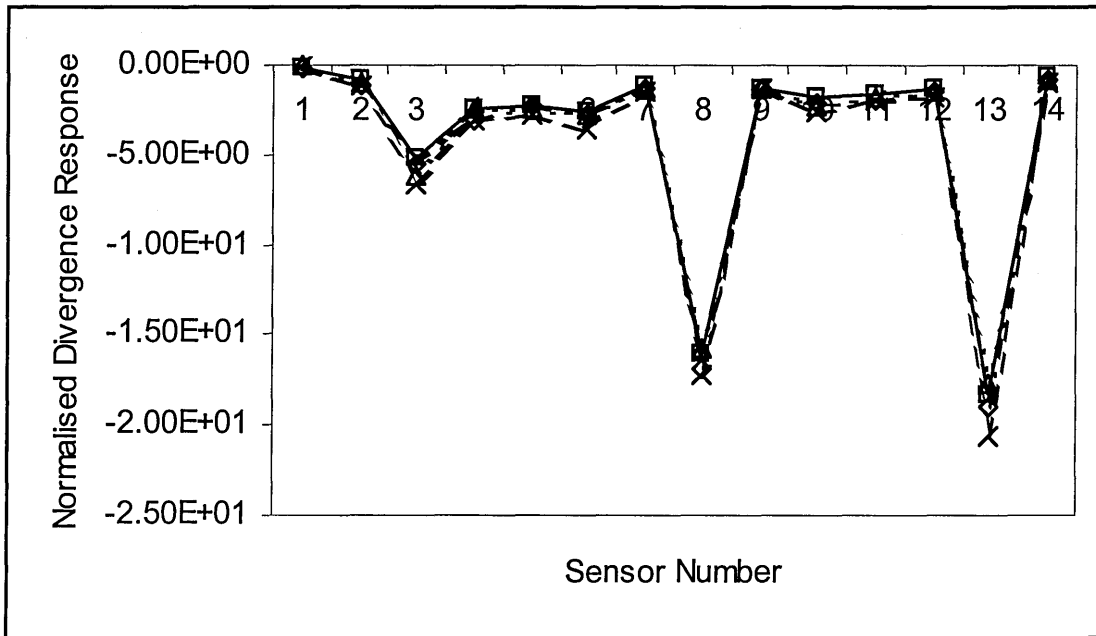
Figure 3.10 Normalised divergence response of the array to volatile production by four replicates of *E. chevalieri* after 48h at 0.90 a_w.
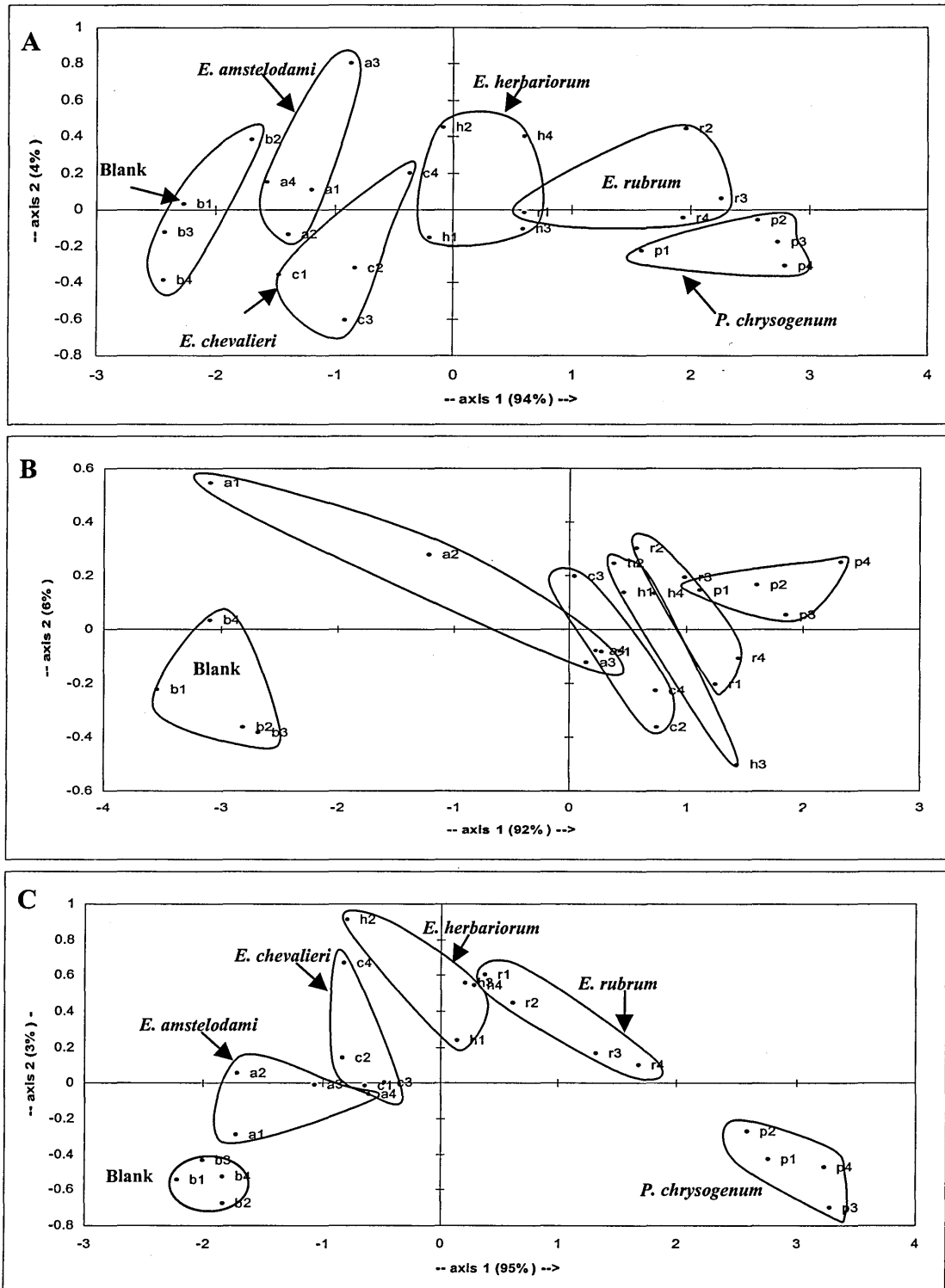
Figure 3.11 Principal component analysis of sensor response to volatiles produced by fungi and control blank at 0.90 a_w after (A) 48h, (B) 120h and (C) 144h growth. Key to treatments: b, control blank agar plates; a, *Eurotium amstelodami*; c, *E. chevalieri*; h, *E. herbariorum*; r, *E. rubrum* and p, *Penicillium chrysogenum*.
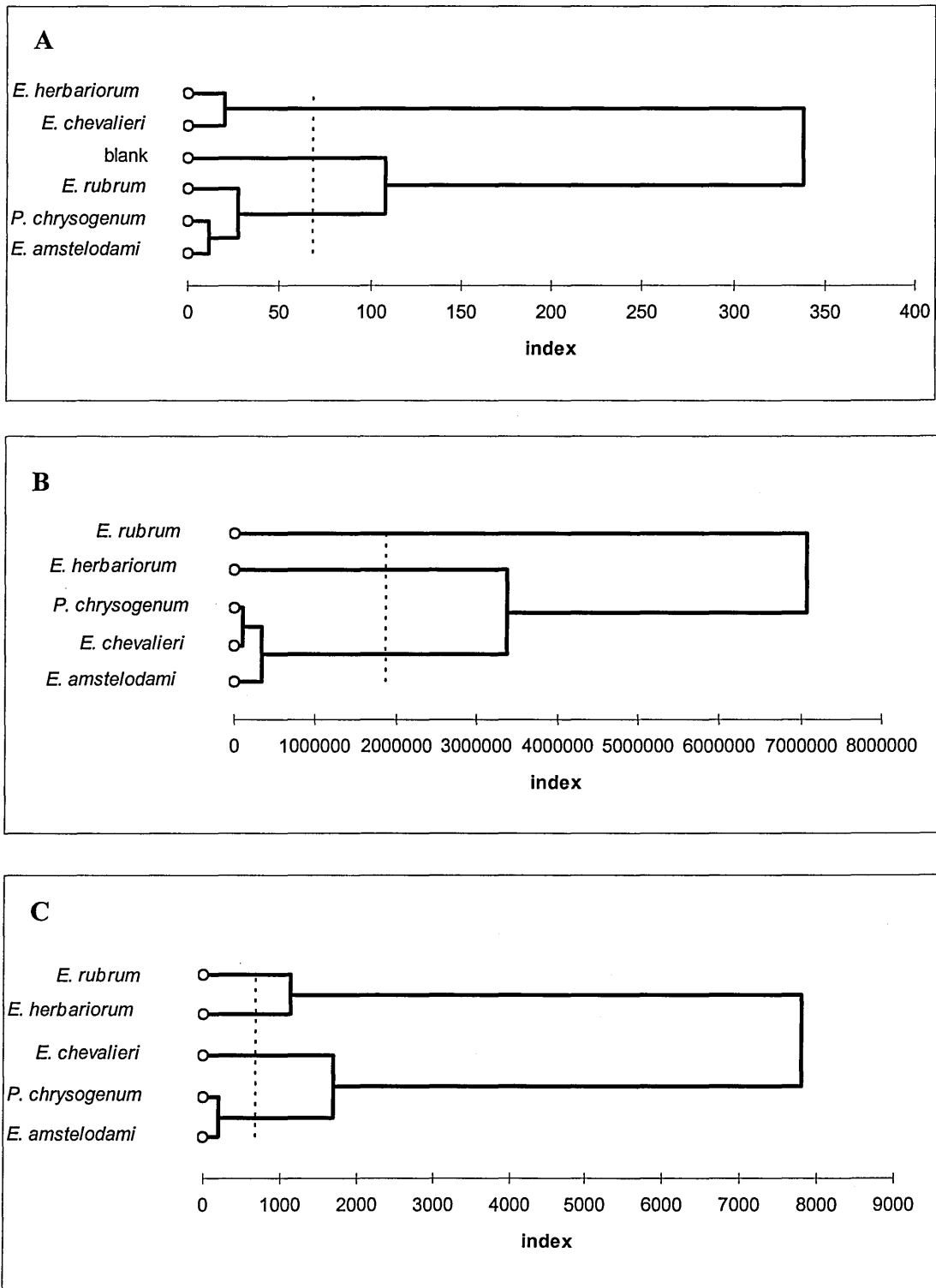
Figure 3.12 Cluster analysis of sensor responses to volatiles produced by blank control and five fungal species after (A) 48h, (B) 48h excluding blank control, (C) 72h growth at 0.90 a_w.
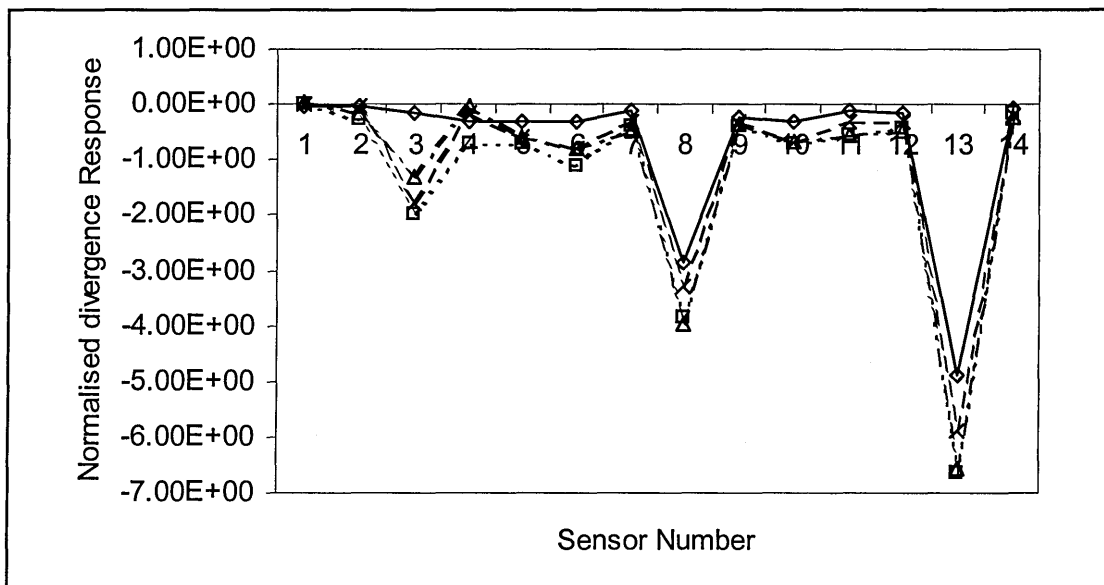
Figure 3.13 Normalised divergence response of the array to volatiles produced by four replicates of *E. chevalieri* after 72h at 0.85a$_w$.
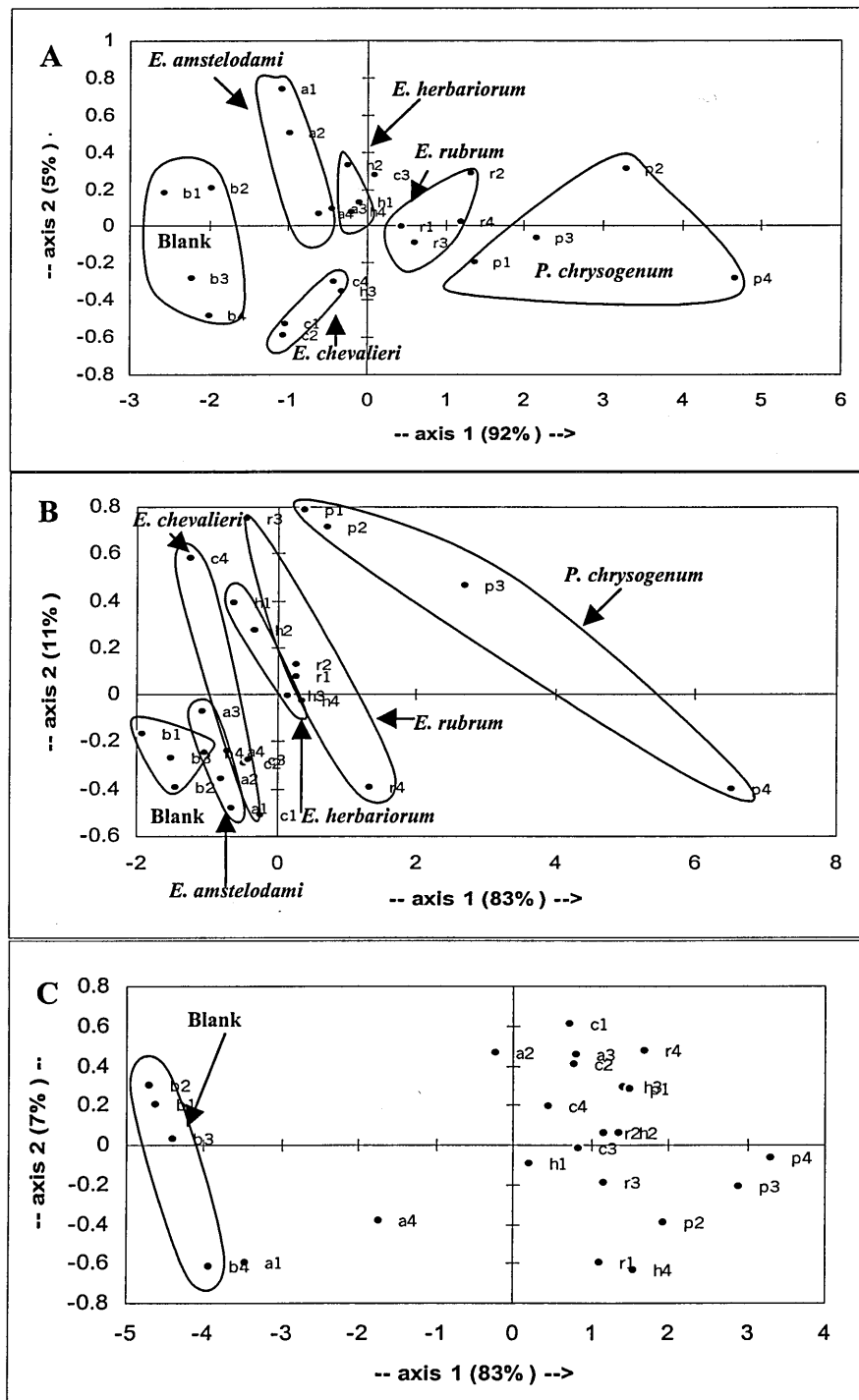
Figure 3.14 PCA of sensor response to volatiles produced by fungi and control blank at 0.85 a_w after (A) 3, (B) 5 and (C) 7 days growth. Key to treatments: b, control blank plates; a, *E. amstelodami*; c, *E. chevalieri*; h, *E. herbariorum*; r, *E. rubrum* and p, *P. chrysogenum*.
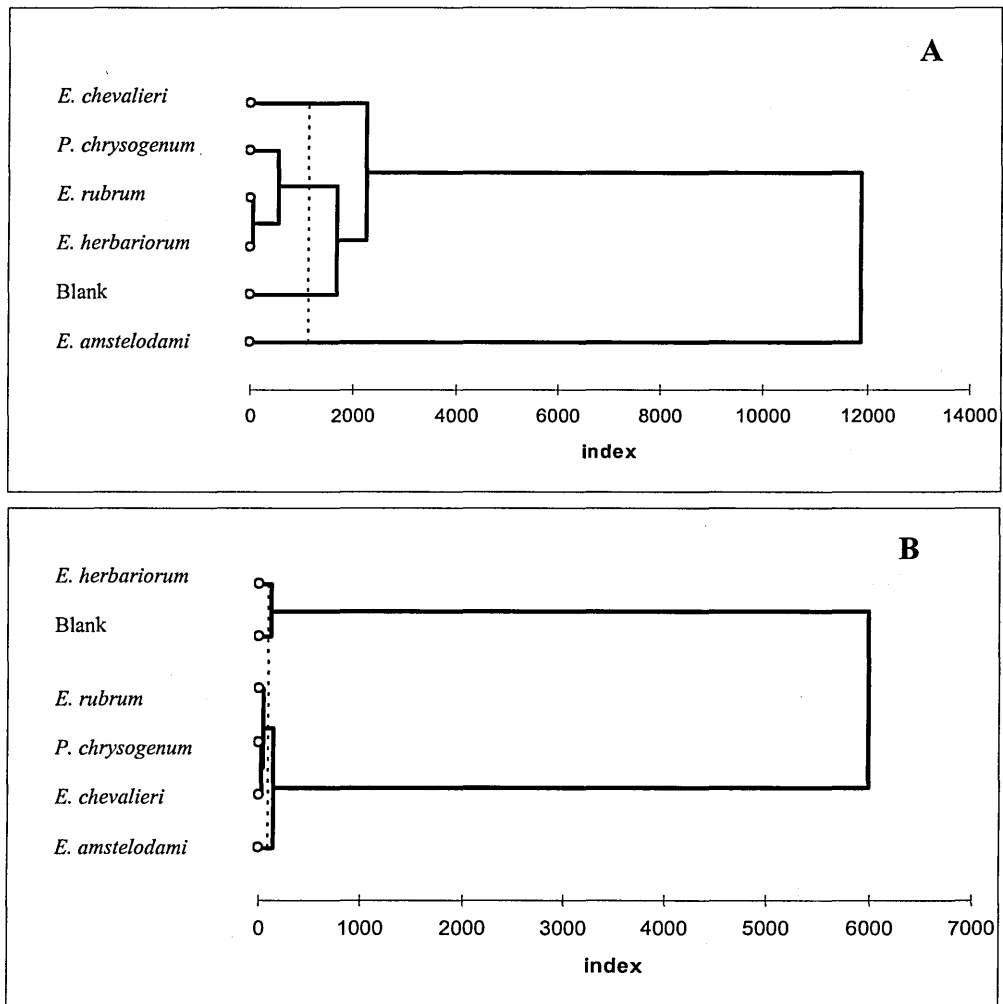
Figure 3.15 Cluster analysis of sensor array response to volatile production of five spoilage fungi and blank control after (A) 5 and (B) 7 days incubation at 0.85 a_w.

The cluster analyses of the data (Figure 3.15) showed discrimination of four groups after 5 days growth with the control blank, *E. amstelodami* and *E. chevalieri* being in separate groups. After 7 days incubation, it was possible to differentiate the control blank, *E. herbariorum* and *E. amstelodami*.

Figure 3.16 shows the PCA for volatile patterns produced by the spoilage fungi and blank control plates after 72h growth at three a_w levels. The clusters for the control blank and the fungal species were found to overlap.

3.3.2 Specific enzyme activity

p-nitrophenol calibration graphs: Calibration graphs of mean absorbance at 405nm against concentration of p-nitrophenol (at pH 5.0 and 4.2) are shown in Figure 3.17. Gradients of 1.8092 and 0.9718 and regression coefficients (r^2) of 0.9627 and 0.9872 were obtained at pH 5.0 and 4.2 respectively.

BSA calibration graph: The calibration curve of mean net absorbance at 550nm against protein concentration is shown in Figure 3.18. The gradient and regression coefficient was 0.0595 and 0.9982 respectively.
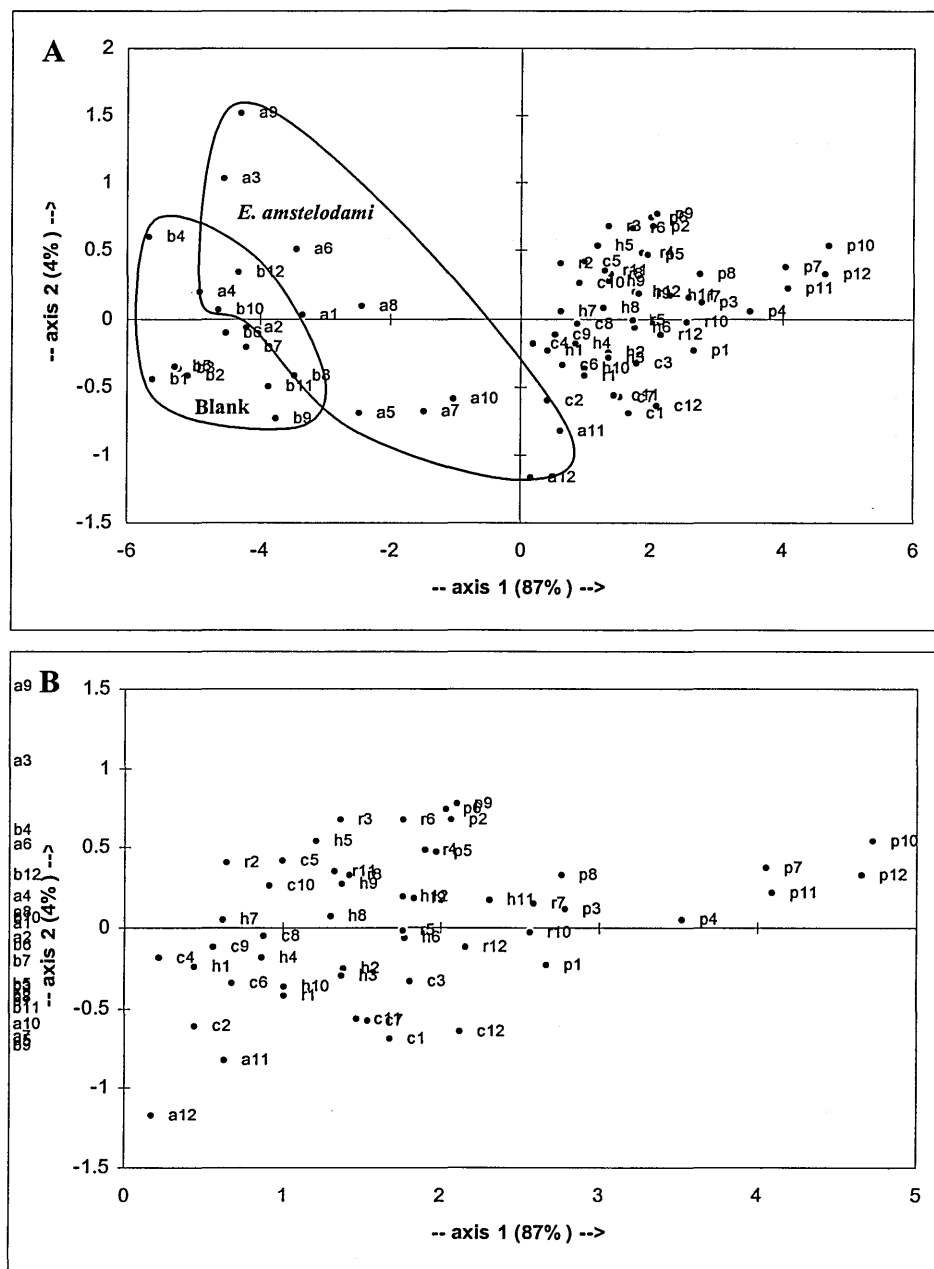
Figure 3.16 PCA of sensor response to volatiles produced by spoilage fungi and blank control at three different a_ws after 72h growth. B is an enlarged scale of A. Key to treatments:

Blank control agar plates	b1-b4, 0.95 a_w; b5-b8, 0.90 a_w and b9-b12, 0.85 a_w.
E. amstelodami	a1-a4, 0.95 a_w; a5-a8, 0.90 a_w and a9-a12, 0.85 a_w.
E. chevalieri	c1-c4, 0.95 a_w; c5-c8, 0.90 a_w and c9-c12, 0.85 a_w.
E. herbariorum	h1-h4, 0.95 a_w; h5-h8, 0.90 a_w and h9-h12, 0.85 a_w.
E. rubrum	r1-r4, 0.95 a_w; r5-r8, 0.90 a_w and r9-r12, 0.85 a_w.
P. chrysogenum	p1-p4, 0.95 a_w; p5-p8, 0.90 a_w and p9-p12, 0.85 a_w.
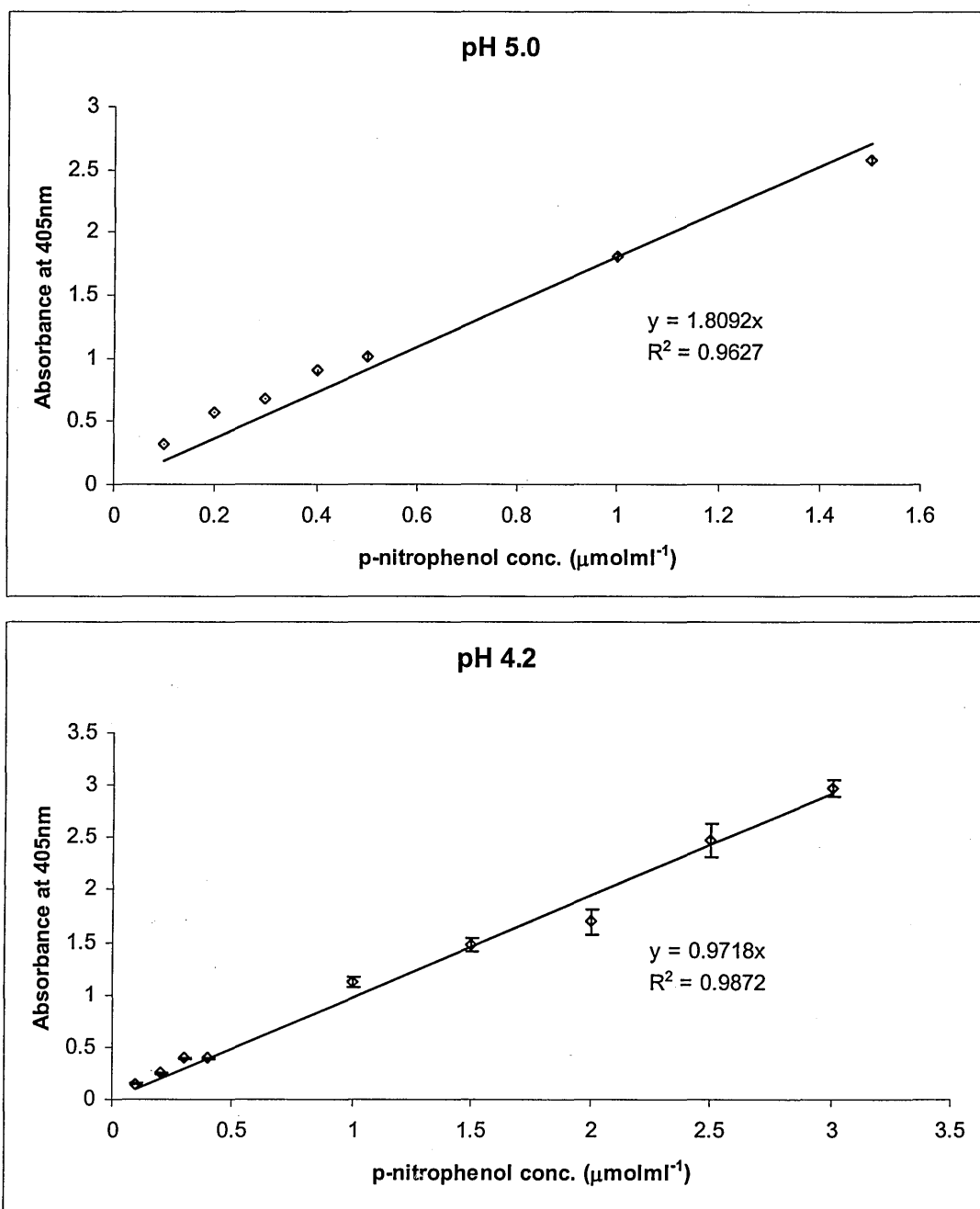
Figure 3.17 Calibration graphs of absorbance at 405nm against p-nitrophenol concentration at pH (a) 5.0 and (b) 4.2. Each data point is a mean of ten replicates. The error bars are shown for each point.
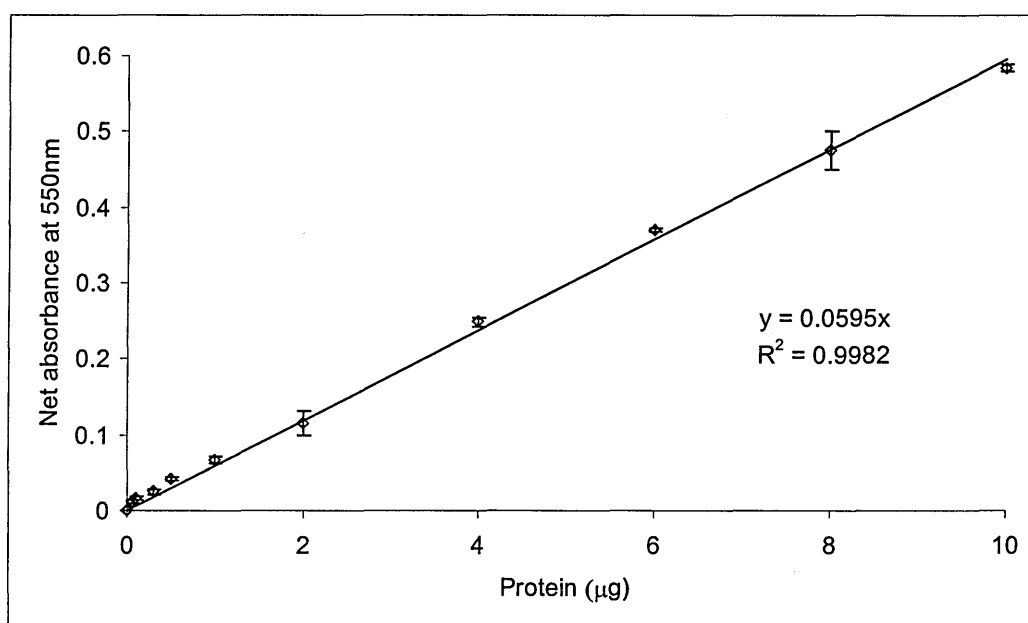
Figure 3.18 Calibration graph of absorbance at 550nm against amount of protein present. Each data point is a mean of ten replicates. The error bars are shown for each point.

0.95 a$_w$: Figure 3.19 shows the specific activities of three hydrolytic enzymes produced by each fungal species on 2% WMA media (using spore lawn inoculation). The specific activity of each enzyme was calculated from the mean absorbance of four replicates.

N-acetyl-β-D-glucosaminidase activity markedly increased for three spoilage fungi. *E. amstelodami* was observed to have the greatest N-acetyl-β-D-glucosaminidase activity after 96h incubation and *E. chevalieri* the least. This increase was significant after 72h for *P. chrysogenum*, and after 96h growth for *E. amstelodami* and *E. chevalieri*.

There was also a significant increase in β-D-glucosidase activity for *P. chrysogenum* after 72h. The activity of α-D-galactosidase was significant for *E. amstelodami* and *E. chevalieri* after 48h growth. However, for *E. chevalieri* the increase was to a lesser extent.

In the case of *P. chrysogenum*, there were significant increases in specific activities of N-acetyl-α-D-glucosaminidase, β-D-fucosidase and β-D-xylosidase after 96h growth (see Appendix G). *E. amstelodami* also had significant N-acetyl-α-D-glucosaminidase activity after 96h incubation. No marked increase in α-D-mannosidase activity was observed.
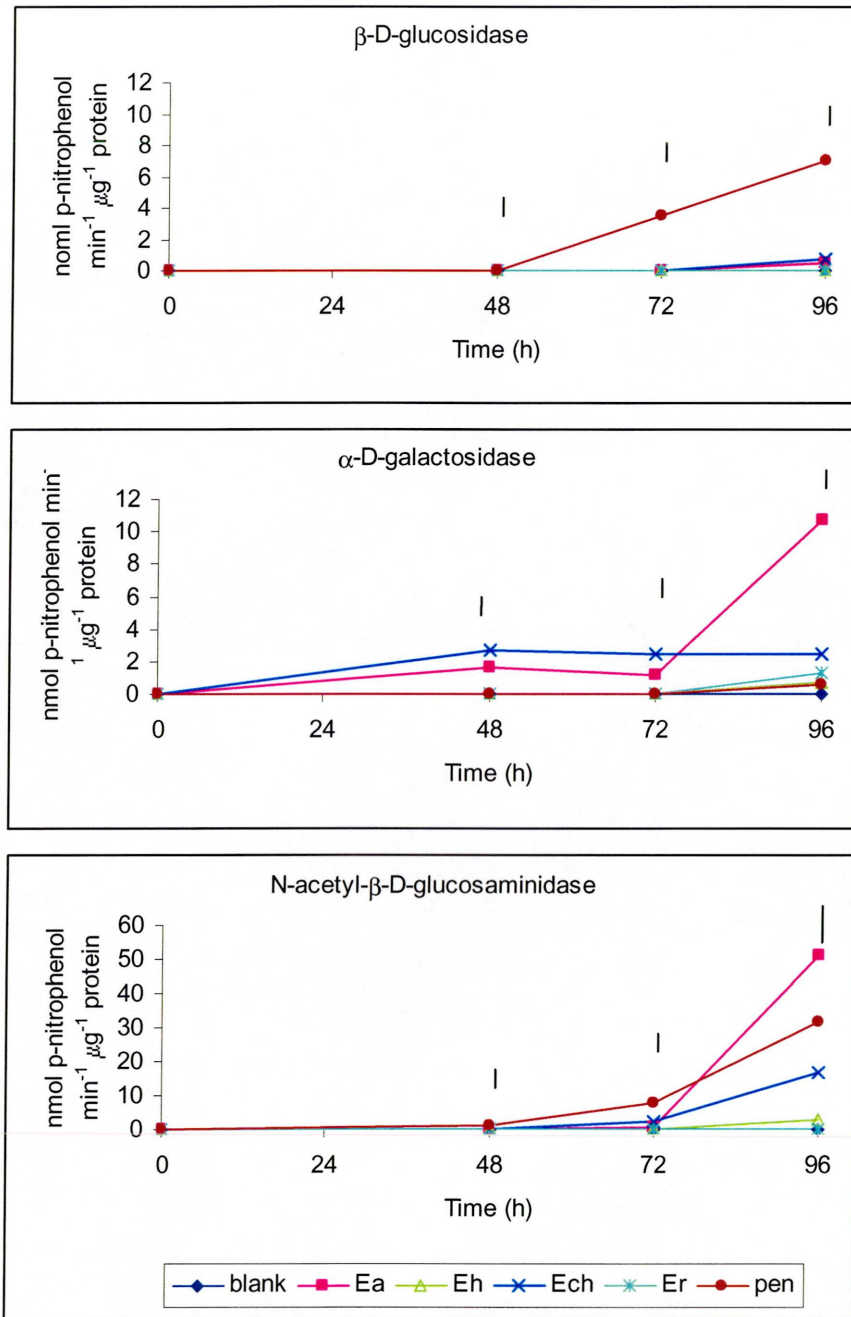
Figure 3.19 Specific activities of β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase produced by five fungi at 0.95 a_w. Note different y axes scales. Key to treatments: blank, blank agar plates; Ea, *E. amstelodami*; Eh, *E. herbariorum*; Ech, *E. chevalieri*; Er, *E. rubrum* and pen, *P. chrysogenum*. The bars indicate the L.S.D. ($P < 0.05$) between fungi at each sampling time.

0.90 a$_w$: Specific enzyme activities for five xerophilic fungi, at 0.90 a$_w$ are shown in Figure 3.20. Only two enzymes were found to significantly increase at lower a$_w$, namely α-D-galactosidase and N-acetyl-β-D-glucosaminidase. A significant increase in α-D-galactosidase activity was observed after 48h for *E. amstelodami*. For *E. chevalieri*, significant increases in α-D-galactosidase and N-acetyl-β-D-glucosaminidase were observed after 72 and 120h respectively. However, the increase in the former enzyme was to a lesser extent for *E. chevalieri*.

No increase in specific activities was observed for the remaining enzymes including β-D-glucosidase (see Appendix G). *E. rubrum*, *E. herbariorum* and *P. chrysogenum* were found to have no significant increase in specific activities of the seven enzymes assayed.

0.85 a$_w$: Figure 3.21 illustrates the specific enzyme activities at a lower a$_w$ of 0.85. Again the same enzymes were found to be predominant as for 0.90 a$_w$ (α-D-galactosidase and N-acetyl-β-D-glucosaminidase). However, the increase in α-D-galactosidase activity was found to be significant only after 11 days of growth for *E. rubrum* and *E. amstelodami*. Again N-acetyl-β-D-glucosaminidase activity was significant for *E. chevalieri* after 11 days. For the remaining fungal species and enzymes assayed no increase was observed to be significant (see Appendix G).
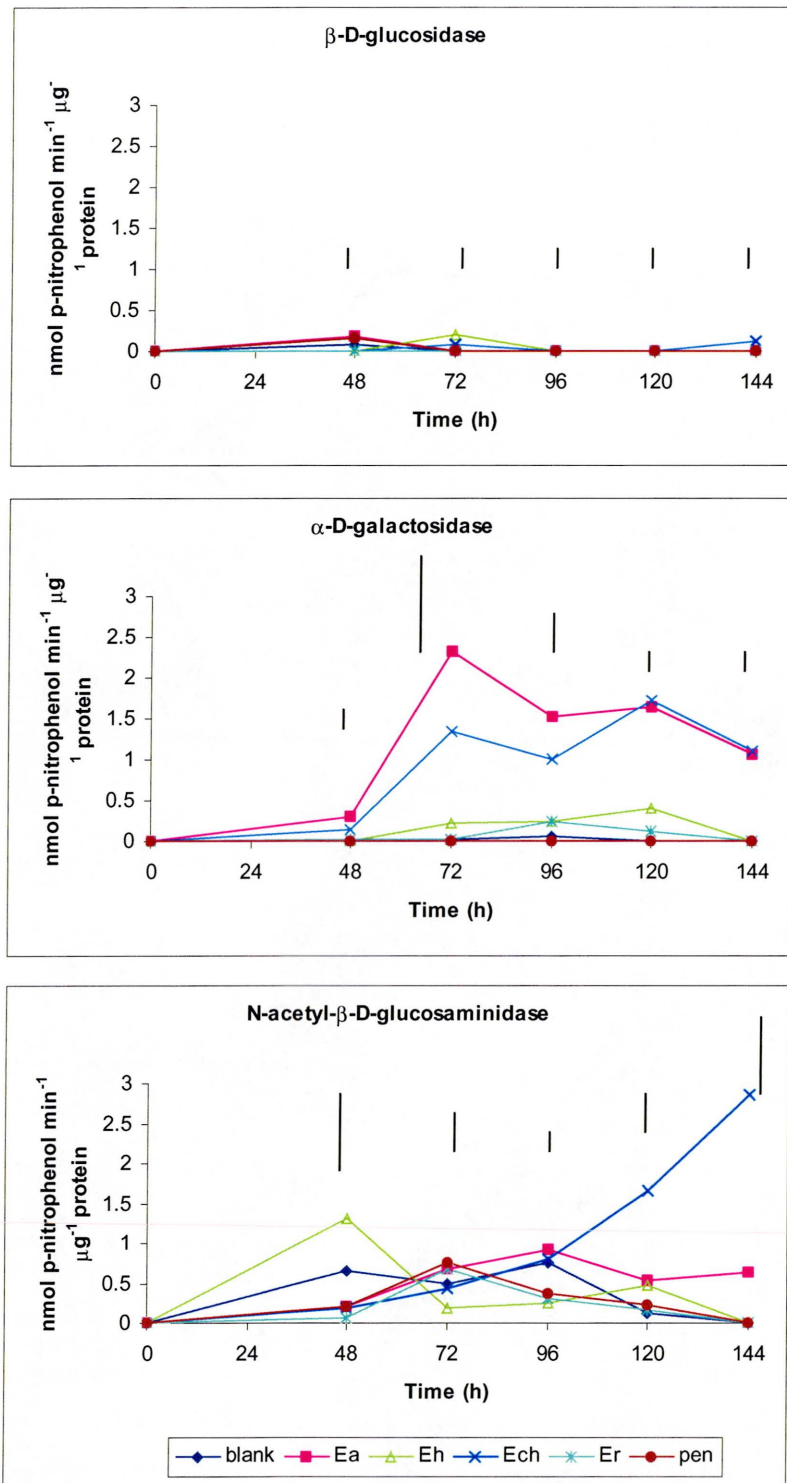
Figure 3.20 Specific activities of β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase produced by five fungi at 0.90 a_w. Key to treatments: blank, blank agar plates; Ea, *E. amstelodami*; Eh, *E. herbariorum*; Ech, *E. chevalieri*; Er, *E. rubrum* and pen, *P. chrysogenum*. The bars indicate the L.S.D. (P < 0.05) between fungi at each sampling time.
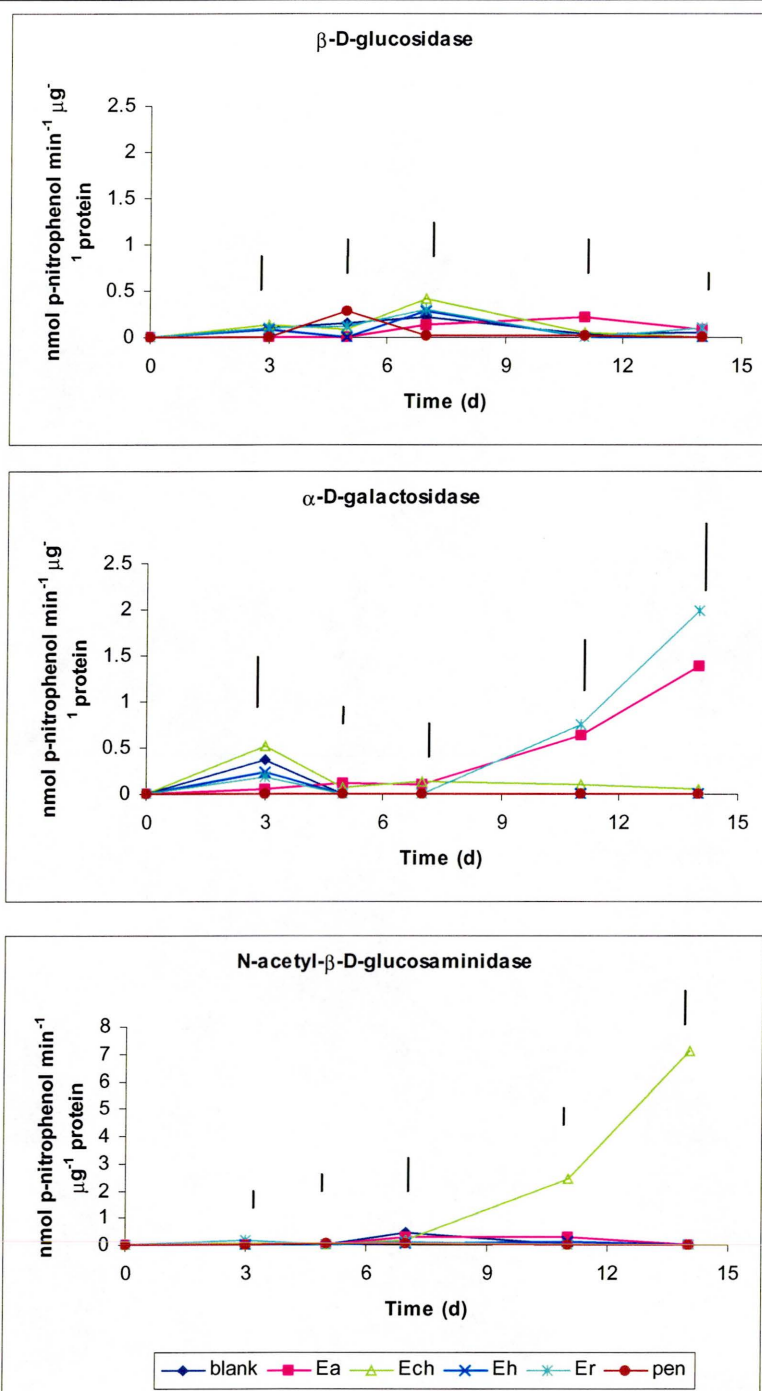
Figure 3.21 Specific activities of β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase produced by spoilage fungi grown on 2% WMA at 0.85 a_w. Note different y axes scales. Key to treatments: blank, blank agar plates; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri*; Eh, *E. herbariorum*; Er, *E. rubrum* and pen, *Penicillium chrysogenum*. The bars indicate the L.S.D. (P <0.05) between fungi at each sampling time.

3.3.3   Growth rate

Growth rate of single colonies of four *Eurotium* species and *P. chrysogenum* were measured at three different a_w (see Appendix H). The growth rate of individual fungi decreased with decreasing a_w (Figure 3.22). At 0.95 a_w, *E. rubrum* was observed to have a faster growth rate. Whereas, *E. herbariorum* had the slowest. The growth rate of *E. chevalieri* and *E. amstelodami* were found to be similar. However, at lower a_w (0.90 a_w) *E. chevalieri* had the faster growth rate while the remaining *Eurotium* spp. had similar growth rates. At 0.85 a_w *E. rubrum* and *E. chevalieri* had similar growth rates, and *E. herbariorum* was noted to have the slowest rate. *P. chrysogenum* grew well at the higher a_w levels tested (0.95 and 0.90).

3.4   COMPARATIVE STUDY OF VOLATILE PATTERNS AND HYDROLYTIC ENZYME PRODUCTION FROM MYCOTOXIGENIC AND NON-MYCOTOXIGENIC FUNGI

3.4.1   Volatile patterns

Three mycotoxigenic fungi (*Aspergillus flavus*, *A. ochraceus*, and *Wallemia sebi*) and two non-mycotoxigenic fungi (*Penicillium chrysogenum* and *A. niger*) were used in this study, utilising the spore lawn inoculation method on 2% WMA media at 0.95 a_w. Volatile measurements were taken after 72 and 120h growth.

Initial visible signs of growth were present after 72h incubation. Figure 3.23 shows the principal component analysis after 72h growth. The analyses used 96% of the information and showed discrimination was possible between the five fungi and control blank media.
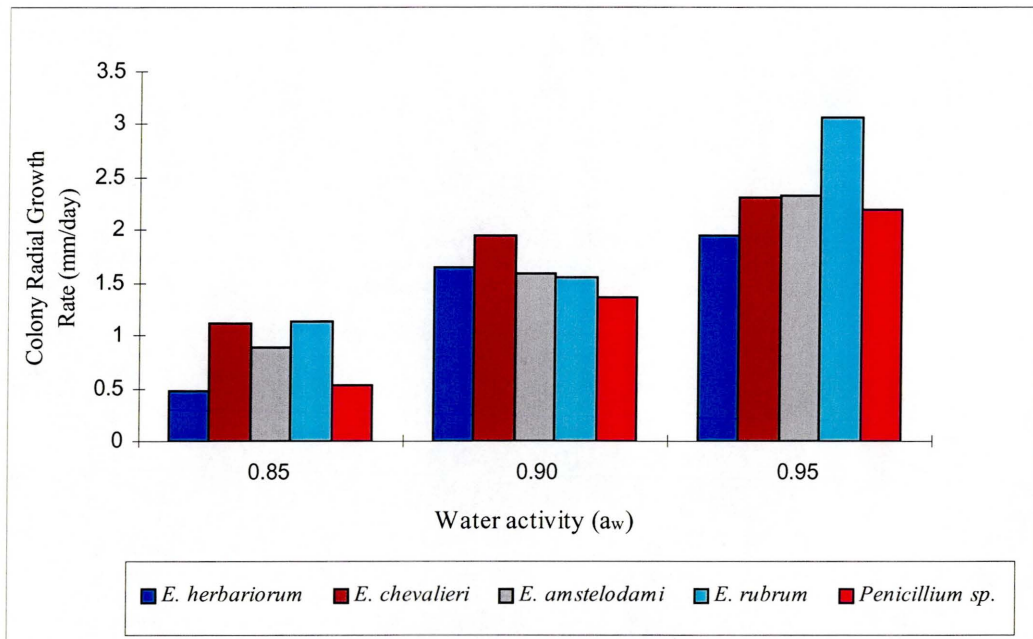
Figure 3.22 Growth rate of four *Eurotium* spp. and *Penicillium chrysogenum* at different water activities: (a) 0.85; (b) 0.90 and (c) 0.95 a_w on 2% wheatmeal agar at 25°C.
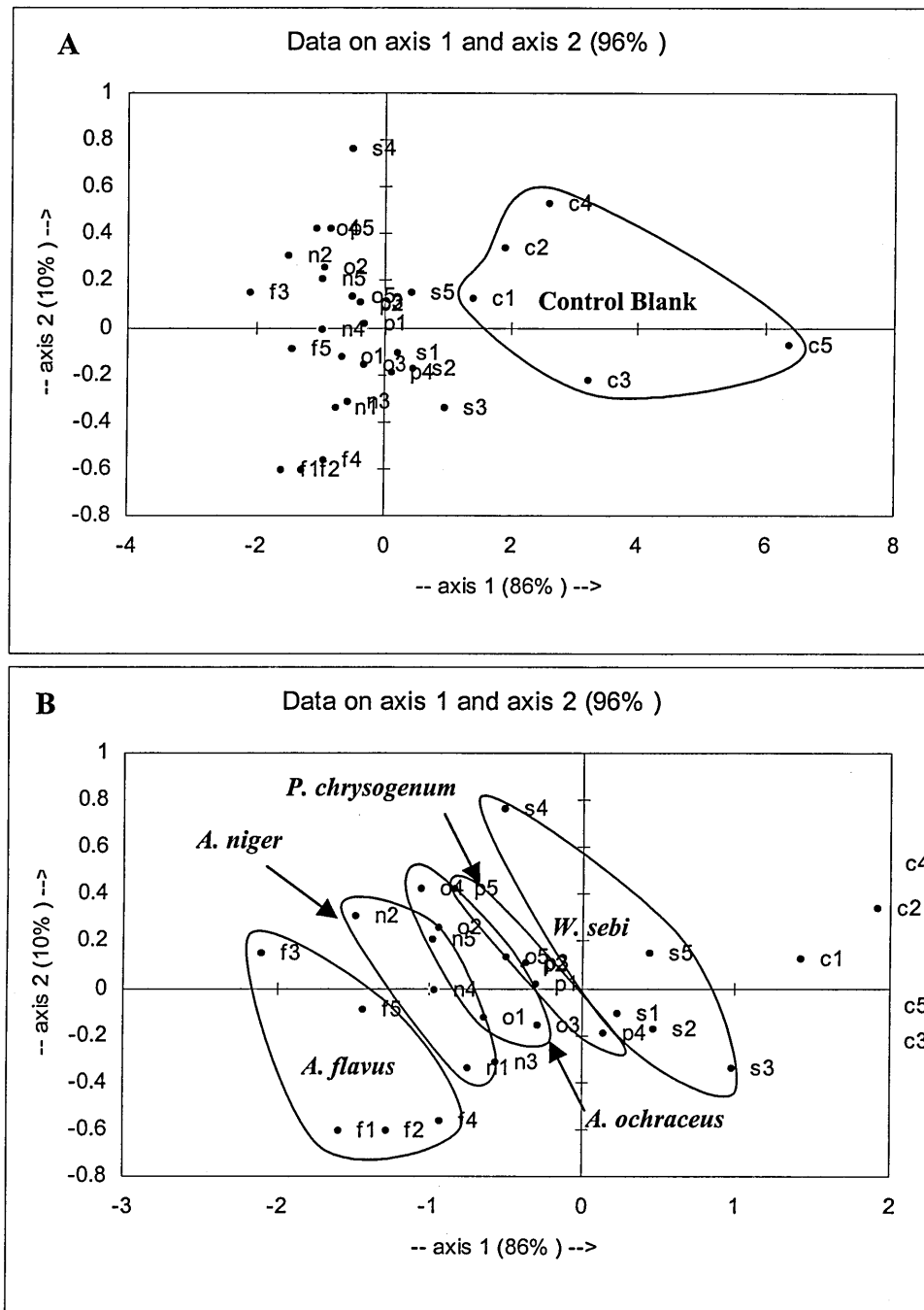
Figure 3.23 Principal component analysis of sensor response to volatiles produced by non-mycotoxigenic and mycotoxigenic fungi and control (blank agar plates) after 72h incubation at 25°C on wheat meal agar media. B is an enlarged scale of A. Key to treatments: f1-f5, *Aspergillus flavus*; n1-n5, *A. niger*; o1-o5, *A. ochraceus*; p1-p5, *Penicillium chrysogenum*; s1-s5, *Wallemia sebi*; and c1-c5, control blank agar plates.

Good separation was achieved for *A. flavus* and *W. sebi* while there was an overlap between *A. niger* (replicate n5), *A. ochraceus* (replicate o5) and *P. chrysogenum*. Sensor drift may be responsible for the overlap observed in the PCA, as the outliers are in batch 5.

After 120h incubation, *A. flavus*, *A. niger* and *A. ochraceus* were sporulating. *P. chrysogenum* and *W. sebi* were observed to be growing more slowly. The PCA after 120h growth (Figure 3.24A) utilised 99% of the information and shows improved separation between the fungal species and control. Figure 3.24B (an enlarged scale of Figure 3.24A) illustrates the discrimination of three fungi, namely *W. sebi*, *P. chrysogenum* and *A. ochraceus*. Some overlap is observed between *A. niger* and *A. flavus*.

The cluster analyses of the sensor responses to fungal volatiles after 72 and 120h are shown in Figures 3.25A and B respectively. After 72h the dendrogram shows three groups. *P. chrysogenum* is defined in one group, *A. niger* and *A. ochraceus* in another and finally *W. sebi*, *A. flavus* and the control blank in the last group. After 120h incubation the cluster analyses demonstrates three groups again. *P. chrysogenum* observed to be in one group. *W. sebi* and *A. niger* in another group and *A. ochraceus*, *A. flavus* and the control media in the third group.
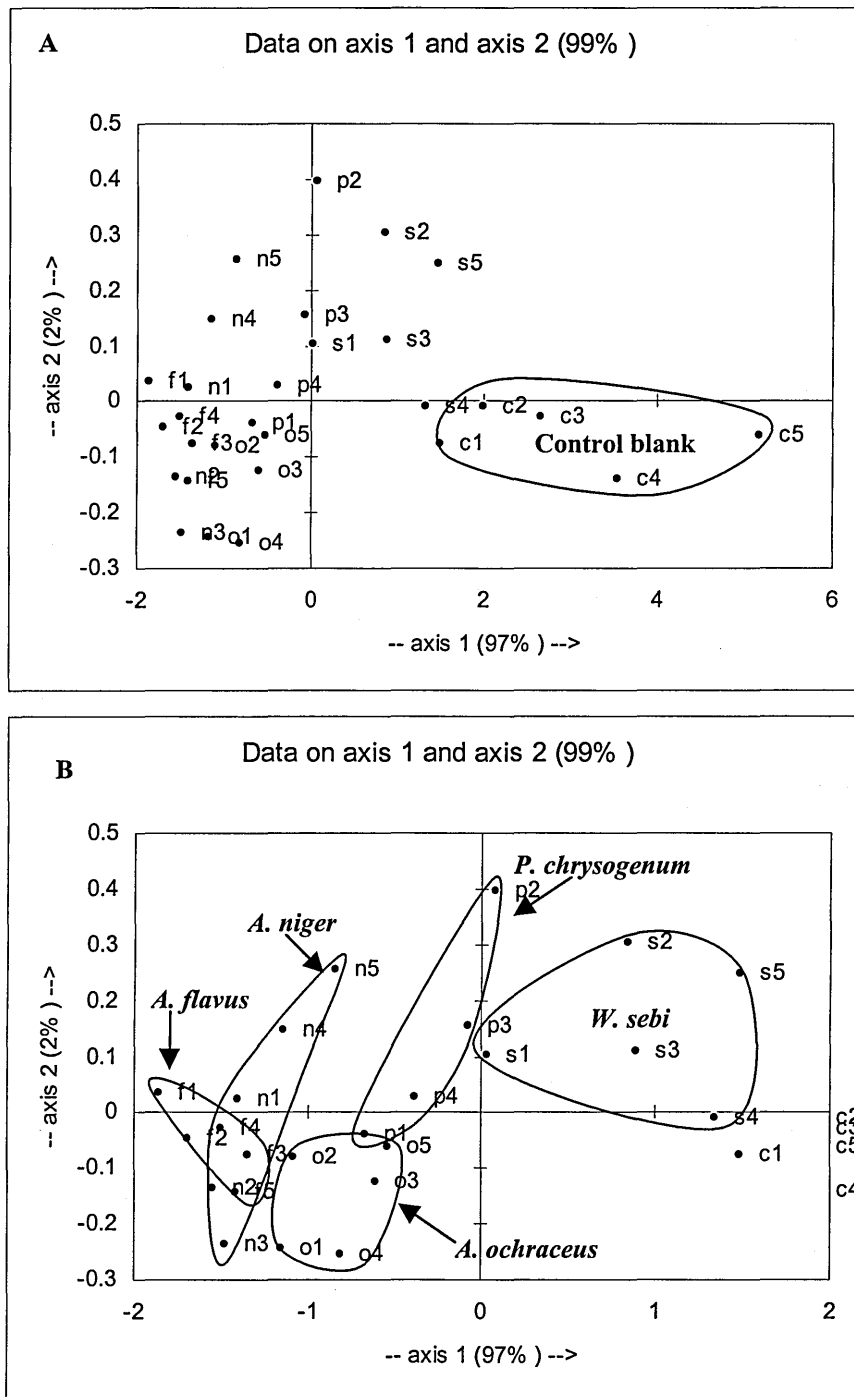
Figure 3.24 PCA showing discrimination of non-mycotoxigenic and mycotoxigenic fungi and control (blank agar plates) after 120h incubation at 25°C. B is an enlarged scale of A. Key to treatments: f1-f5, *Aspergillus flavus*; n1-n5, *A. niger*; o1-o5, *A. ochraceus*; p1-p5, *Penicillium chrysogenum*.; s1-s5, *Wallemia sebi*; and c1-c5, control blank agar plates.
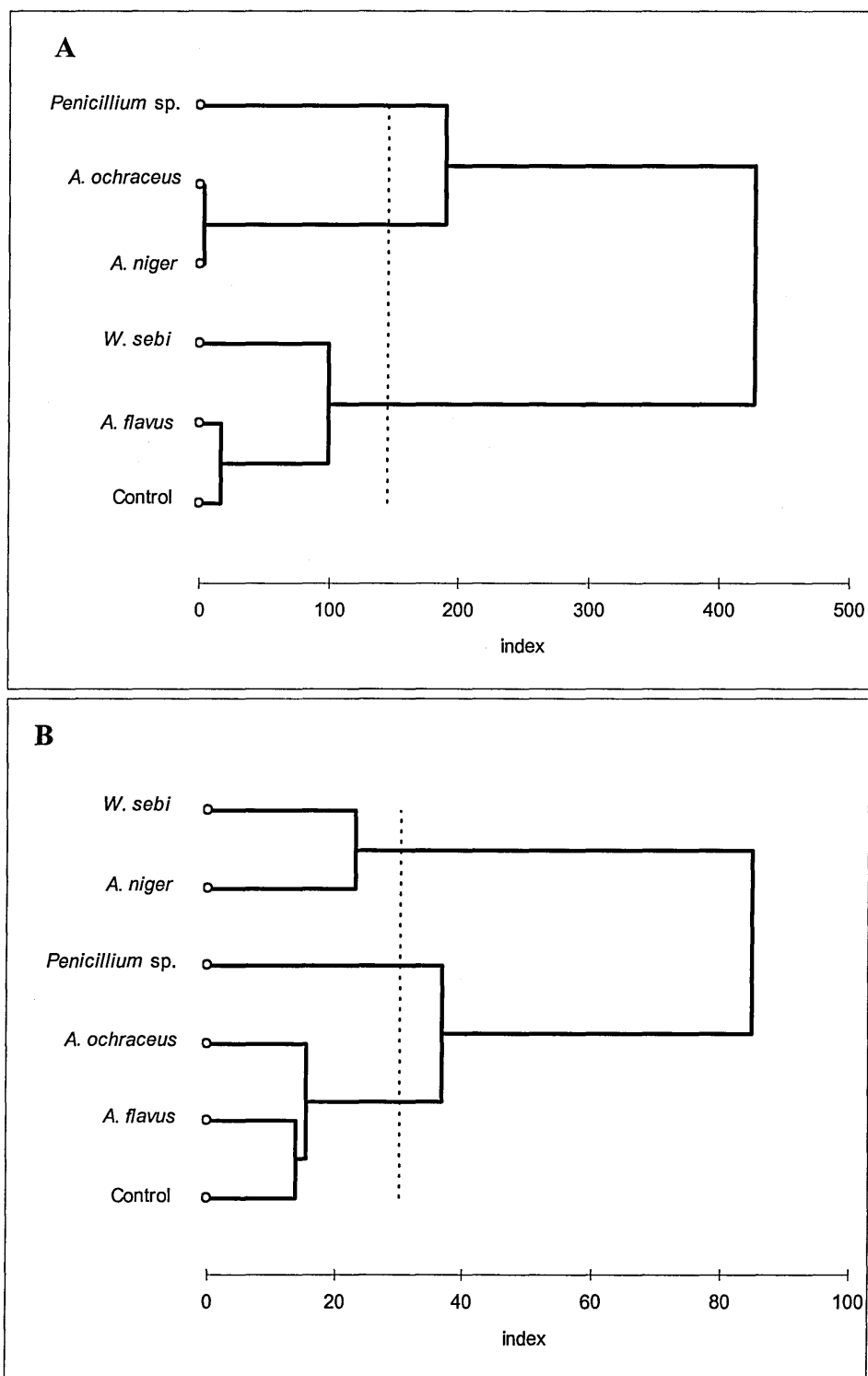
Figure 3.25 Cluster analyses of the volatile profiles produced by mycotoxigenic and non-mycotoxigenic fungi after (A) 72 and (B) 120h incubation. The controls used are blank agar plates.

3.4.2   Enzyme activity

Figure 3.26 shows specific activities of three hydrolytic enzymes. The least significant difference (L.S.D.) are plotted as bars on the graphs. The blank control agar plates were found to have some activity however, this was relatively low.

Three enzymes were found to be predominant namely, β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase. Statistical analyses showed the activities of these enzymes to increase over time and were found to be significantly increased after 120h when visible signs of growth occurred. *Aspergillus niger* was observed to have the highest β-D-glucosidase and N-acetyl-β-D-glucosaminidase activity in comparison to that for *A. flavus*. Whereas only *A. ochraceus* had significant α-D-galactosidase activity after 120h growth. The activities of the remaining four enzymes (α-D-mannosidase, β-D-xylosidase, β-D-fucosidase and N-acetyl-α-D-glucosaminidase) were found to be not significant (see Appendix G).

For *P. chrysogenum* and *W. sebi* no significant enzyme activities were observed for the seven hydrolytic enzymes assayed.
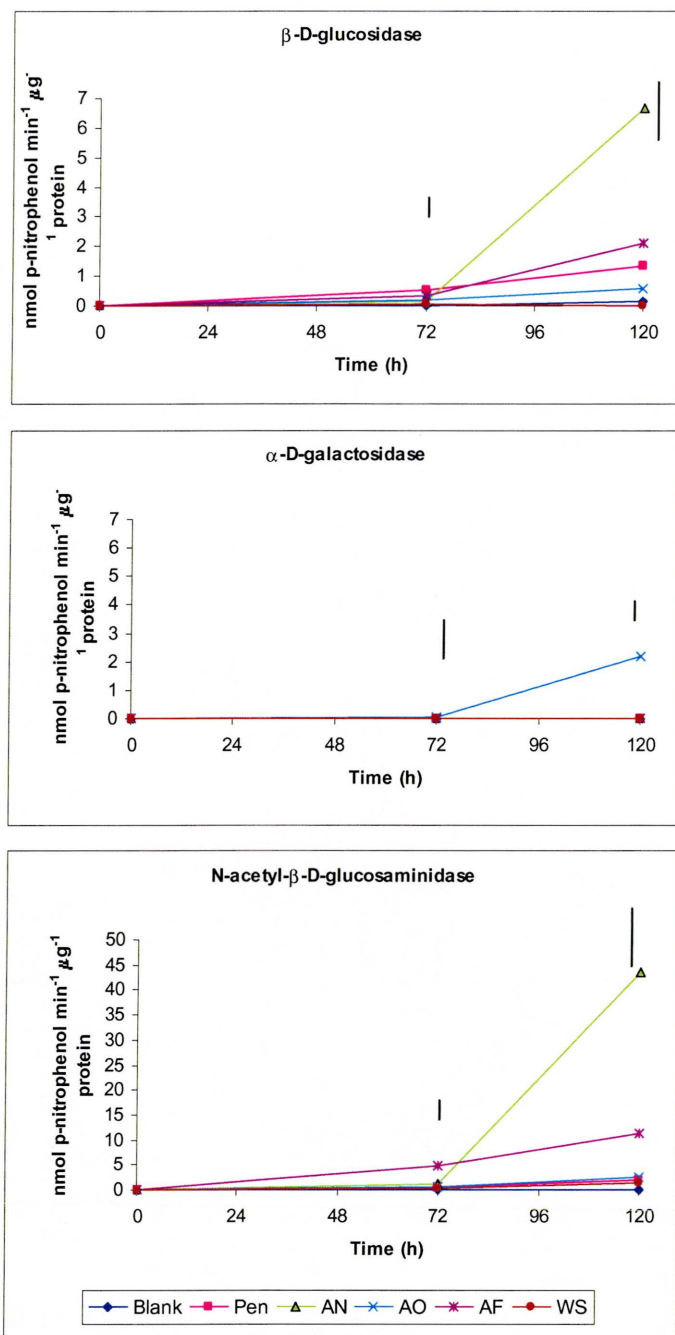
Figure 3.26 Specific activities (nmol p-nitrophenol min^{-1} μg^{-1} protein) of β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase produced by different non-mycotoxigenic and mycotoxigenic fungal species. Note different y axes scales. Key to fungi: blank, blank agar plates; Pen, *Penicillium chrysogenum*; AN, *Aspergillus niger*; AO, *A. ochraceus*; AF, *A. flavus*; and WS, *Wallemia sebi*. The bars indicate the L.S.D. (P <0.05) between fungi at each sampling time.

3.5 DETECTION AND DIFFERENTIATION BETWEEN MYCOTOXIGENIC AND NON-MYCOTOXIGENIC STRAINS OF TWO *FUSARIUM* SPP. USING VOLATILE PROFILES AND ENZYME ACTIVITIES.

3.5.1 Volatile profiles

Figure 3.27 shows an example of the normalized divergence responses for the sensor array for four replicate samples of germinating spore lawn cultures of one *F. moniliforme* strain (48N), which were very reproducible with only a small standard error, in most cases <10%.

Principal component analyses were used in an attempt to separate the non-producer isolates from the other three mycotoxigenic strains after 48, 72 and 96h of incubation. Figure 3.28 shows that for strains of *F. moniliforme*, it was possible to use 99% and 93% of the data for separation of the control blanks from the non-mycotoxigenic (6N), and also to obtain some discrimination between the others. The separation between strains was better after 72 and 96h incubation when visible mycelial growth had occurred on the agar plates. For *F. proliferatum*, separation between strains was also achieved and could account for 83, 93 and 96% after 48, 72 and 96h, respectively (Figure 3.29). The non-mycotoxigenic strain (20N) could be differentiated from the mycotoxigenic strains, and from the uninoculated controls.
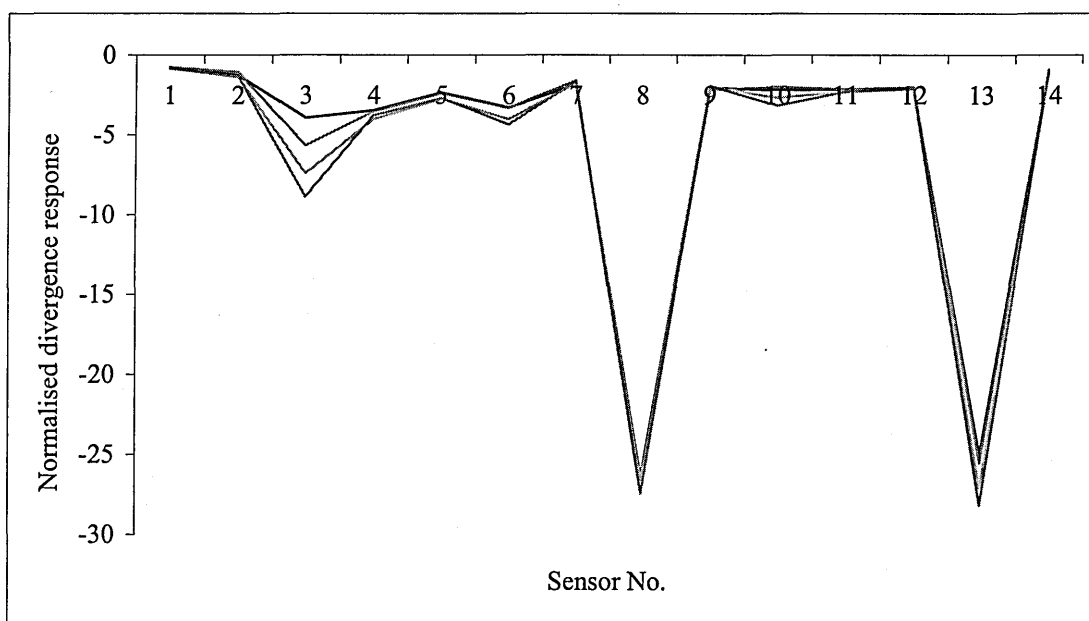
Figure 3.27 Normalised divergence responses showing reproducibility of the sensor array to fungal volatiles produced by four replicates of germinating spore cultures of *Fusarium moniliforme* after 48h of incubation at 25°C on a wheat meal agar medium.
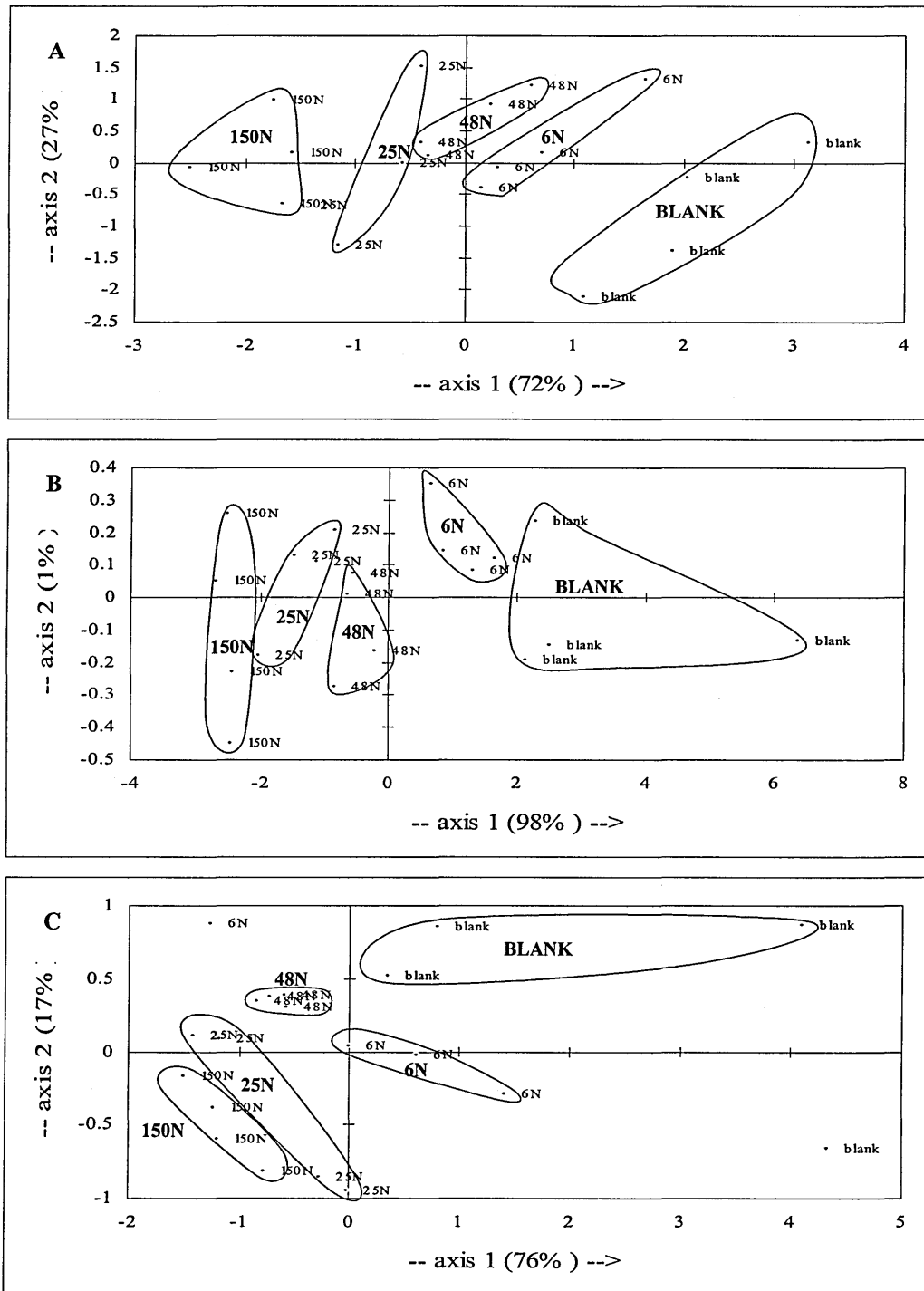
Figure 3.28 Principal component analysis of volatiles produced by four isolates of *Fusarium moniliforme* and control (blank agar plates) after (a) 48h, (b) 72h and (c) 96h growth at 25°C on a wheat meal agar medium.
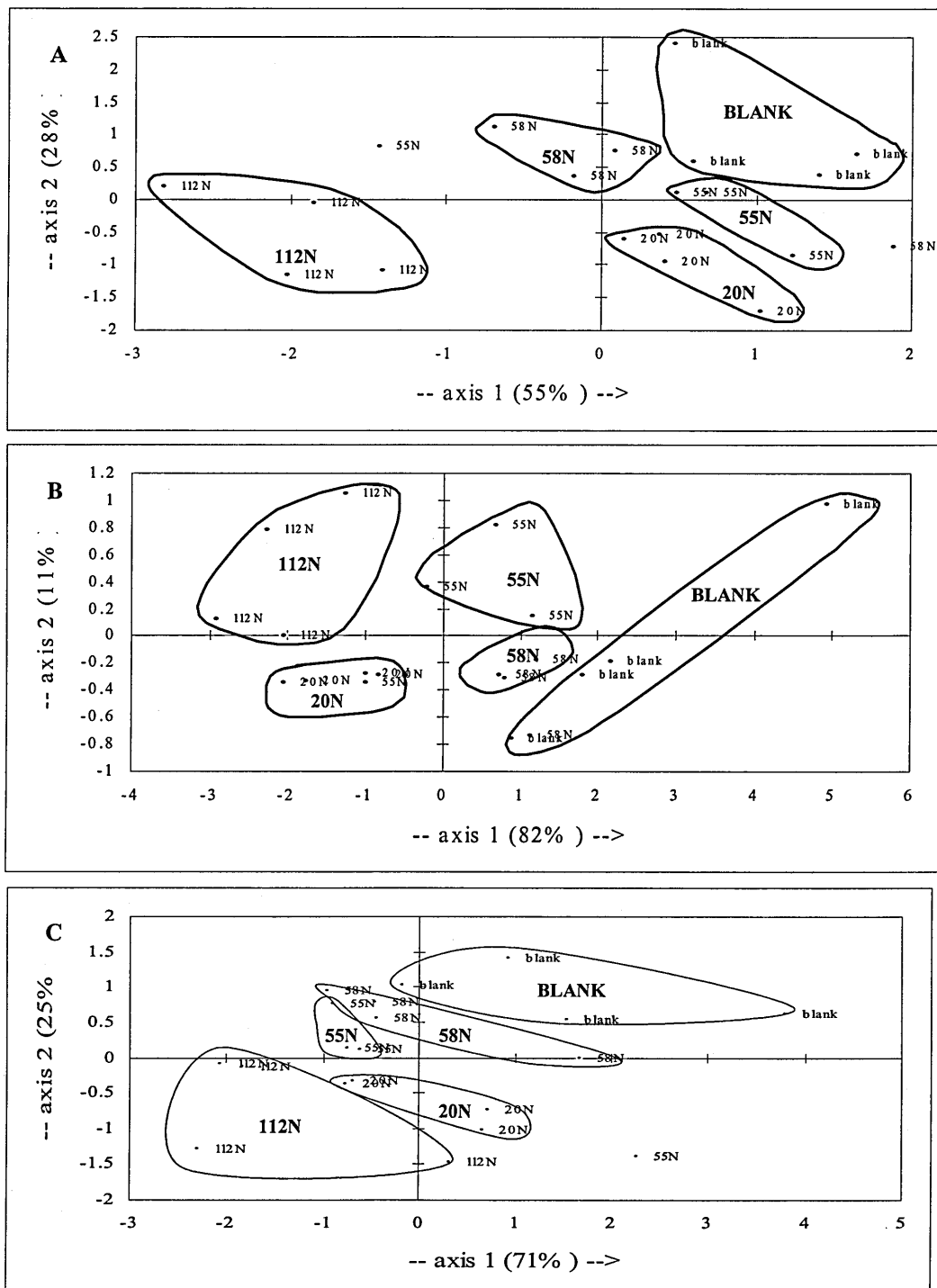
Figure 3.29 Principal component analysis of volatiles produced by four isolates of *Fusarium proliferatum* and control blanks after (a) 48h, (b) 72h and (c) 96h growth at 25°C on a wheat meal agar medium.

As illustrated in Figure 3.30, differentiation of the control blank from the *Fusarium* strains was possible after 48h growth. It was possible to use cluster analyses to separate some of the strains when the control blanks were excluded from the analyses (Figure 3.31). After 48h, the dendrograms show that for both *F. moniforme* and *F. proliferatum* it was possible to distinguish the non-mycotoxigenic strains from the others. For *F. moniliforme*, there was some overlap between strains 25N (fumonisin producer) and 150N (zearelenone and fumonisin producer). For *F. proliferatum*, there was some overlap between strains 55N (trichothecene and fumonisin producer) and 112N (fumonisin producer).

3.5.2 Hydrolytic enzyme activity

The hydrolytic enzymes produced by germlings and by growing mycelium were quantified after 48, 72 and 96h. Figure 3.32 compares the temporal changes in specific activity of three enzymes produced by each of the four isolates of *F. moniliforme*. Statistical analyses showed that the specific activities of β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase significantly increased over time in all isolates of *F. moniliforme* and *F. proliferatum*, while that of the other four enzymes (α-D-mannosidase; β-D-xylosidase; β-D-fucosidase and N-acetyl-α-D-glucosaminidase) were unaffected or changed little during the experiments. It shows that significantly more β-D-glucosidase and N-acetyl-β-D-glucosaminidase enzymes were produced by the non-mycotoxigenic strain (6N) than by the other three mycotoxigenic strains after 48, 72 and 96h. For α-D-galactosidase, there was only a significant difference after 96h of growth.
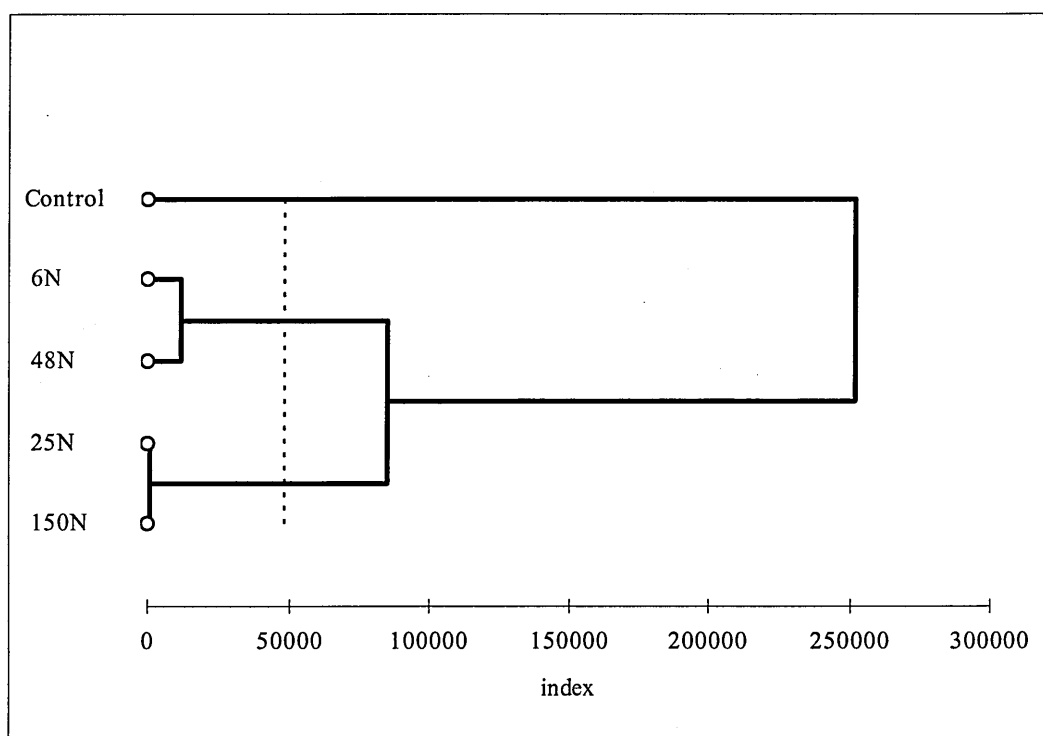
Figure 3.30 Cluster analysis of sensor array responses to volatiles produced by *Fusarium moniliforme* after 48h showing the separation of control agar blanks.
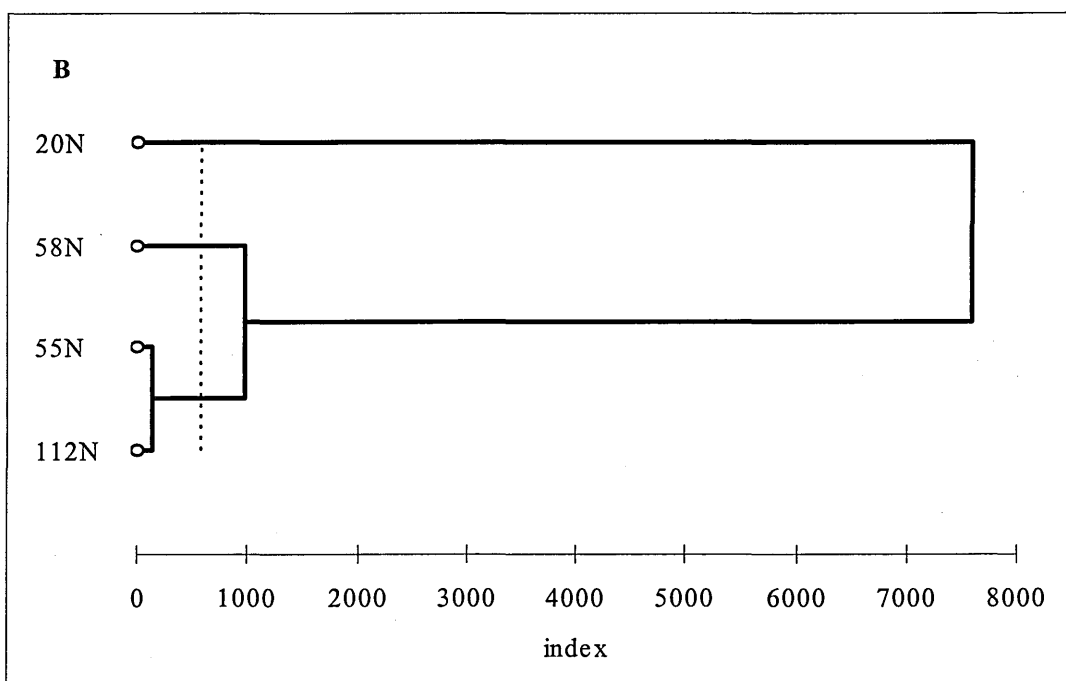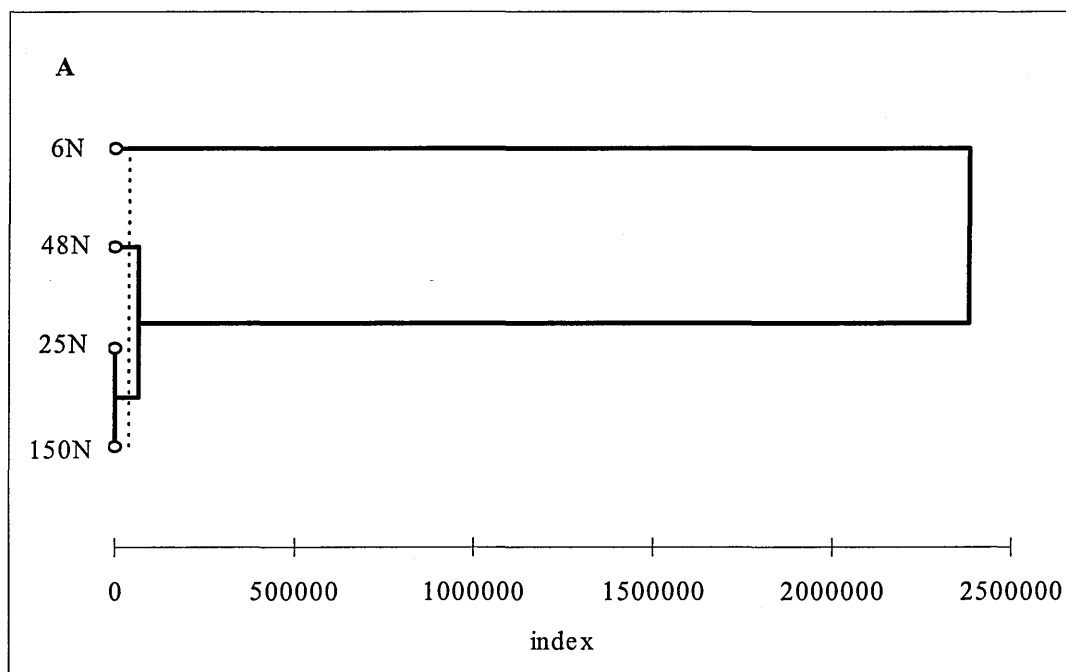
Figure 3.31 Cluster analyses of sensor array responses to volatiles produced by (a) *Fusarium moniliforme* and (b) *Fusarium proliferatum* after 48h. The agar blanks have been excluded from the analysis.
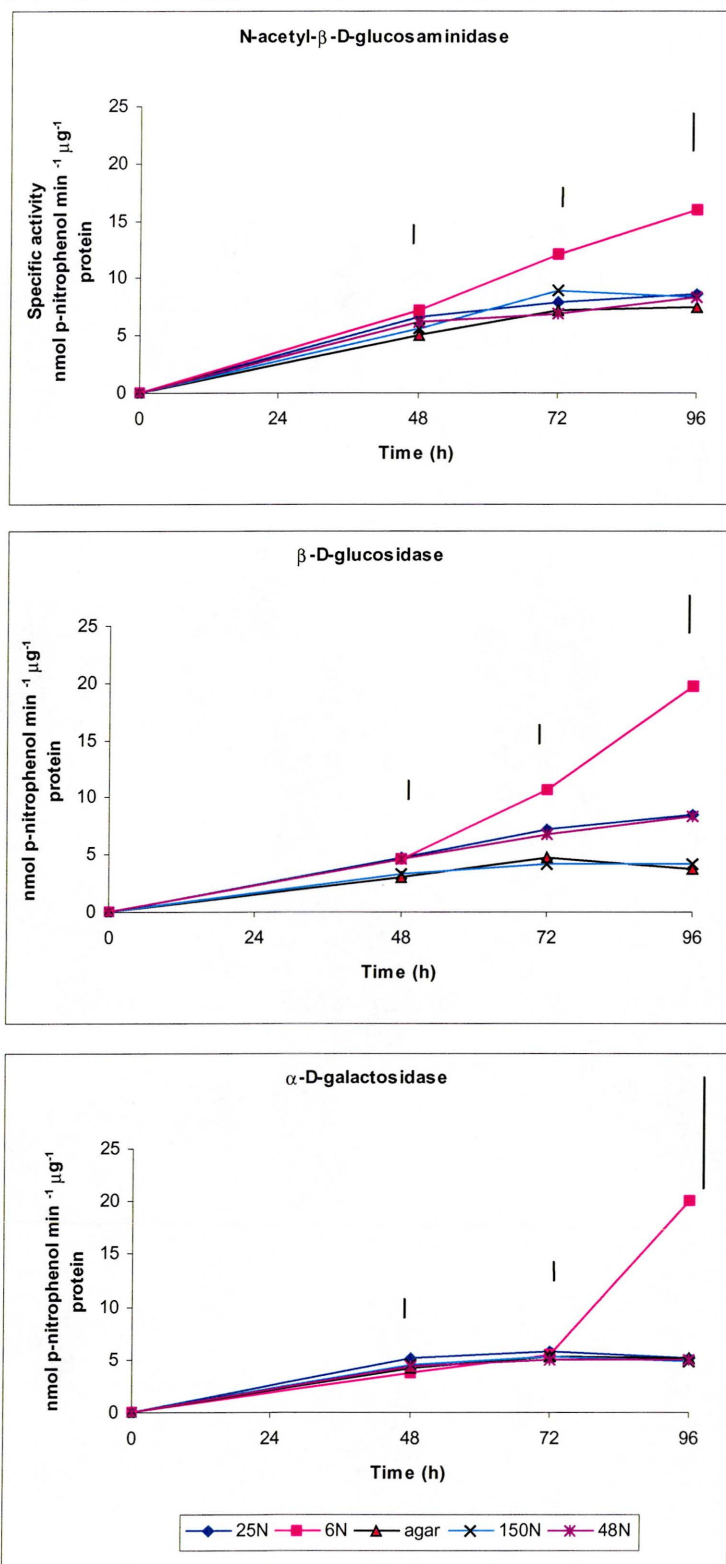
Figure 3.32 Specific activities (nmol p-nitrophenol min^{-1} µg^{-1} protein) of N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase produced by *Fusarium moniliforme*. The bars indicate the L.S.D. (P <0.05) between isolates at each sampling time.

The specific activities of the remaining four enzymes were found to be not significant (see Appendix G).

For *F. proliferatum*, differences were less clear based on the specific enzyme activity, although there was a significant increase in activity of α-D-galactosidase after 72h and of N-acetyl-β-D-glucosaminidase after 96h (Figure 3.33). The non-mycotoxigenic strain 20N had a higher activity of N-acetyl-β-D-glucosaminidase than the other strains. Highest β-D-glucosidase activity was observed for strains 55N (trichothecene and fumonisin producer) and 112N (fumonisin producer). The remaining four enzymes were not found to be significant (see Appendix G).

3.6 EARLY DETECTION OF FUNGAL GROWTH ON MODIFIED BREAD SUBSTRATE BASED ON THE VOLATILE PRODUCTION PATTERNS, ENZYMIC STUDY AND TOTAL FUNGAL POPULATIONS

3.6.1 Volatile production patterns

Figure 3.34 shows an example of the divergence response of six replicates of *E. chevalieri* after 26h incubation, grown on bread substrate modified to 0.95 a_w. Good reproducibility was obtained with only a small variation in these patterns between replicates of the same treatment.

Principal component analysis (PCA) was utilized to discriminate between fungi and the control blank. Figures 3.35 and 3.36 illustrate the analyses after 26, 40, 48 and 72h incubation, the data on axes 1 and 2 representing 99, 98, 94 and 94% of the information utilized respectively.
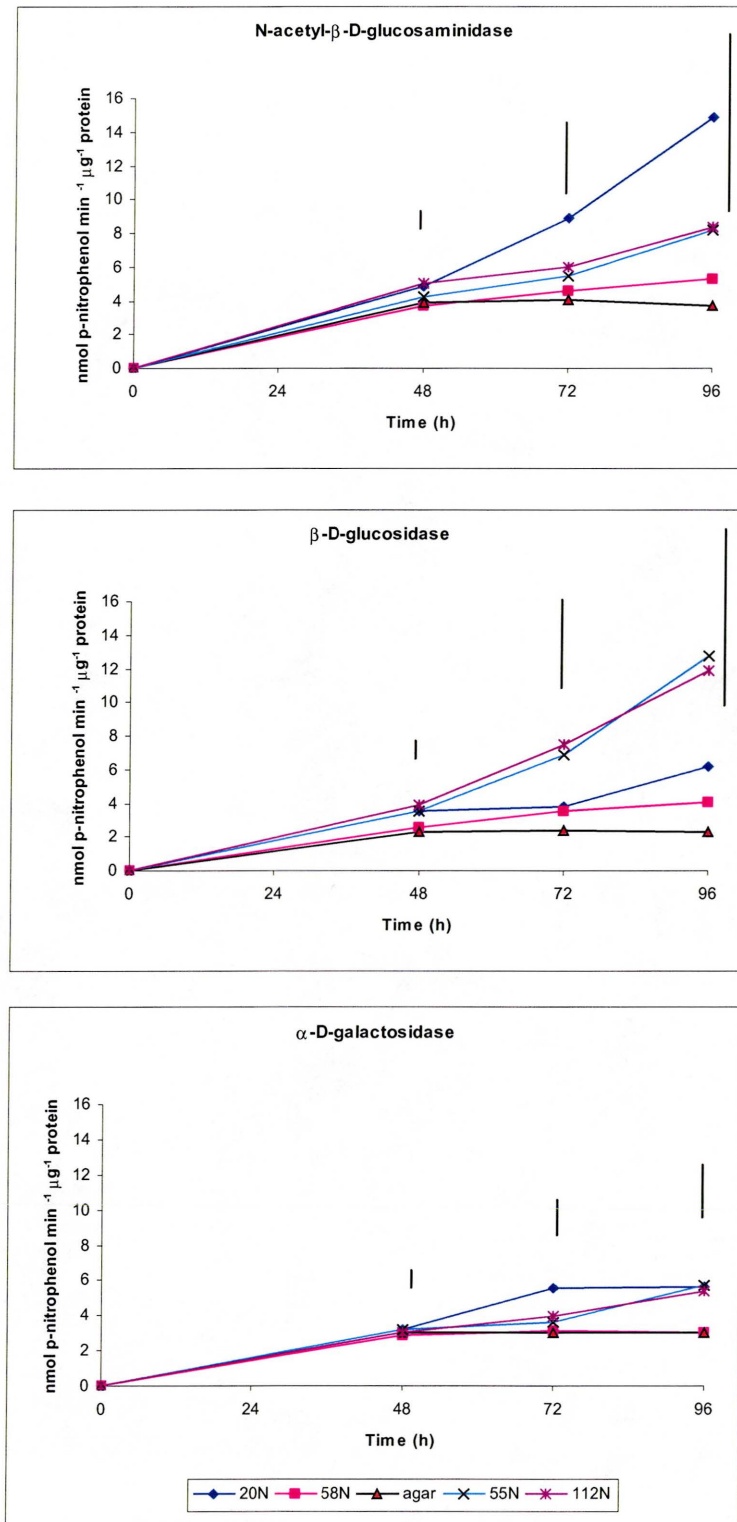
Figure 3.33 Specific activities (nmol p-nitrophenol min^{-1} μg^{-1} protein) of N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase produced by *Fusarium proliferatum*. The bars indicate the L.S.D. (P <0.05) between isolates at each sampling time.
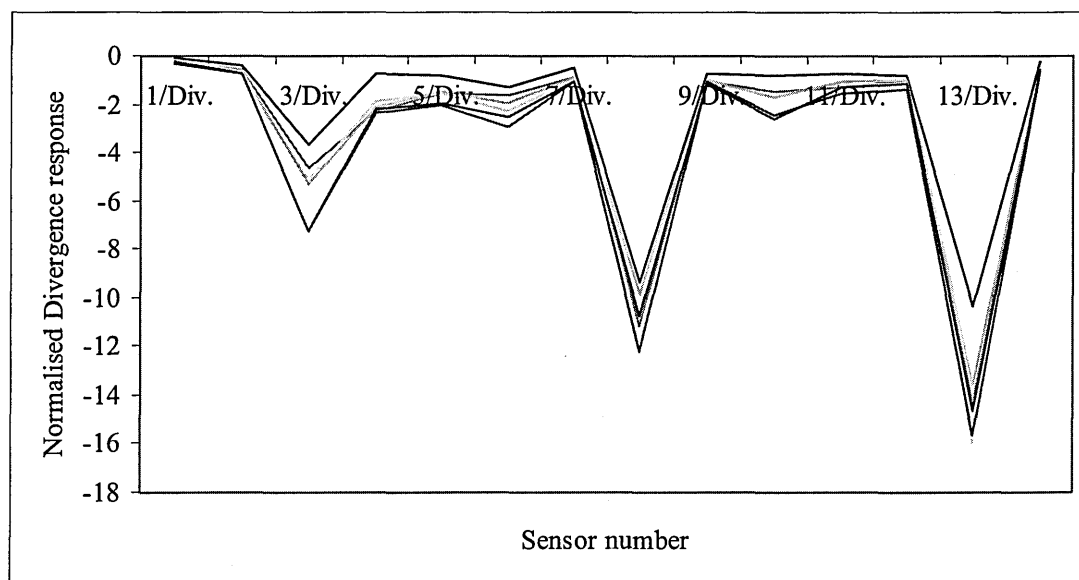
Figure 3.34 Normalised divergence responses showing reproducibility of 14 conducting polymer sensors to six replicates of *E. chevalieri* on bread analogue (0.95 a_w) after 26h growth at 25°C.
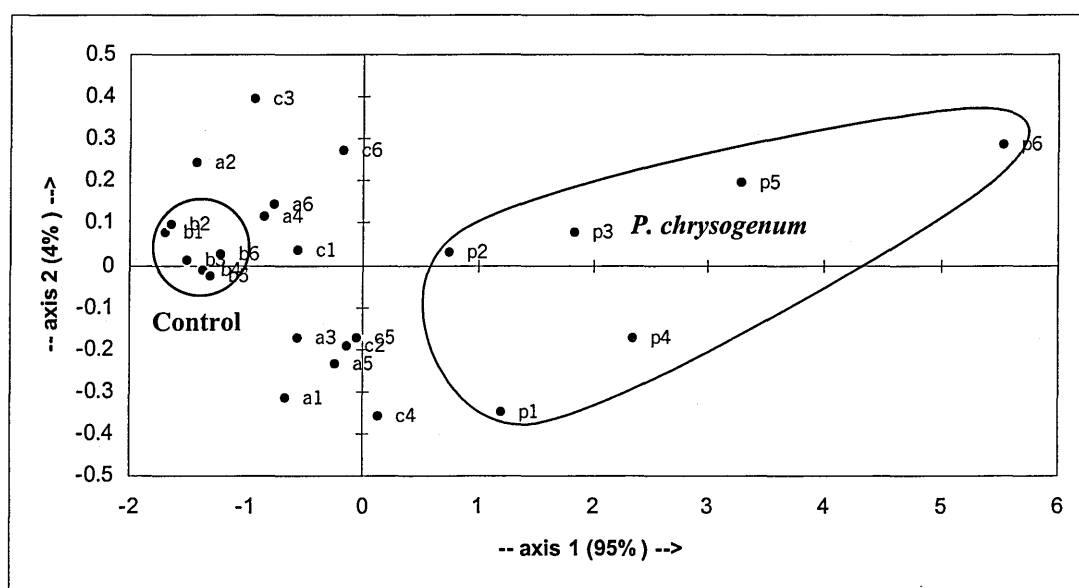
Figure 3.35 Principal component analysis of volatiles produced by spoilage fungi on bread analogue after 26h growth at 25°C. Data on axis 1 and 2 shows 99% of the information. Key to treatments: b1-b6, blank bread slices; a1-a6, *E. amstelodami*; c1-c6, *E. chevalieri* and p1-p6, *P. chrysogenum*.
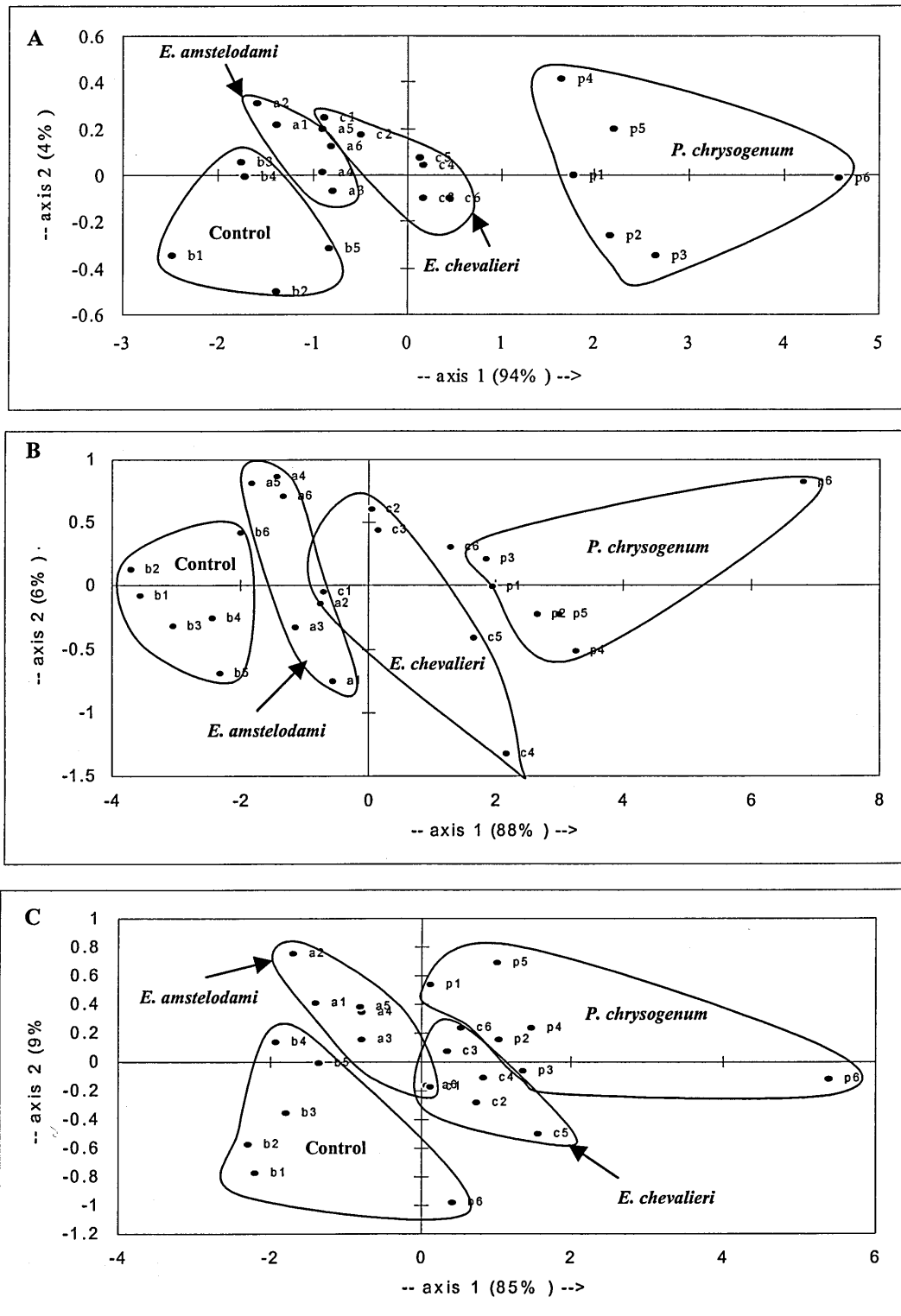
Figure 3.36 Principal component analysis of volatiles produced by spoilage fungi on bread analogue after (A) 40h, (B) 48h and (C) 72h growth at 25°C. Key to treatments: b1-b6, blank bread slices; a1-a6, *E. amstelodami*; c1-c6, *E. chevalieri* and p1-p6, *P. chrysogenum*.

As seen from the PCA (Figure 3.35) after 26h growth, there was a close cluster for the control untreated bread, and a clear difference for *P. chrysogenum*, prior to visible signs of growth. However, for the *Eurotium* species there was less discrimination. After 40h, discrimination between the growth of *Eurotium* spp., *P. chrysogenum* on bread substrate, and the control was obtained (Figure 3.36). After 48h this was even more distinct.

The cluster analysis of the data illustrates that after 40h growth, it was possible to differentiate the control untreated bread and *P. chrysogenum* but not the *Eurotium* spp. (Figure 3.37).

3.6.2 Enzyme activity

Seven hydrolytic enzyme activities were assayed and Figure 3.38 shows the specific activities of three of these enzymes. Each point on the graph represents the mean of four replicates and the least significant differences (L.S.D.) are shown as line bars on the graphs. For all the enzymes, the background levels in the control were very low. Significant increases in specific enzyme activities were observed for three enzymes after 48h, when initial visible signs of growth were observed. These were N-acetyl-β-D-glucosaminidase, α-D-galactosidase and β-D-glucosidase. Generally the activity of the first two enzymes was significantly higher for the *Eurotium* spp., while for the latter enzyme, the activity was significantly higher in bread colonised by *P. chrysogenum*.
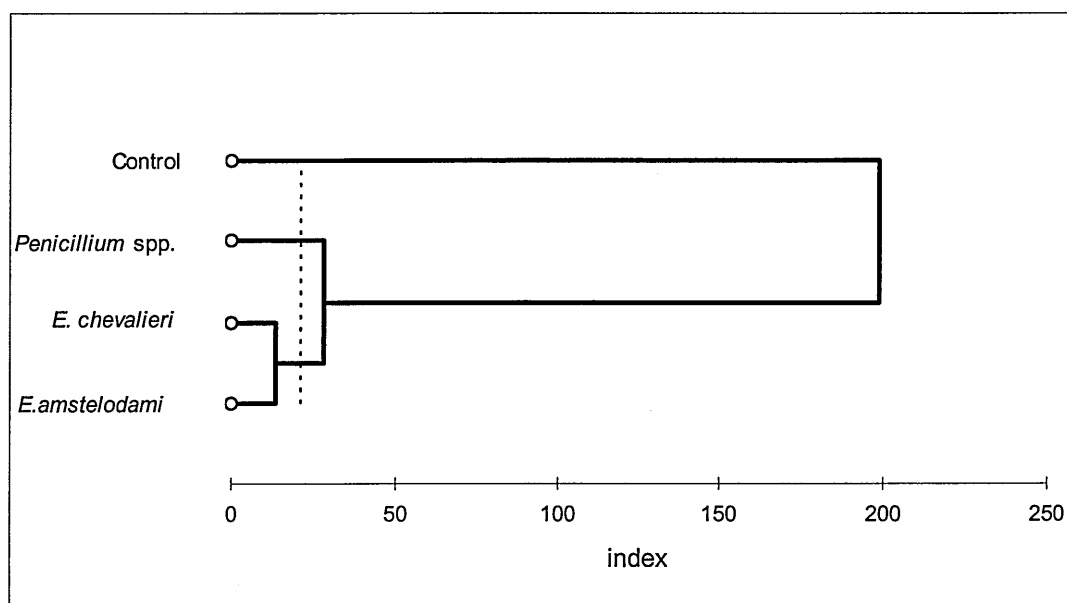
Figure 3.37 Cluster analyses of sensor array response data to volatiles produced by spoilage fungi after 40h inoculation on bread analogue showing the discrimination between the untreated control bread sample, *Penicillium chrysogenum* and the similarity between the two *Eurotium* spp.
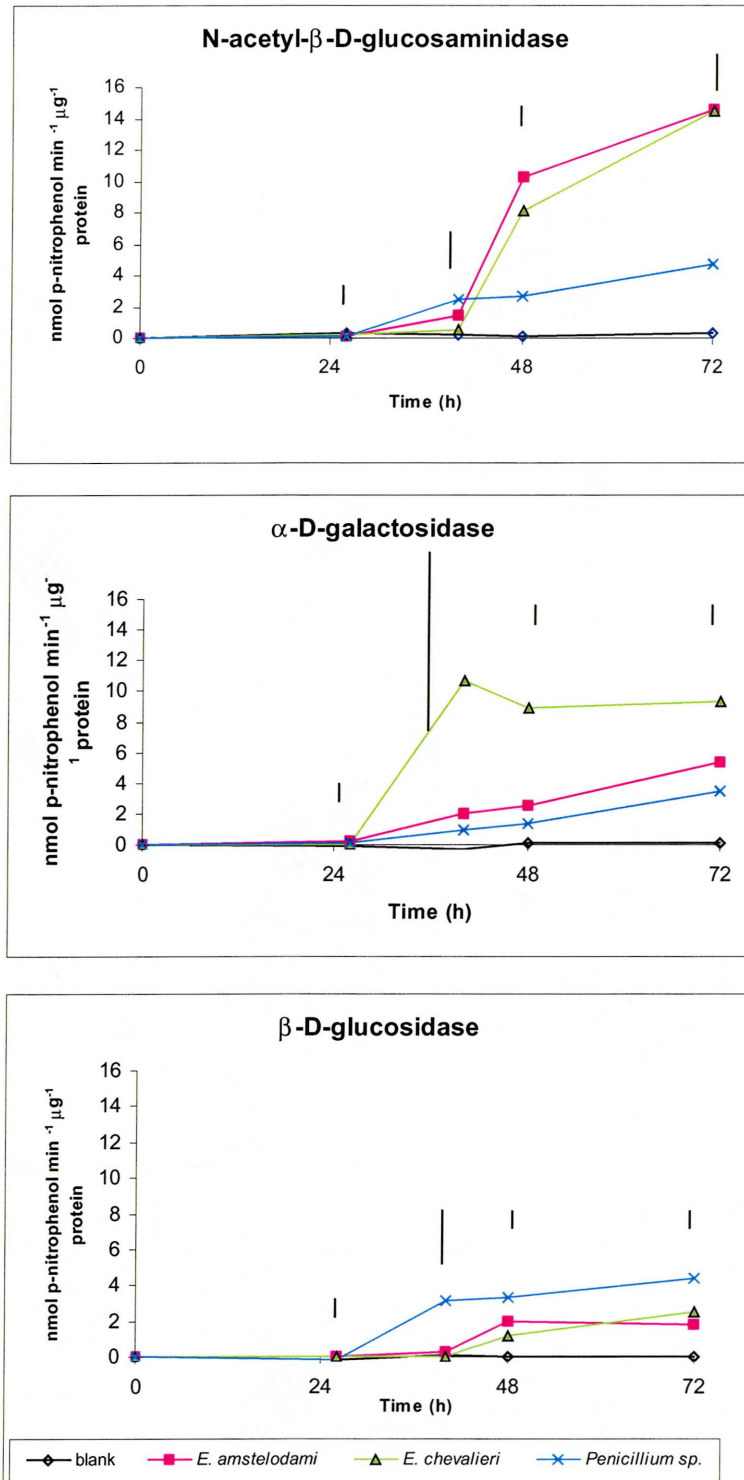
Figure 3.38 Temporal changes in specific activities of three enzymes produced by *E. amstelodami*, *E. chevalieri* and *P. chrysogenum* on bread analogue slices ($0.95a_w$). The blank control is the uninoculated bread analogue slices. The bars at each time interval indicates the least significant difference ranges ($P < 0.05$).

The activity of β-D-xylosidase and N-acetyl-α-D-glucosaminidase changed significantly after 48h and 72h respectively, for both *P. chrysogenum* and *E. amstelodami* (see Appendix G). Furthermore, an increase in enzyme activity was also found to be significant for α-D-mannosidase for the latter *Eurotium* spp. and β-D-fucosidase for *P. chrysogenum* species after 72h.

3.6.3 Fungal population changes

The change in the fungal population over a period of 72h is shown in Figure 3.39. Generally, the increase in populations of *Eurotium* spp. was slower than that of *P. chrysogenum*. Some microscopic growth was present after 40h in the *Penicillum* species treatments but not the *Eurotium* inoculated bread. However, over the incubation period, by 72h at 25°C, the inoculated bread treatments all carried populations $>10^6$ CFUs g^{-1} fresh weight bread.

3.7   DETERMINATION OF THE THRESHOLD LIMIT OF DETECTION OF FUNGI ON BREAD SUBSTRATE USING THE ELECTRONIC NOSE

3.7.1   Volatile patterns

Three inoculum concentrations (10^2, 10^4 and 10^6 spores ml^{-1}) of a *Penicillium* sp. and an *Eurotium* sp. were used. The volatile patterns produced by the species grown on bread analogue (0.95 a_w) were measured using the electronic nose to determine the threshold detection limit.
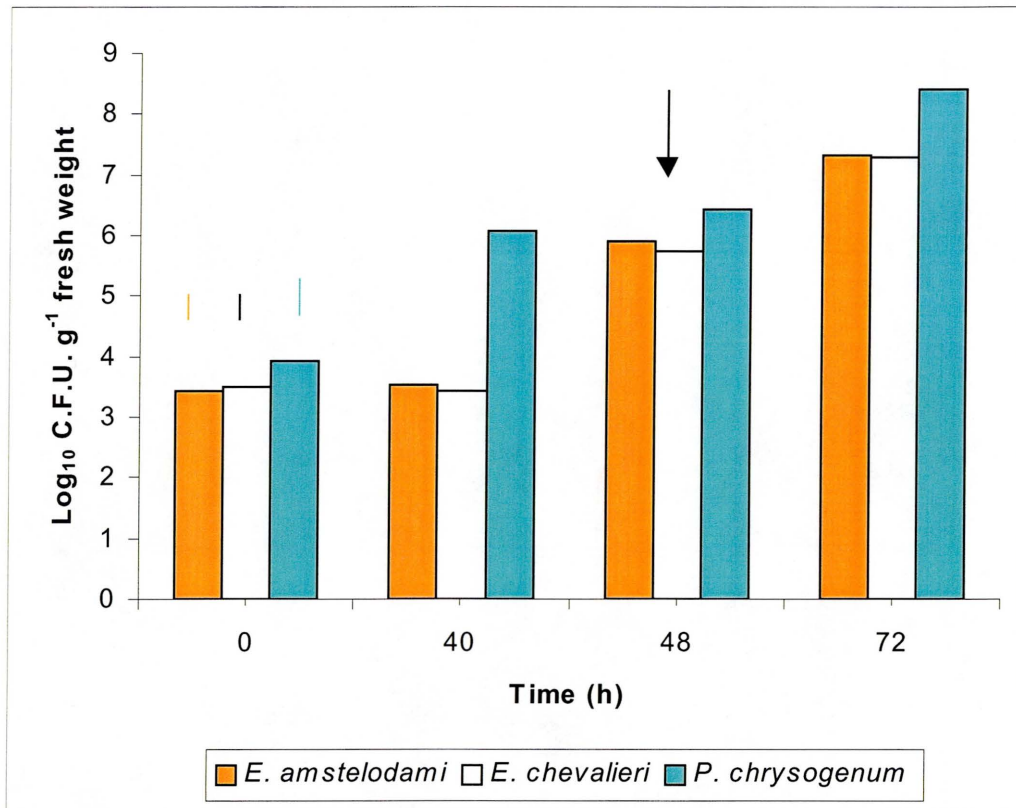
Figure 3.39 Temporal changes in $\log_{10}$ colony forming units (CFUs) for three fungal species on bread analogue (0.95 a_w) over the experimental period. Bars indicate least significant difference (L.S.D.) for each treatment (P <0.05) over time. Arrow indicates when visible growth was observed.

Figure 3.40 illustrates the array response to volatiles produced by four replicates of *P. chrysogenum* grown on bread analogue after 24h incubation. Reasonable reproducibility was obtained for the three inoculum concentrations. However, it must be noted that the sensor array had to be rebalanced by Bloodhound Sensors Ltd. at this stage of the project.

Figure 3.41 shows the principal component analysis of sensor response to fungal volatiles after 24h growth, prior to visible signs of growth. It was possible to differentiate the uninoculated bread analogue from that colonised by the spoilage fungal species (Figure 3.41 A). The individual PCA analyses of the separate species and the blanks are shown in Figures 3.41 B and C. For *E. chevalieri*, 10^6 spores ml^{-1} treatment could be differentiated, whereas the 10^4 and 10^2 spores ml^{-1} treatments clusters were closer together. In the case of *P. chrysogenum*, the difference was less clear. However, it was still possible to differentiate 10^6 spores ml^{-1} treatment, and an overlap was seen between 10^4 and 10^2 spores ml^{-1} treatments.

The cluster analysis of the individual species are shown in Figures 3.42 A and B. It was possible to differentiate 10^2 and 10^6 spores ml^{-1} treatments in the case of *P. chrysogenum*. It was not possible to separate out the blank and the 10^4 spores ml^{-1} treatments. In contrast, for *E. chevalieri*, it was possible to differentiate 10^4 and 10^6 spores ml^{-1} treatments.
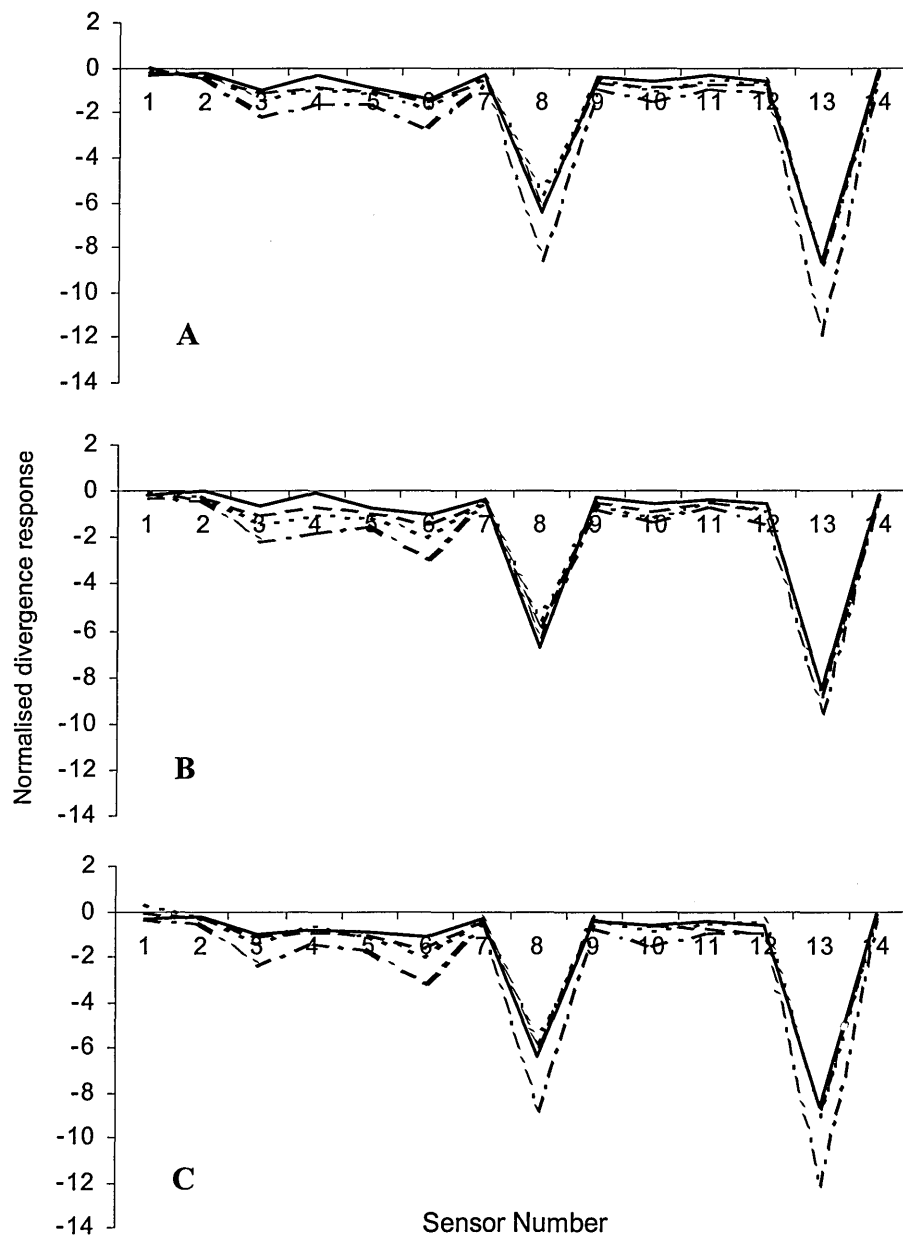
Figure 3.40 Sensor responses produced by volatiles from four replicates of *P. chrysogenum* of different initial inocula concentrations (A) 10^6, (B) 10^4 and (C) 10^2 spores ml^{-1} after 24h growth.
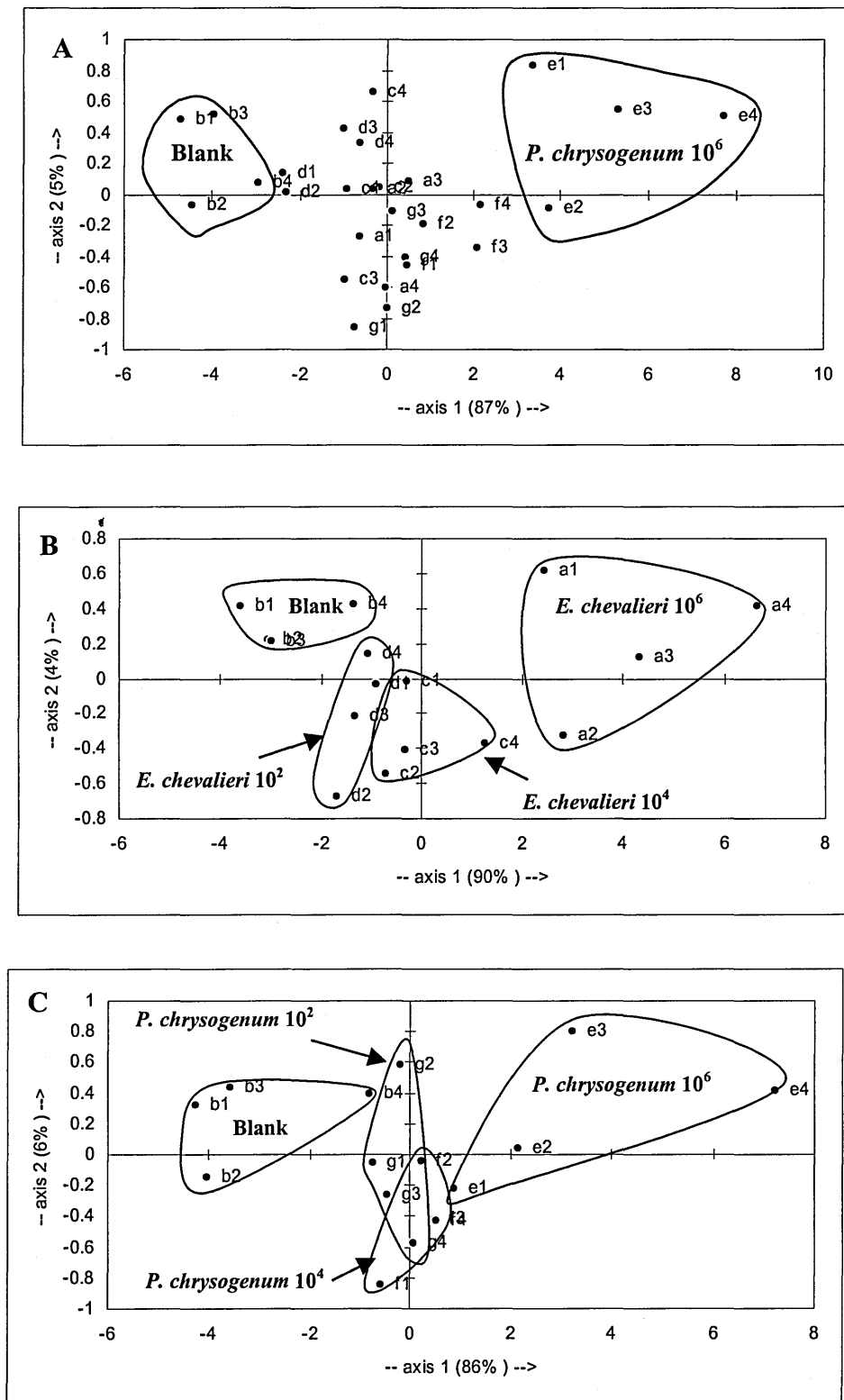
Figure 3.41 Principal component analysis of volatiles produced by *E. chevalieri* and *P. chrysogenum* at three different initial inocula concentrations (10^6, 10^4 and 10^2 spores ml^{-1}) after 24h growth. B and C show the PCA of the individual species and the control blank.
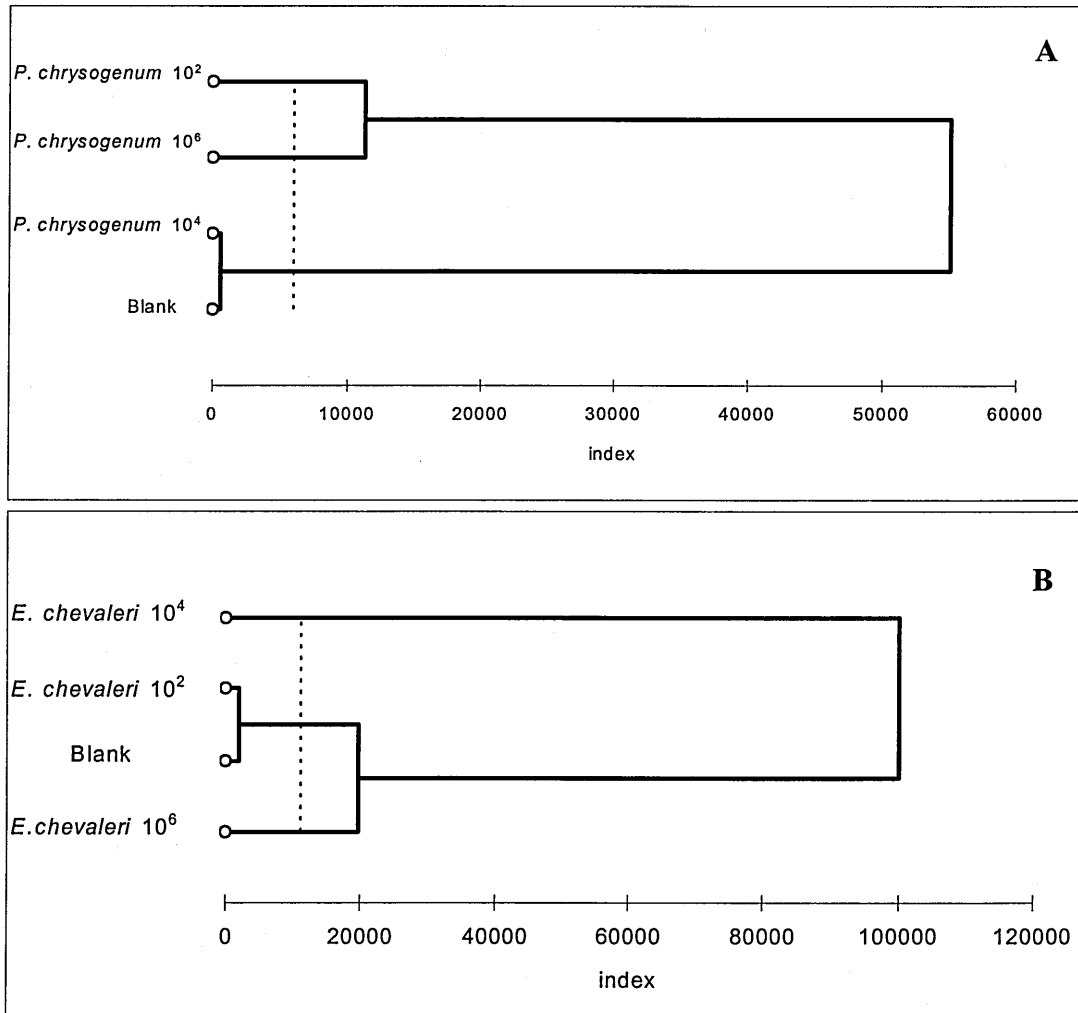
Figure 3.42 Cluster analyses of the sensor response data to volatiles produced by (A) *P. chrysogenum* and (B) *E. chevalieri* after 24h growth on bread analogue showing the differentiation of the different inoculum concentrations.

Better separation was achieved for *P. chrysogenum* after 72h when visible growth was present (Figure 3.43 A). The PCA showed clear clusters for 10^6 spores ml^{-1} treatment and the blank controls, and close clusters for 10^4 and 10^2 spores ml^{-1} treatments. In the case for *E. chevalieri* (Figure 3.43 B), the uninoculated blank was separate from the *Eurotium* species and some overlap was seen between the three inoculum concentrations treatments.

The cluster analyses after 48 and 72h are shown in Figure 3.44. It was possible to differentiate the uninoculated bread analogue and 10^6 spores ml^{-1} treatment for *P. chrysogenum* after 48h (Figure 3.44 A) when visible signs of growth is present. However, for *E. chevalieri* the cluster analysis result was similar to that seen after 24h (Figure 3.44 B) at initial visible growth. After 72h, it was possible to discriminate 10^4 and 10^6 spores ml^{-1} treatments for *E. chevalieri* (Figure 3.44 C). For *P. chrysogenum*, 10^6 spores ml^{-1} treatment and the blanks could be discriminated (Figure 3.44 D).

3.7.2 Total fungal population

Figure 3.45 shows the temporal population changes for the two fungal species over the experimental period. The increase in population was found to be slower for *E. chevalieri* in comparison to that for *P. chrysogenum*. For all treatments, initial visible growth was observed after 48h growth. All inoculated treatments carried populations 10^6 CFUs g^{-1} fresh weight bread after 48 and 72h incubations for the *Penicillium* sp. and the *Eurotium* sp. respectively.
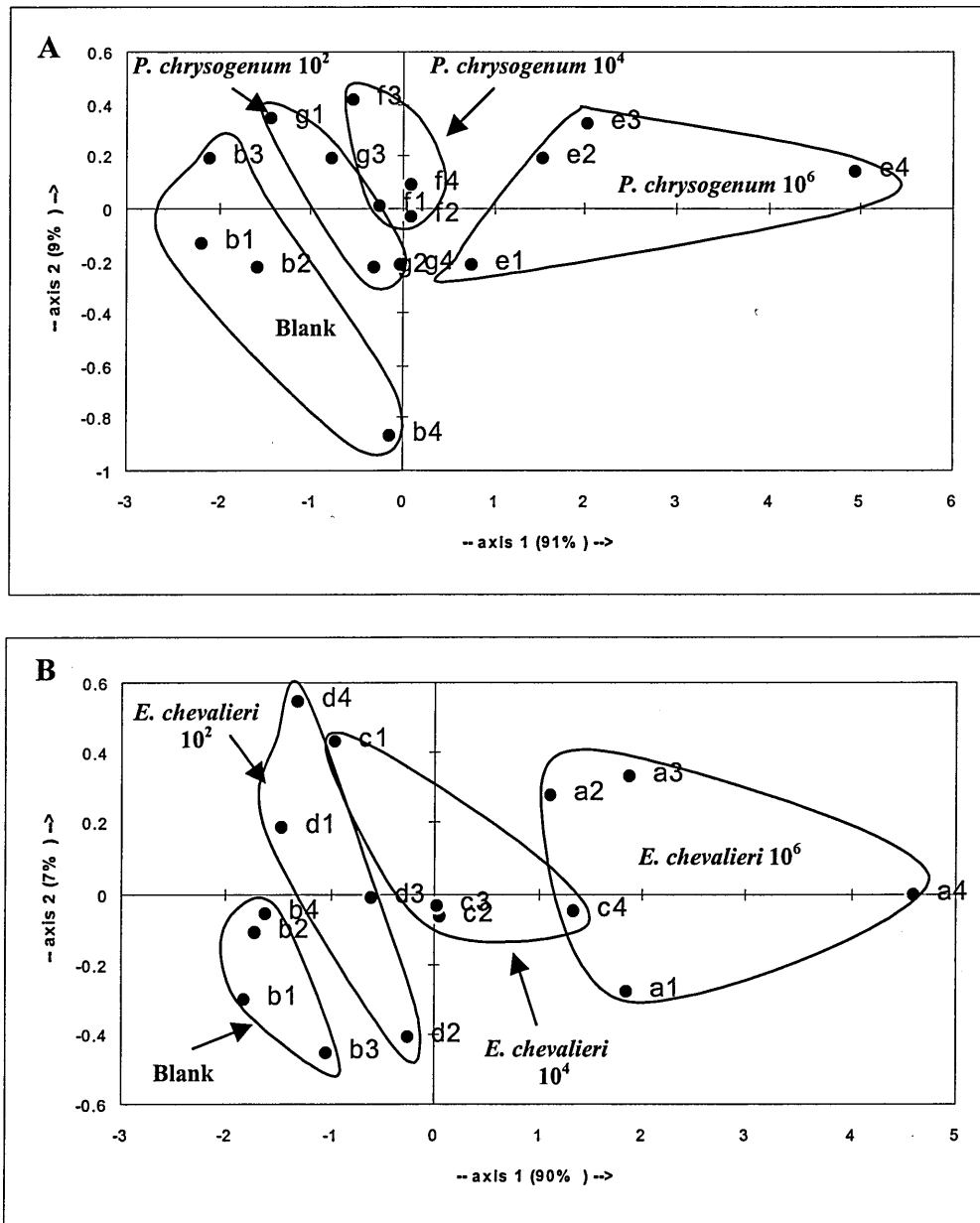
Figure 3.43 Principal component analysis showing the discrimination of (A) *P. chrysogenum* and (B) *E. chevalieri* at three initial inoculum concentrations (10^2, 10^4 and 10^6 spores ml^{-1}) after 72h incubation at 25°C. The blanks are the uninoculated bread analogue.
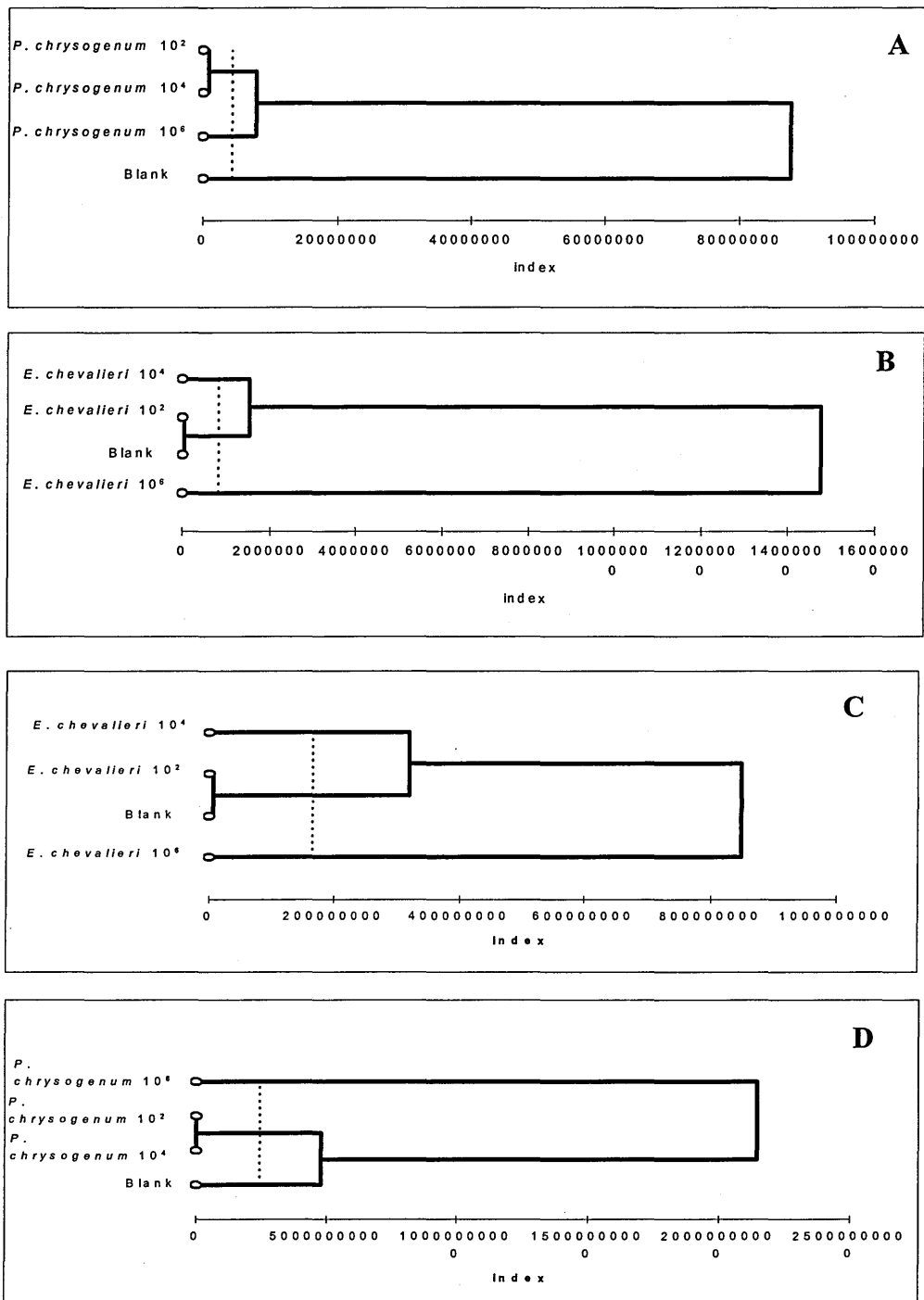
Figure 3.44 Cluster analysis showing the differentiation of *P. chrysogenum* and *E. chevalieri* at three initial inoculum concentrations (10^2, 10^4 and 10^6 spores ml^{-1}) after 48h (A and B), and 72h (C and D) incubation at 25°C. The blanks are the uninoculated bread analogue.
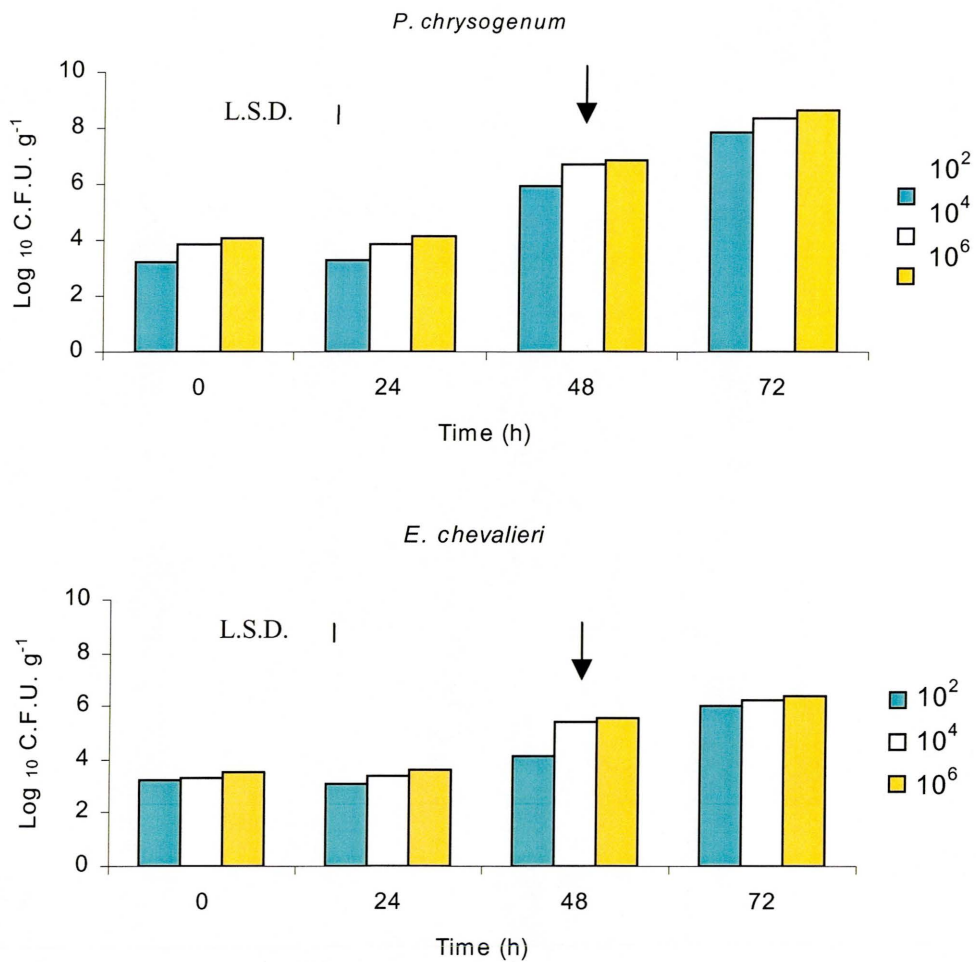
Figure 3.45 Temporal population changes for *P. chrysogenum* and *E. chevalieri* on bread analogue over the experimental period at initial inoculum concentrations 10^2, 10^4 and 10^6 spores ml^{-1}. Bars indicate least significant difference (L.S.D.) for each treatment ($P < 0.05$) over time. Arrow indicates when visible growth was observed.

3.8 EFFECT OF POTASSIUM SORBATE PRESERVATIVE ON FUNGAL VOLATILE PATTERNS, HYDROLYTIC ENZYMES, AND TOTAL POPULATIONS

3.8.1 Plates

Plate 3.1 shows the colonisation of *P. chrysogenum* on modified bread substrate containing different levels of potassium sorbate after 48h incubation. The most growth was observed in Plate 3.1 A (in the absence of potassium sorbate) and the least in C containing 0.3% potassium sorbate. Plate 3.2 illustrates growth of *E. amstelodami, E. chevalieri* and *P. chrysogenum* after 72h incubation. Although it is less clear in Plate 3.2, fungal colonisation was observed to increase with decreasing potassium sorbate content for each fungal species.

3.8.2 Volatile patterns

Volatile patterns produced by three spoilage fungi grown on bread analogue in the absence and presence of potassium sorbate were measured using an electronic nose. Figure 3.46 shows the sensor response to volatiles produced by four replicates of *P. chrysogenum* after 24h incubation. Better reproducibility was obtained for the 0% and 0.3% potassium sorbate treatments.
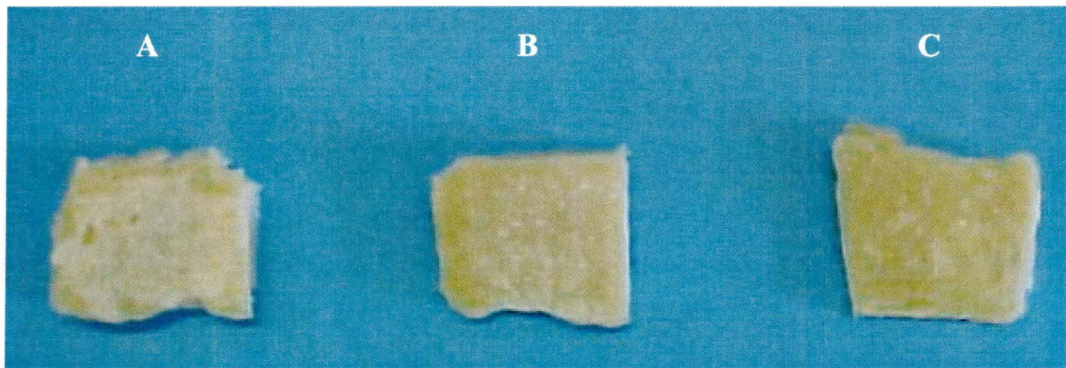
Plate 3.1 Growth of *Penicillium chrysogenum* after 48h, on bread analogue in the (A) absence of potassium sorbate, and presence of (B) 0.15% and (C) 0.3% potassium sorbate.
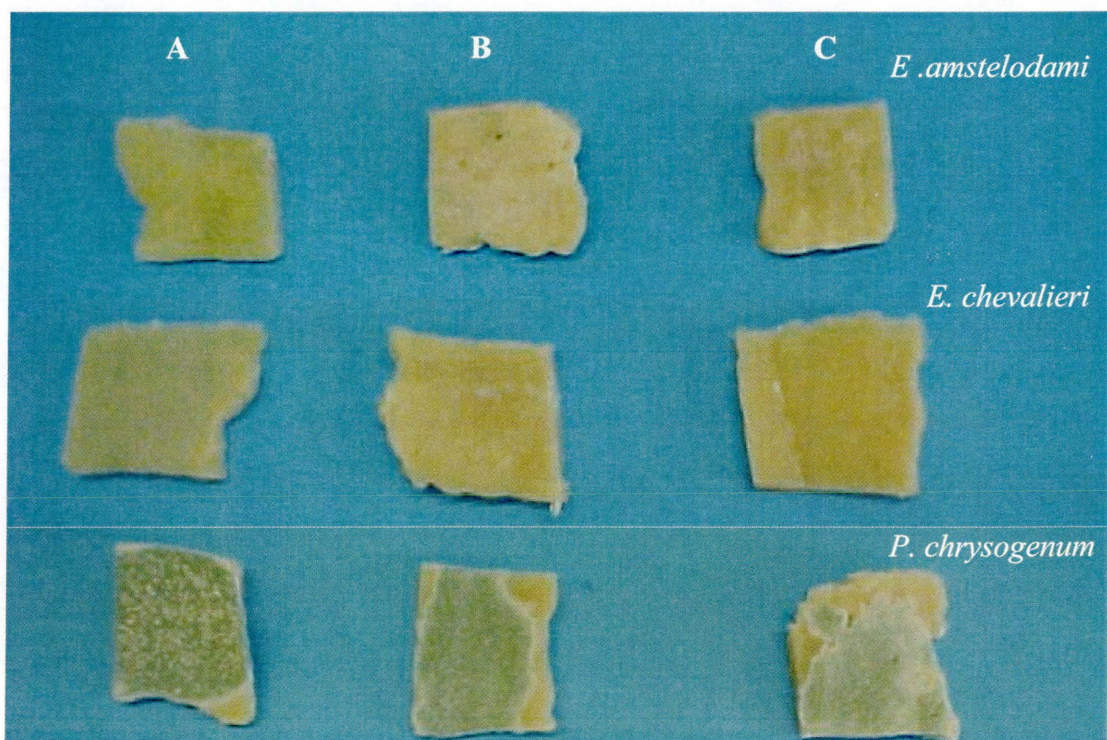
Plate 3.2 Growth of three fungi (*E. amstelodami*, *E. chevalieri* and *P. chrysogenum*) after 72h, on bread analogue in the (A) absence of potassium sorbate, and presence of (B) 0.15% and (C) 0.3% potassium sorbate.
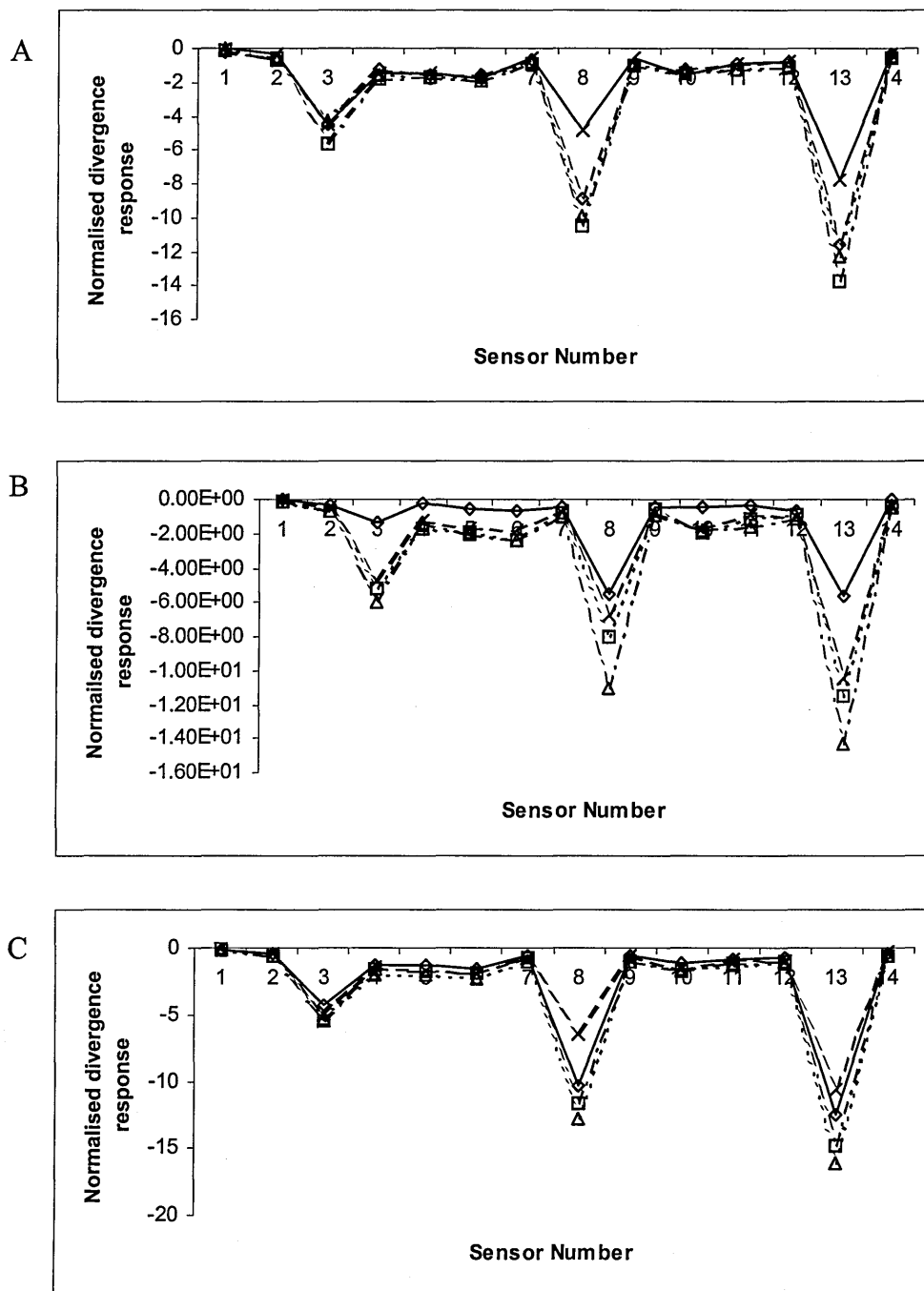
Figure 3.46 Reproducibility of the sensor array to volatiles produced by four replicates of *P. chrysogenum* grown on bread analogue modified to 0.95 a_w (A) in the absence of potassium sorbate; (B) in the presence of 0.15% and (C) 0.3% potassium sorbate after 24h incubation.

Principal component analyses were performed and are shown in Figure 3.47. After 24h, it was possible to see clusters for blank (no potassium sorbate), blank 0.15% and *P. chrysogenum* 0.3% potassium sorbate treatments, prior to visible growth. Better separations were seen after 48 and 72h. The control treatment (no sorbate) were observed to have visible growth after 48h. Whereas, only *P. chrysogenum* 0.15% and 0.3% potassium sorbate treatments were noted to have initial visible and microscopic growth, respectively. After 72h, visible growth was noted for all treatments with the exception of the two *Eurotium* 0.3% potassium sorbate treatments, which had microscopic signs of growth. From the PCA, it was possible to discriminate the uninoculated bread analogues, *P. chrysogenum* and *E. chevalieri* in the absence and presence of potassium sorbate (0.15 and 0.3%). No differentiation was seen for *E. amstelodami* treatments. The cluster analyses after 24h growth showed four groups with the differentiation of the uninoculated bread analogues in one group (Figure 3.48).

3.8.3   Hydrolytic enzymes

Figure 3.49 shows the activity of three enzymes produced by fungi grown on bread analogue substrate containing different levels of potassium sorbate. Specific activities of all seven enzymes assayed were found to change significantly over a period of time. Highest N-acetyl-β-D-glucosaminidase and α-D-galactosidase activities were observed for the *Eurotium* spp. and β-D-glucosidase activity for the *Penicillium* sp.. Generally the enzyme activity decreased for the *Eurotium* spp. and increased for the *Penicillium* sp., with increasing levels of potassium sorbate.
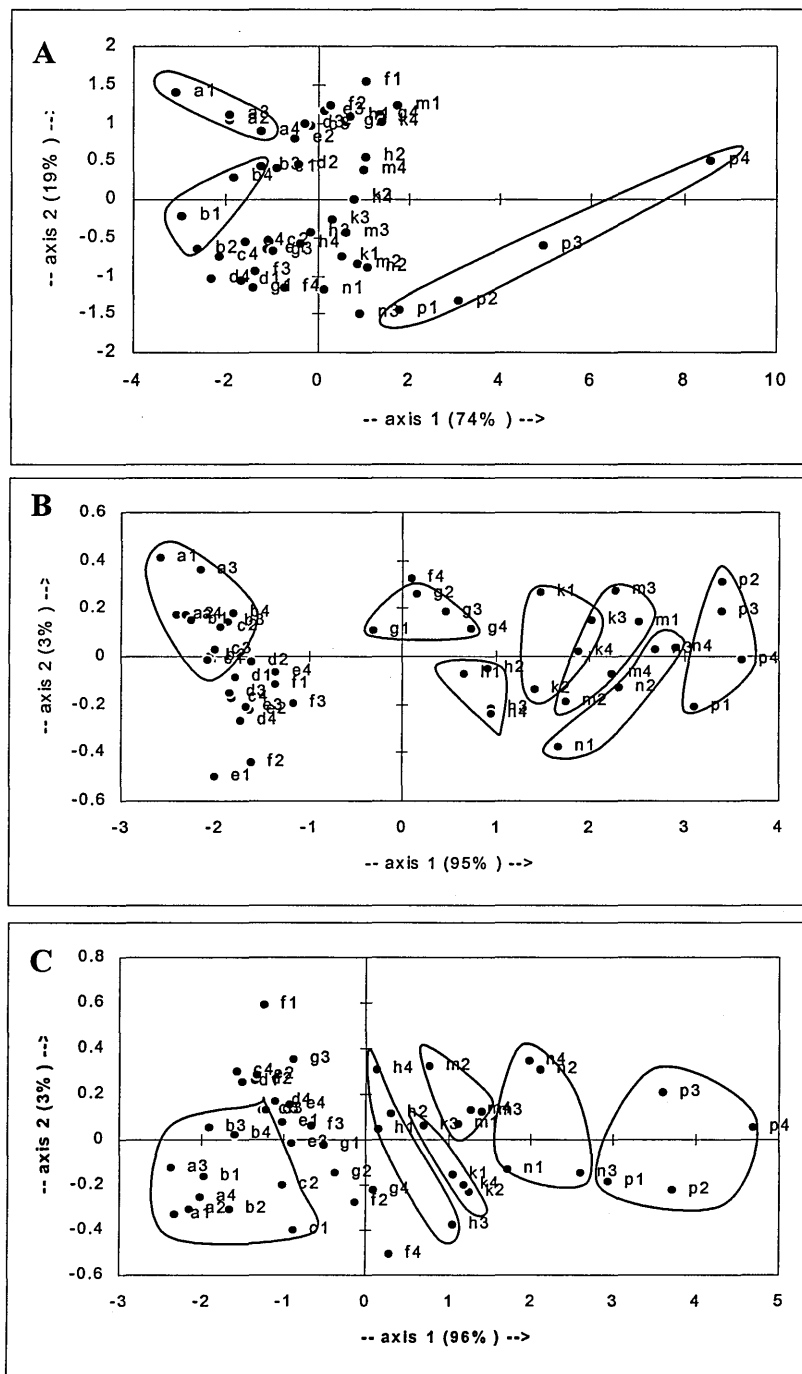
Figure 3.47 Principal component analysis of sensor response to fungal volatiles and blank bread analogues after (A) 24h, (B) 48h and (C) 72h growth in the absence and presence of potassium sorbate. Key to treatments:

Blank bread analogue	a1-a4, 0%; b1-b4, 0.15% and c1-c4, 0.30% sorbate.
Eurotium amstelodami	d1-d4, 0%; e1-e4, 0.15% and f1-f4, 0.30% sorbate.
E. chevalieri	g1-g4, 0%; h1-h4, 0.15% and k1-k4, 0.3% sorbate.
Penicillium chrysogenum	m1-m4, 0%; n1-n4, 0.15% and p1-p4, 0.30% sorbate.
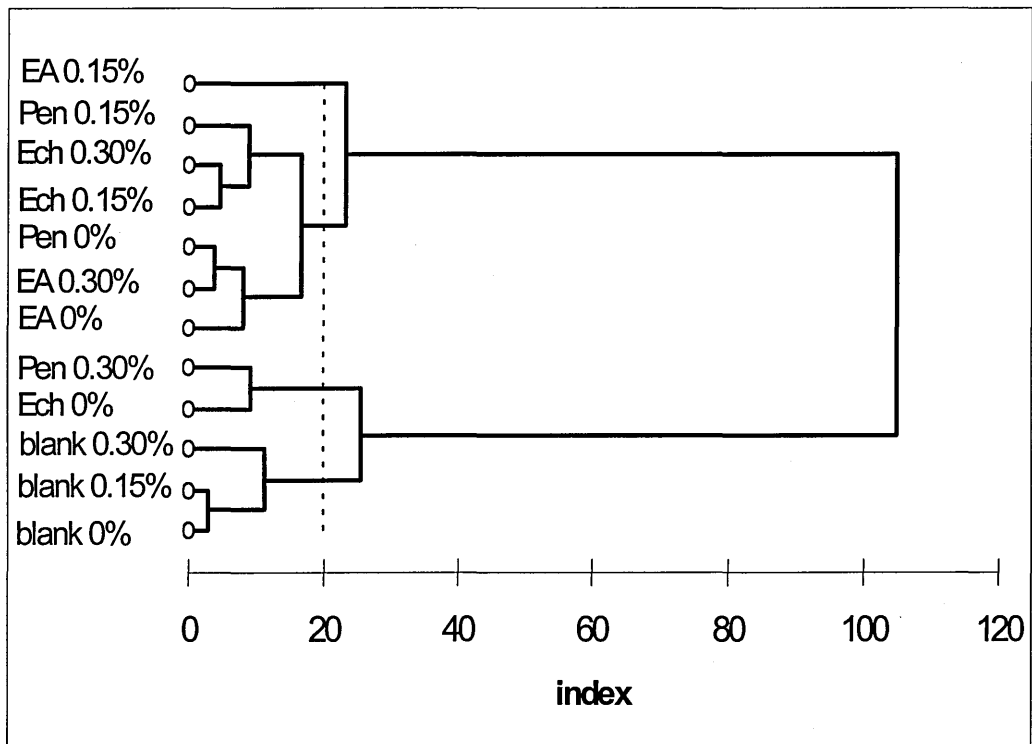
Figure 3.48 Cluster analysis after 24h showing the separation of uninoculated blank bread analogues (containing 0, 0.15% and 0.30% potassium sorbate) from that colonised by the spoilage fungi. Key to treatments: Pen, *P. chrysogenum*; Ech, *E. chevalieri* and EA, *E. amstelodami*.
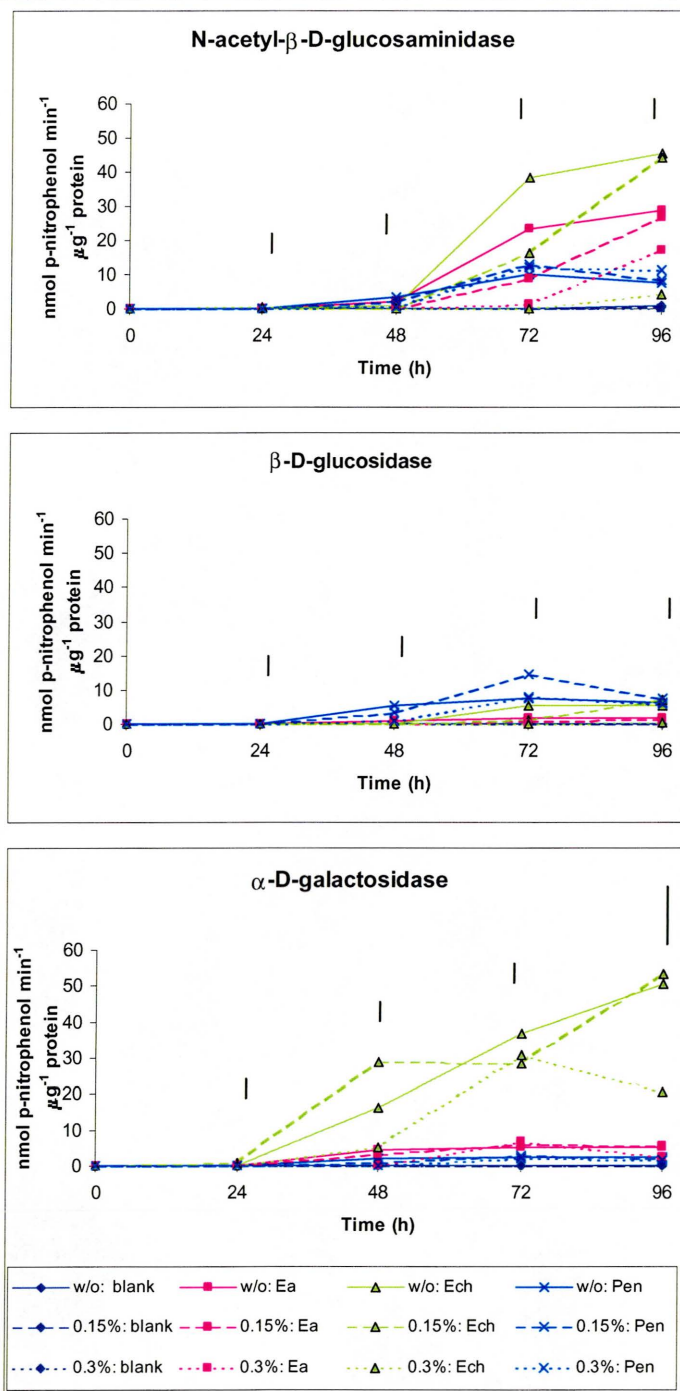
Figure 3.49 Temporal changes in specific activities of three enzymes produced by *E. amstelodami*, *E. chevalieri* and *P. chrysogenum* on bread analogue (0.95a$_w$) in the absence and presence of potassium sorbate. Key to treatments: w/o, 0% potassium sorbate; 0.15%, 0.15% potassium sorbate; 0.30%, 0.30% potassium sorbate; Ea, *E. amstelodami*; Ech, *E. chevalieri* and Pen, *P. chrysogenum*. The blank control is the uninoculated bread analogue. The bars at each time interval indicates the least significant difference ranges ($P < 0.05$).

Activity of N-acetyl-β-D-glucosaminidase and β-D-glucosidase significantly increased for the spoilage fungi colonising bread analogues both in the absence and presence of potassium sorbate. However, the increase was observed 24h later in the presence of potassium sorbate compared to that for the control treatment (no sorbate). In the case for the *Eurotium* spp., changes in the two enzyme activity were observed for the sub-optimal concentration of 0.15% potassium sorbate after 72 and 96h respectively. Whereas, changes for both enzyme activities were significant for *P. chrysogenum* 0.3% potassium sorbate treatment after 72h. Furthermore, *E. chevalieri* was found to have the highest and significant α-D-galactosidase activity after 48h, regardless of the levels of potassium sorbate present. However, the increase was observed 24h later for *E. amstelodami* 0.3% potassium sorbate treatment.

Colonisation of bread analogue by *P.chrysogenum* resulted in significant increases in β-D-fucosidase and β-D-xylosidase activity in the absence and presence of potassium sorbate (see Appendix G). However, in the presence of potassium sorbate the increase was observed 24h later compared to that for the control treatment (no sorbate). In contrast, *E. chevalieri* had significant enzyme activities after 72h only in the absence of potassium sorbate.

Significant increases in N-acetyl-α-D-glucosaminidase and α-D-mannosidase activity were noted for *E. amstelodami* in the presence of potassium sorbate (see Appendix G). However, for *P. chrysogenum* only the former enzyme activity was found to be significant in the presence of potassium sorbate. In addition, the latter enzyme activity was observed to increase 24h earlier in the presence of potassium sorbate compared to that for *P. chrysogenum* control treatment. *E. chevalieri* was

found to have an increase in the former enzyme activity 24h later in the presence of potassium sorbate and the latter enzyme activity only in the absence of potassium sorbate.

3.8.4   Total fungal population

The change in fungal population over time was determined for all the treatments used in this study and are shown in Figure 3.50. The increase in population was found to be the greatest for the control treatments (no sorbate) for all fungi after 48h, when visible signs of growth were present. However, the *Penicillium* sp. was observed to have the greatest increase in population in comparison to the *Eurotium* spp. in general. Furthermore, *P. chrysogenum* growing in the absence of potassium sorbate was noted to have populations $>10^6$ CFUs g^{-1} fresh weight bread after 48h. Whereas, all the remaining treatments carried populations $>10^6$ CFUs g^{-1} fresh weight bread after 72h for *Penicillium* sp., and after 96h in the case of the *Eurotium* spp.
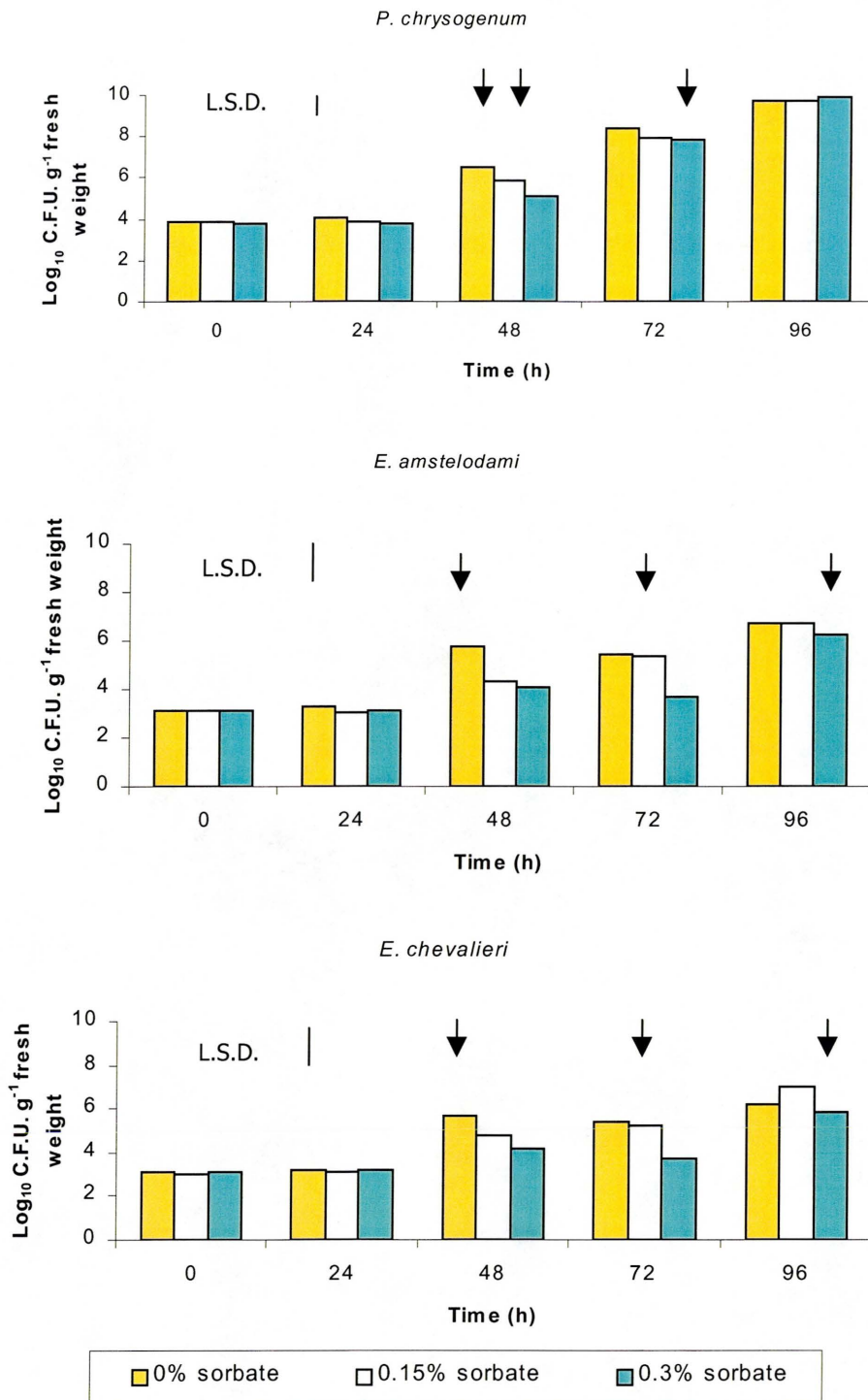
Figure 3.50 Log_{10} colony forming units (CFUs) for (A) *P. chrysogenum*, (B) *E. amstelodam*i and (C) *E. chevalieri* in the absence and presence of 0.15% and 0.3% potassium sorbate. Bars indicate least significant difference (L.S.D.) for each treatment (P <0.05). Arrows indicate when visible growth was observed.

3.9 EFFECT OF CALCIUM PROPIONATE PRESERVATIVE ON FUNGAL VOLATILE PROFILES, ENZYME ACTIVITIES AND TOTAL POPULATIONS

3.9.1 Volatile profiles

Volatile profiles from xerophilic spoilage fungi grown on bread analogues over a period of time were measured using the electronic nose. Figure 3.51 shows the array response to volatiles produced by four replicates of *P. chrysogenum* after 24h incubation on bread analogues modified with different levels of calcium propionate. Reproducibility of the array to fungal volatiles in the presence of calcium propionate was found to be poor.

The PCA of the data after 24 and 72h are shown in Figure 3.52. The PCA for 48h was found to be similar to that obtained for 24h. As seen by the PCA, after 24h it was possible to discriminate uninoculated bread analogues in the absence and presence of calcium propionate (0.15 and 0.3%), prior to visible growth. With the exception of *E. amstelodami* which had microscopic growth, all the control treatments (no propionate) were noted to have visible signs of growth, after 48h. In contrast, for the sub-optimal calcium propionate treatments (0.15%), only *E. chevalieri* and *P. chrysogenum* had microscopic and initial visible growth, respectively. For 0.3% treatments, only *P. chrysogenum* was observed to have initial visible growth. However, after 72h, uninoculated bread analogues containing no propionate (a1-a4) were found to be in a separate cluster compared to the other treatments, when visible growth for the three spoilage species was observed.
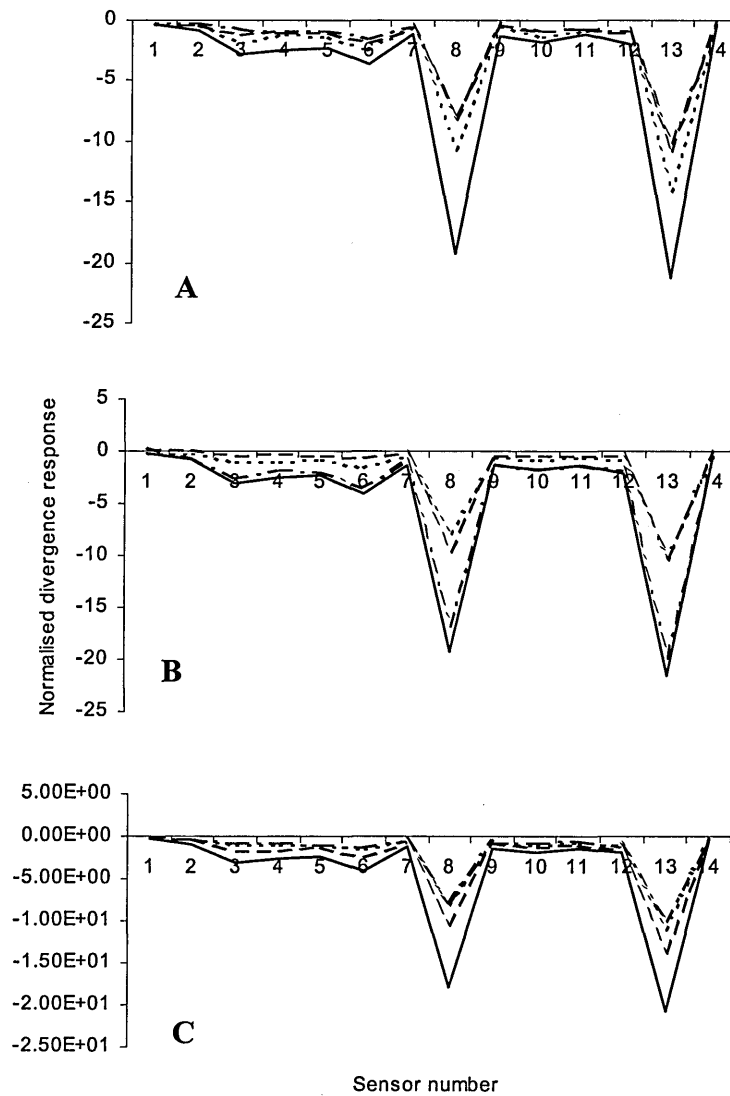
Figure 3.51 Reproducibility of the sensor array to volatiles produced by four replicates of *P. chrysogenum* grown on bread analogue modified to 0.95 a_w (A) in the absence of calcium propionate; (B) in the presence of 0.15% and (C) 0.3% calcium propionate after 24h incubation.
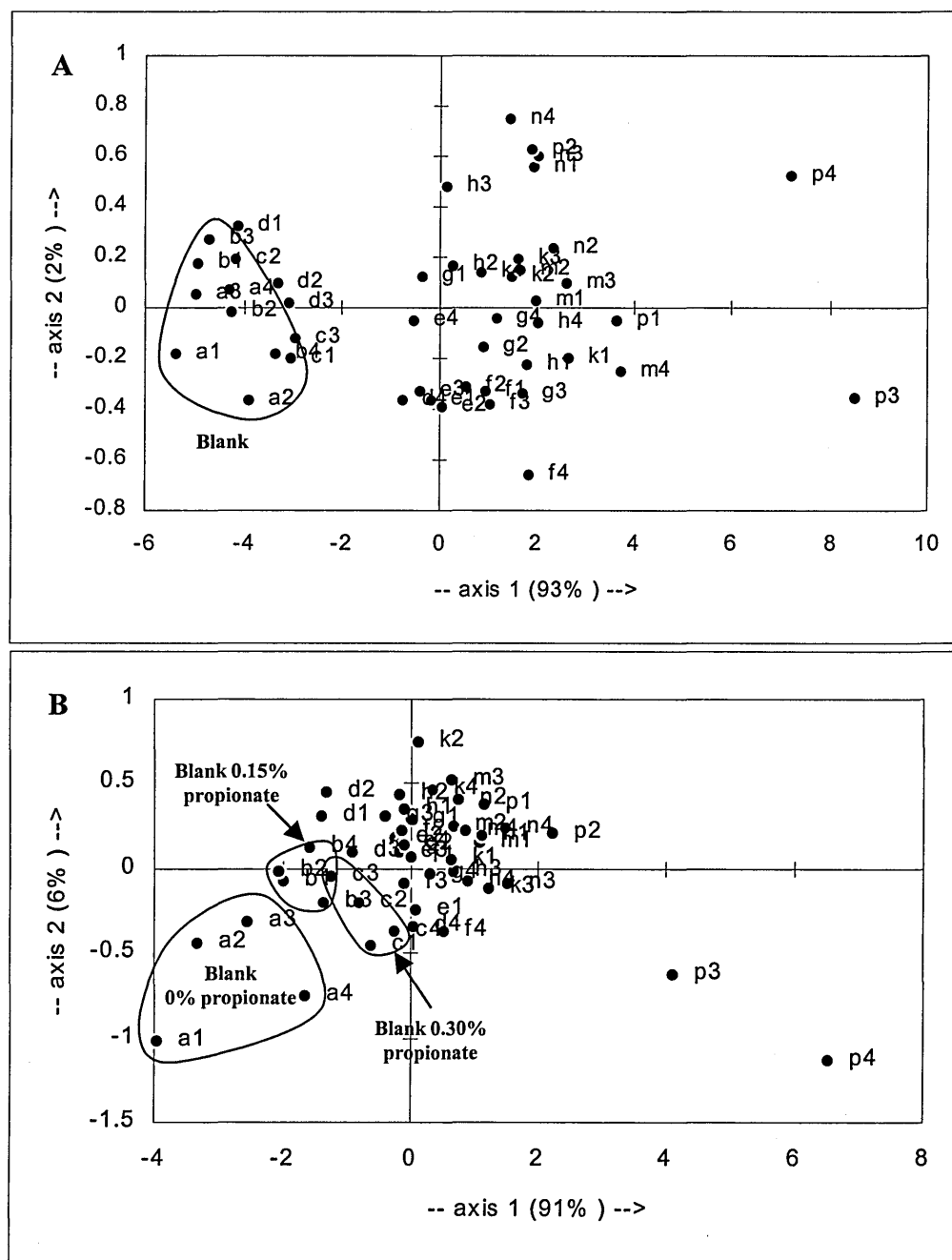
Figure 3.52 Principal component analysis of sensor response to fungal volatiles and blank bread analogues after (A) 24h and (B) 72h growth in the absence and presence of calcium propionate. Key to treatments:

Blank bread analogues   a1-a4, 0%; b1-b4, 0.15% and c1-c4, 0.3% propionate.

Eurotium amstelodami   d1-d4, 0%; e1-e4, 0.15% and f1-f4, 0.3% propionate.

E. chevalieri   g1-g4, 0%; h1-h4, 0.15% and k1-k4, 0.3% propionate.

Penicillium chrysogenum   m1-m4, 0%; n1-n4, 0.15% and p1-p4, 0.3% propionate.

The cluster analysis after 24h growth (Figure 3.53) also showed the discrimination of uninoculated bread analogues in the absence and presence of calcium propionate (0.15 and 0.3%).

3.9.2 Enzyme activities

Activities of three enzymes produced by spoilage fungi colonising bread analogues of different calcium propionate levels, were measured over a period of time and are illustrated in Figure 3.54. There were three predominant enzymes. Generally the specific activities of α-D-galactosidase and N-acetyl-β-D-glucosaminidase were higher for *E. chevalieri*, while for *P. chrysogenum*, β-D-glucosidase activity was observed to be the highest.

Activities of β-D-glucosidase and N-acetyl-β-D-glucosaminidase were found to be significant after 48h for *P. chrysogenum* colonising bread analogue in the absence and presence of calcium propionate. However, the highest activities were observed in the presence of calcium propionate (0.15% calcium propionate treatment). In the case of the *Eurotium* spp. the enzyme activities decreased with increasing calcium propionate levels. Furthermore, the activities increased significantly in the absence of calcium propionate for *E. chevalieri* and *E. amstelodami* and *E. chevalieri* 0.15% calcium propionate treatment. After 72h the activity of the latter enzyme was found to be significant for the *Eurotium* spp. for all treatments.
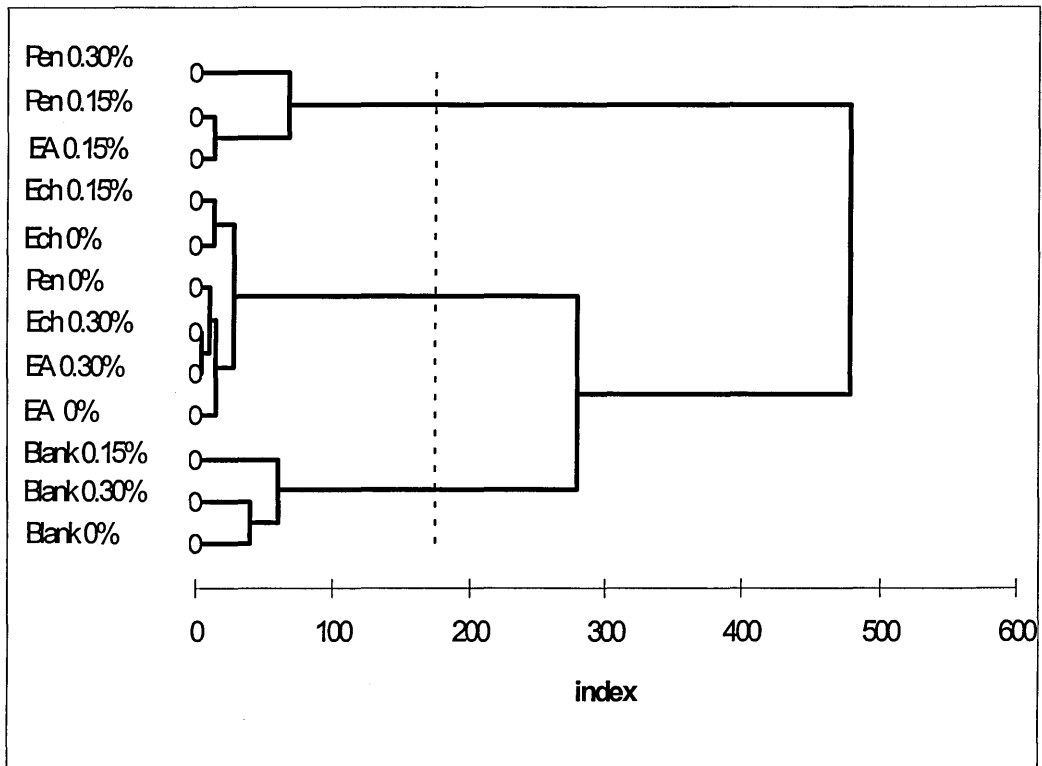
Figure 3.53 Cluster analysis after 24h showing the separation of uninoculated blank bread analogues (containing 0, 0.15% and 0.3% calcium propionate) from the spoilage fungi. Key to treatments: Pen, *P. chrysogenum*; Ech, *E. chevalieri* and EA, *E. amstelodami*.
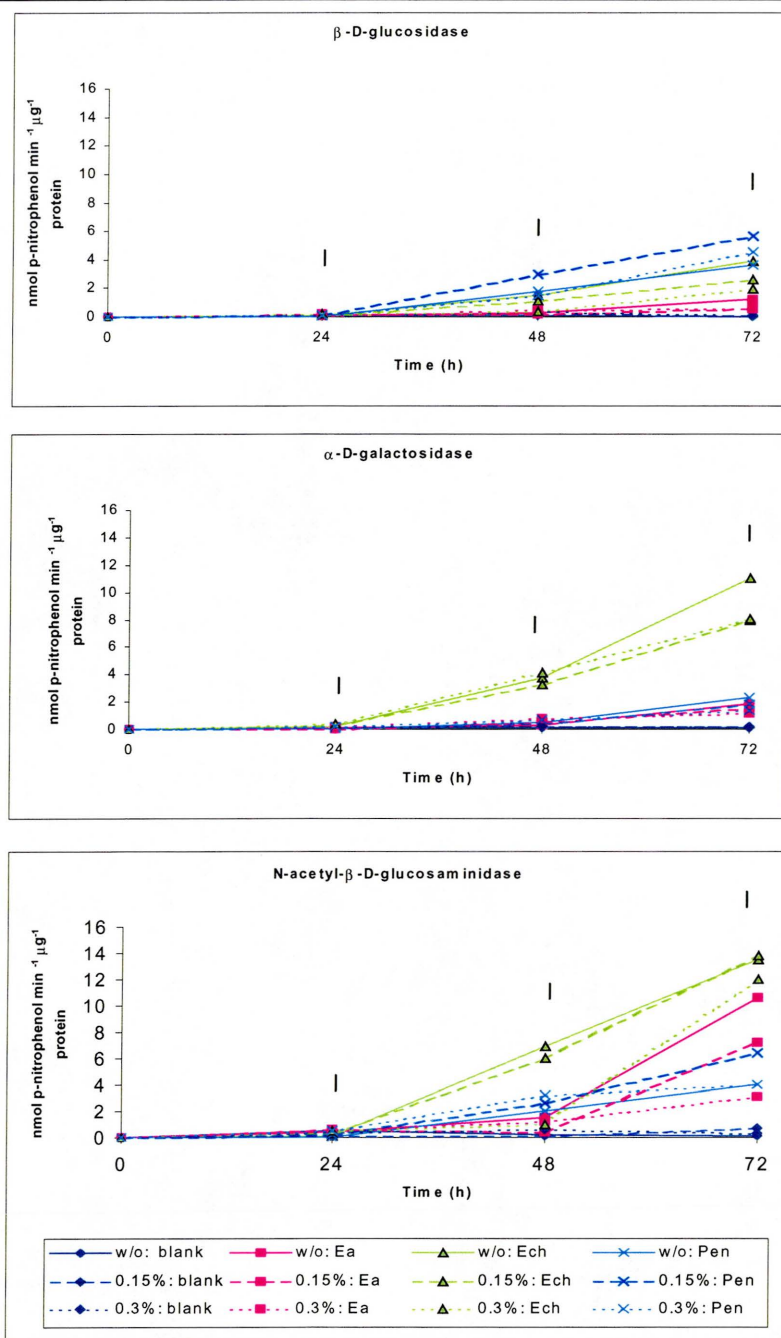
Figure 3.54 Temporal changes in specific activities (nmol p-nitrophenol min^{-1} µg^{-1} protein) of three enzymes produced by *E. amstelodami*, *E. chevalieri* and *P. chrysogenum* on bread analogue slices (0.95a$_w$) in the absence and presence of calcium propionate. Key to treatments: w/o, 0% calcium propionate; 0.15%, 0.15% calcium propionate; 0.30%, 0.3% calcium propionate; Ea, *E. amstelodami*; Ech, *E. chevalieri* and Pen, *P. chrysogenum*. The blank control is the uninoculated bread analogue slices. The bars at each time interval indicates the least significant difference ranges (P <0.05).

Significant α-D-galactosidase activity was found for *E. chevalieri* after 48h and for the three spoilage fungi after 72h in the absence and presence of calcium propionate. Generally the highest activity was observed for *E. chevalieri* in the absence of propionate.

Furthermore colonisation of bread analogue by *P. chrysogenum* resulted in an increase in β-D-xylosidase activity in the absence and presence of calcium propionate after 72h (see Appendix G). Whereas, for *E. chevalieri* the increase was only significant in the absence of calcium propionate. The activities of β-D-fucosidase and N-acetyl-α-D-glucosaminidase increased for *P. chrysogenum* in the presence of calcium propionate (0.3% propionate). *E. chevalieri* was also observed to have significant activity of the former enzyme in the presence of calcium propionate. No significant α-D-mannosidase activity was observed in the absence or presence of calcium propionate (see Appendix G).

3.9.3 Total fungal population

Figure 3.55 shows the change in fungal populations over time for all treatments used in this study. Overall the *Penicillium* sp. was noted to have the greatest population increase. Furthermore, greatest increase was observed in the control treatment (no propionate) for all three species.
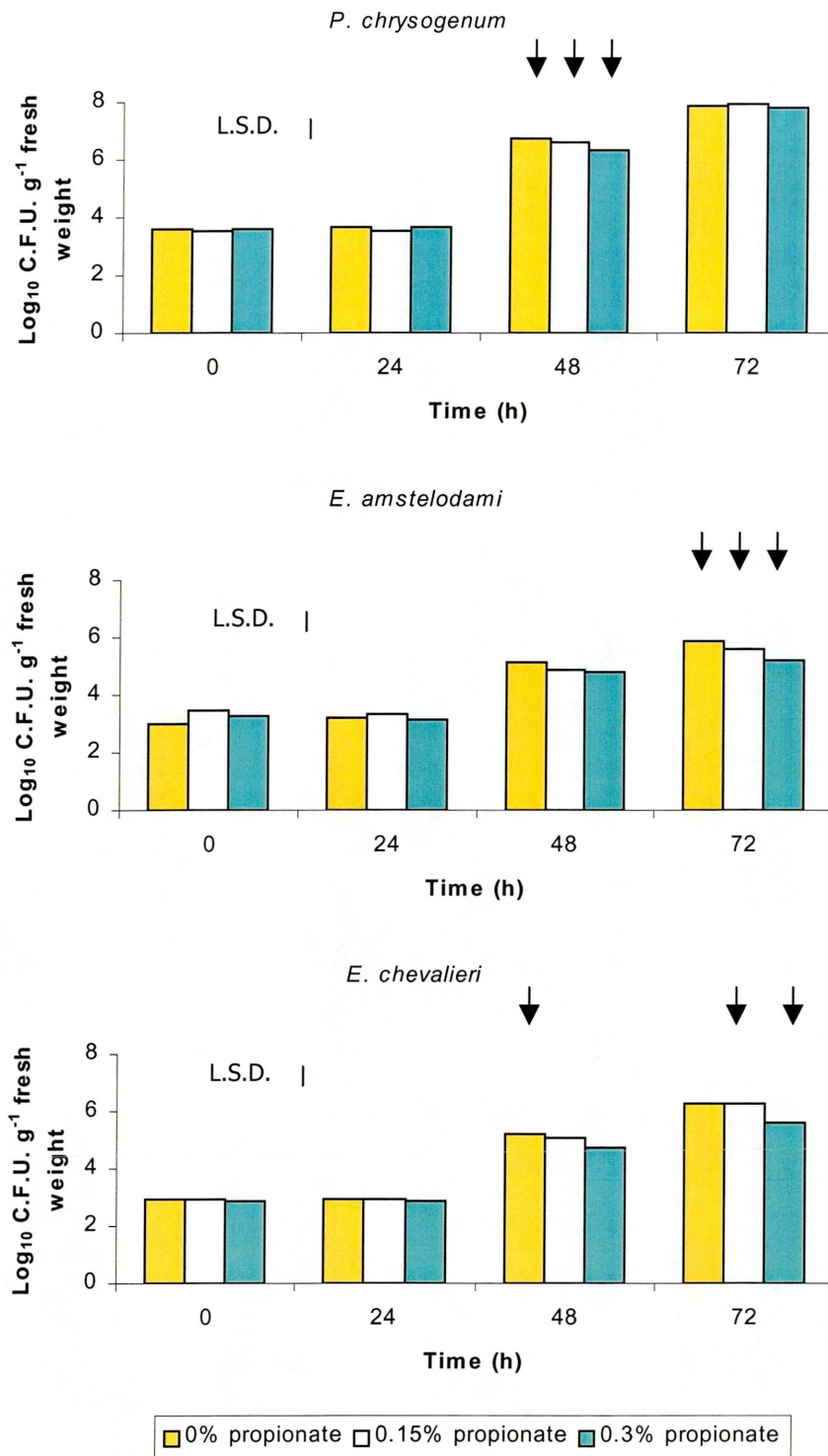
Figure 3.55 Log_{10} colony forming units (CFUs) for (A) *P. chrysogenum*, (B*) E. amstelodam*i and (C) *E. chevalieri* in the absence and presence of 0.15% and 0.3% calcium propionate. Bars indicate least significant difference (L.S.D.) for each treatment (P <0.05). Arrows indicate when visible growth was observed.

P. chrysogenum was found to have populations $>10^6$ CFUs g^{-1} fresh weight bread after 48h in the absence and presence of calcium propionate. Whereas *E. amstelodami* and *E. chevalieri* in the absence of calcium propionate, and with sub-optimal concentration of 0.15% propionate had populations $>10^6$ CFUs g^{-1} fresh weight after 72h.

3.10   DETECTION AND DIFFERENTIATION OF SPOILAGE FUNGI ON NATURAL BREAD SUBSTRATE USING THE ELECTRONIC NOSE

3.10.1 Volatile patterns

Figure 3.56 shows normalised divergence sensor response to volatiles produced by four replicates of *E. chevalieri*, grown on natural bread substrate after 24h incubation at 25°C. The sensor array was found to show good reproducibility for replicates of the same treatment.

PCA analyses were performed to try to discriminate between the fungi and the uninoculated bread substrate. Figure 3.57 shows the analyses after 24, 48 and 72h growth, utilising 95, 99 and 97% of the information respectively. After 24h growth, it was possible to differentiate the uninoculated bread substrate and *P. chrysogenum*, prior to visible signs of growth. Whereas for the *Eurotium* spp. some overlap was observed and the separation did not improved after 48 and 72h growth.

The cluster analyses of the data shown in Figure 3.58, illustrates the differentiation of the uninoculated control bread substrate and the *Penicillium* sp. but not the *Eurotium* spp. after 48 and 72h incubation.
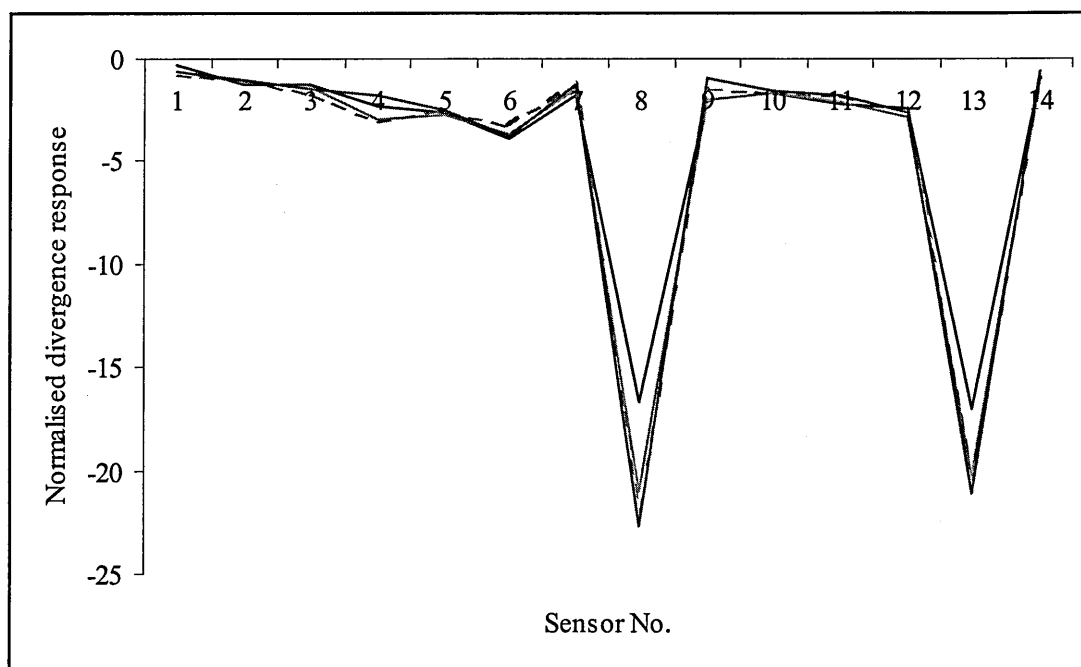
Figure 3.56 Normalised divergence responses showing reproducibility of 14 conducting polymer sensors to four replicates of *E. chevalieri* on natural bread substrate after 24h growth at 25°C.
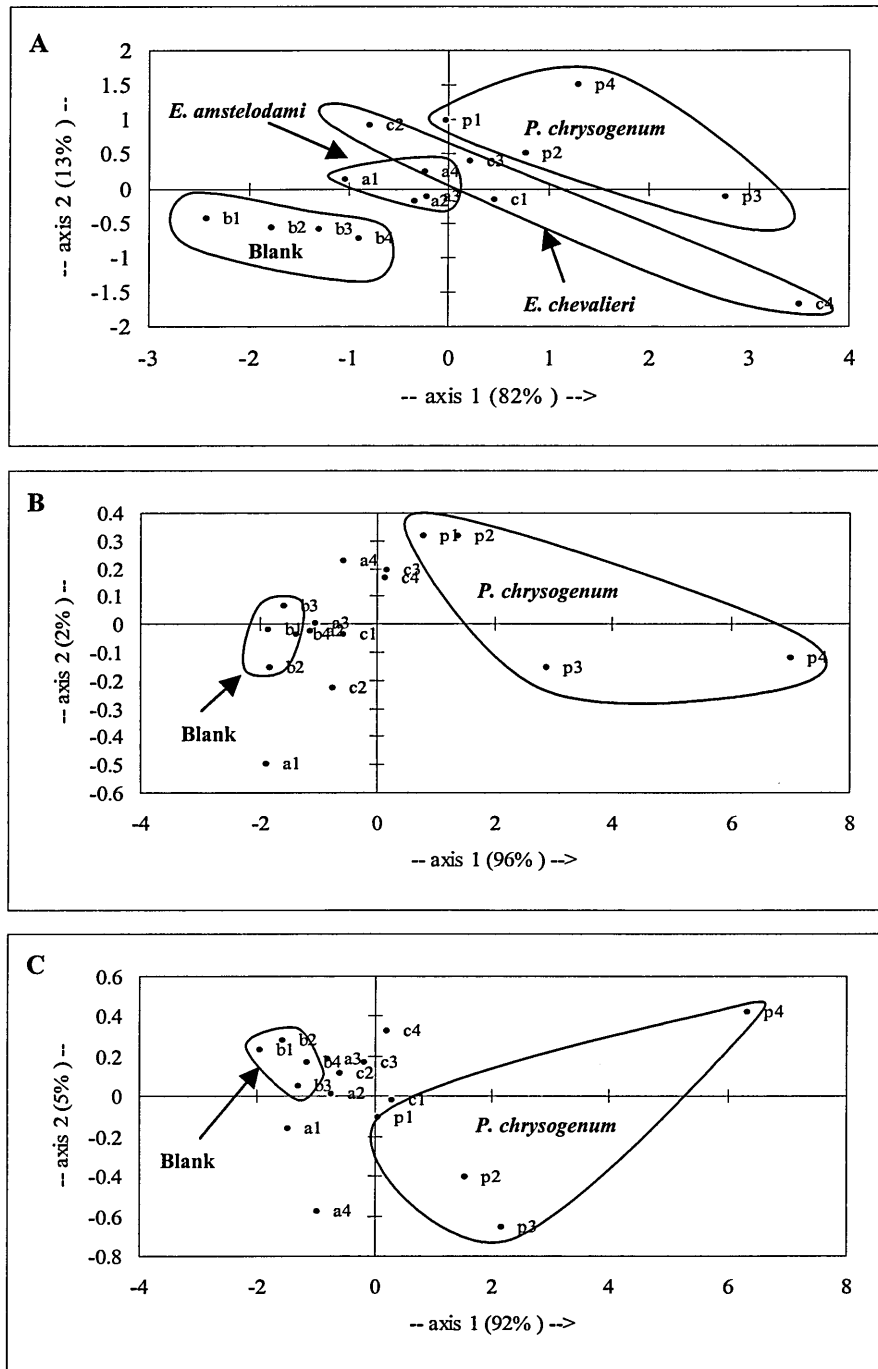
Figure 3.57 Principal component analysis of volatiles produced by spoilage fungi on natural bread substrate after (A) 24h, (B) 48h and (C) 72h growth at 25°C. Key to treatments: b, blank bread slices; a, *E. amstelodami*; c, *E. chevalieri* and p, *P. chrysogenum*.
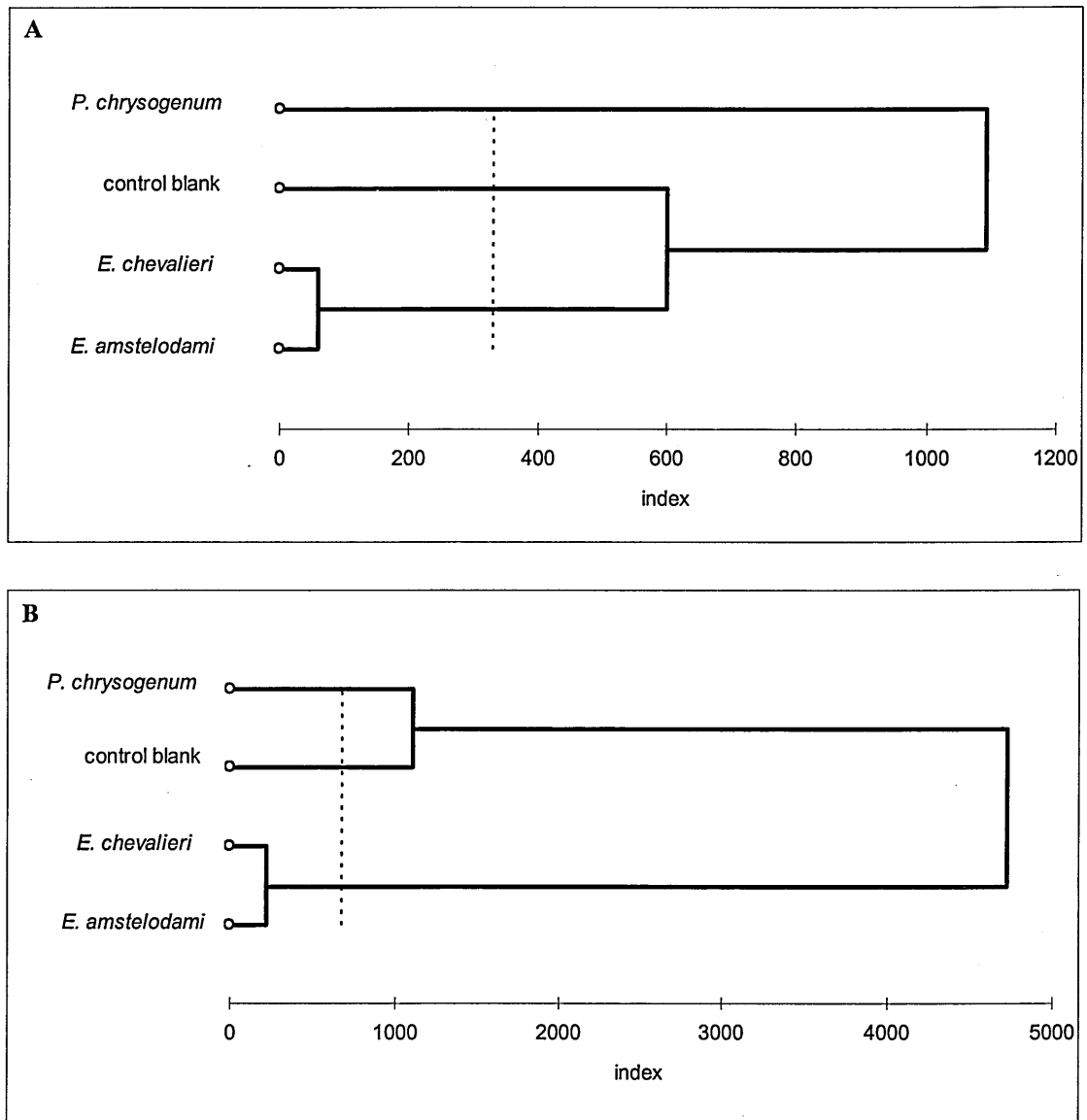
Figure 3.58 Cluster analyses of sensor array response data to volatiles produced by spoilage fungi after (A) 48h and (B) 72h inoculation on natural bread substrate showing the discrimination between the control bread sample, *Penicillium chrysogenum* and the similarity between the two *Eurotium* spp.

3.10.2 Enzyme activity

Seven hydrolytic enzymes produced by spoilage fungi colonising natural bread substrate were assayed and found to changed significantly over the period of incubation (Figure 3.59).

Control bread slices had very low activity of the enzyme assayed. However, activity of N-acetyl-β-D-glucosaminidase and α-D-galactosidase significantly increased in bread slices inoculated with *P. chrysogenum* and the *Eurotium* spp. after 48 and 72h respectively. *E. chevalieri* having the highest activity for both these enzymes.

Colonisation of bread by *P. chrysogenum* also resulted in an increase in β-D-glucosidase, α-D-mannosidase, β-D-xylosidase, β-D-fucosidase and N-acetyl-α-D-glucosaminidase activity after 72h (see Appendix G). Furthermore, significant increases in activities of the latter two enzymes were also observed for the *Eurotium* spp.

3.10.3 Total fungal population

Figure 3.60 illustrates the change in the fungal population over a period of time. The increase in population was found to be slower for the *Eurotium* spp. compared to that for *P. chrysogenum*. For all inoculated bread treatments some microscopic growth was observed after 48h incubation. Furthermore, all inoculated treatments carried populations $>10^6$ CFUs g^{-1} fresh weight bread after 72h incubation at 25°C.
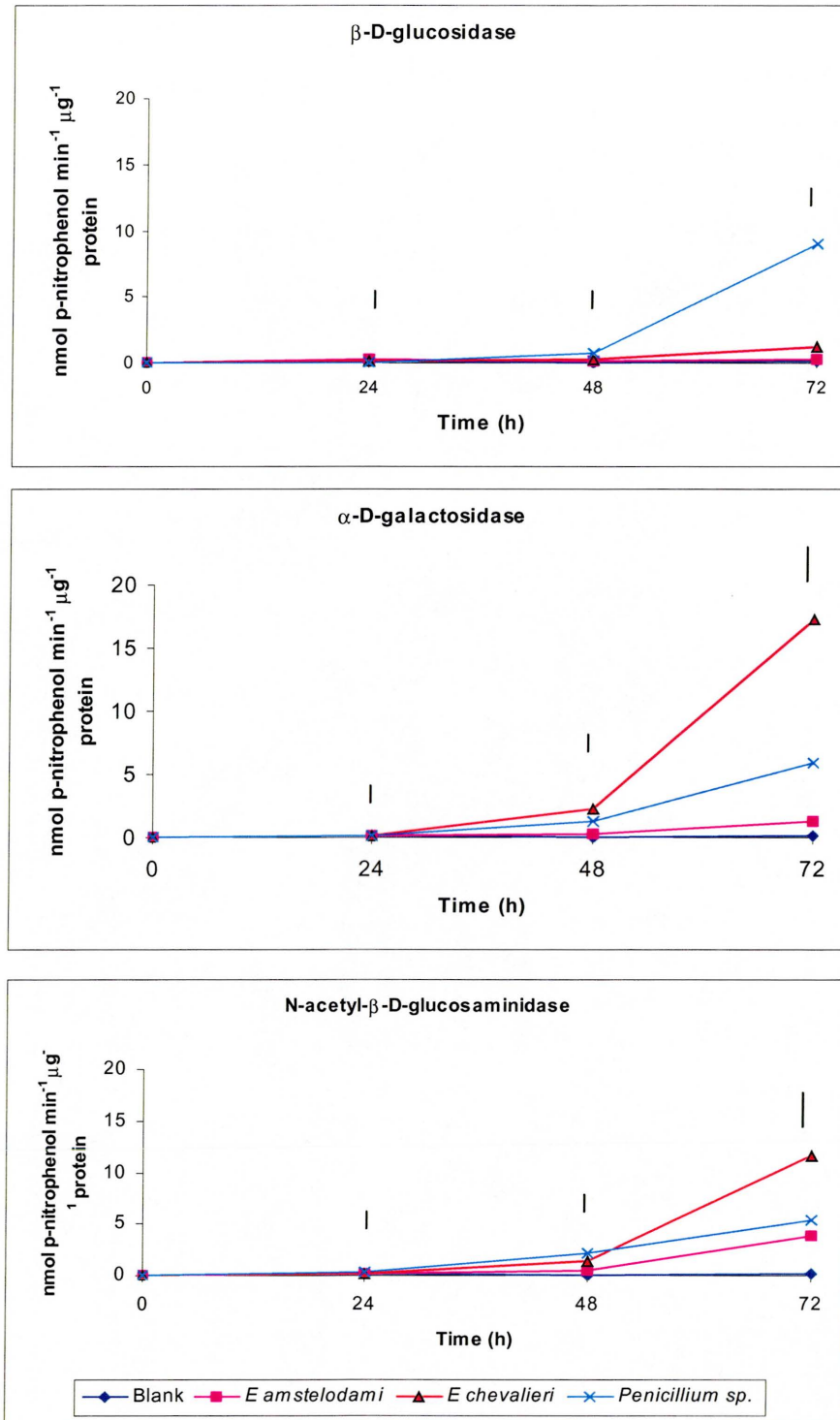
Figure 3.59 Temporal changes in specific activities (nmol p-nitrophenol min^{-1} μg^{-1} protein) of three enzymes produced by *E. amstelodami*, *E. chevalieri* and *P. chrysogenum* grown on natural bread substrate. The blank control is the uninoculated bread slices. The bars at each time interval indicates the least significant difference ranges (P <0.05).
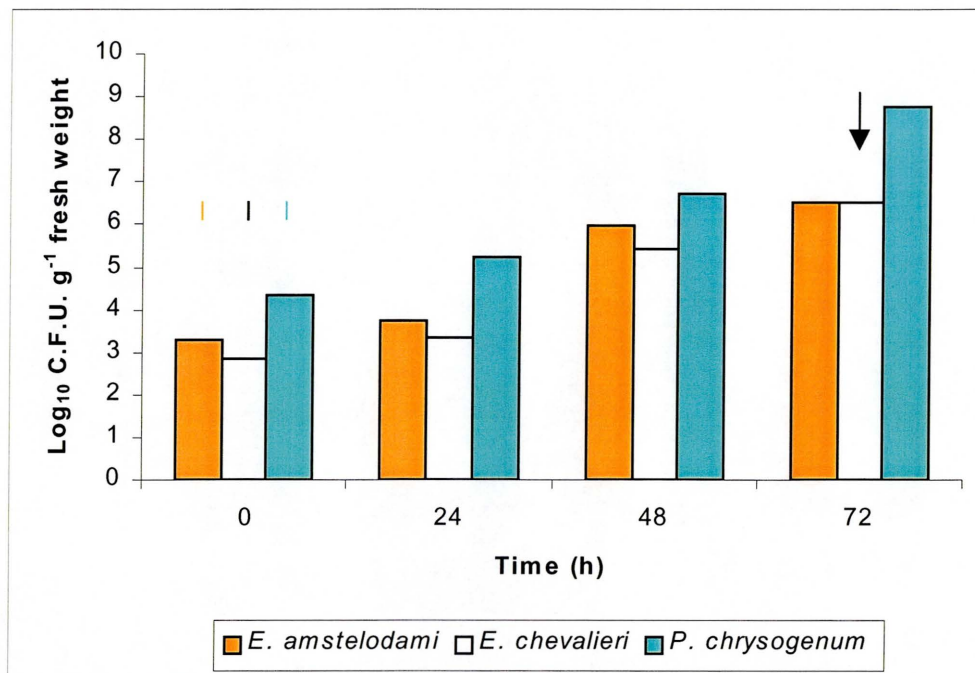
Figure 3.60 Temporal changes in $\log_{10}$ colony forming units (CFUs) for three fungal species on natural bread substrate over the experimental period. Bars indicate least significant difference (L.S.D.) for each treatment (P <0.05) over time. Arrow indicates when visible growth was observed.

Chapter 4

Current and perspective uses of e-nose technology for food mycology

CURRENT AND PERSPECTIVE USES OF E-NOSE TECHNOLOGY FOR FOOD MYCOLOGY

There is significant interest in developing methods for the early detection of fungi in food products. The available techniques used in food mycology have already been discussed in Chapter 1 in some detail. The objective of this chapter is to give an evaluation of the current techniques with electronic nose (e-nose) technology and discuss the future of this technology. Several factors need to be considered concerning the suitability of a method.

4.1 LABOUR TIME TO PERFORM THE ASSAY AND TIME REQUIRED TO OBTAIN A RESULT

The conventional methods used in the food industry have many drawbacks including being labour intensive and time consuming (Figure 4.1). Furthermore, the Hazard Analysis Critical Control Point (HACCP) approach requires corrective action to be taken in real-time. The traditional mycological plating techniques can take up to 5-7 days incubation, by which time the precautionary steps to avoid further fungal contamination in the bakery plant may be too late (Twiddy & Phillips, 1995). Most other techniques still take up to a day to perform the assay and obtain the results. In contrast, the e-nose system can operate 24h a day taking sample volatile patterns every 5-10 minutes, providing rapid qualitative results (Zubritsky, 2000).
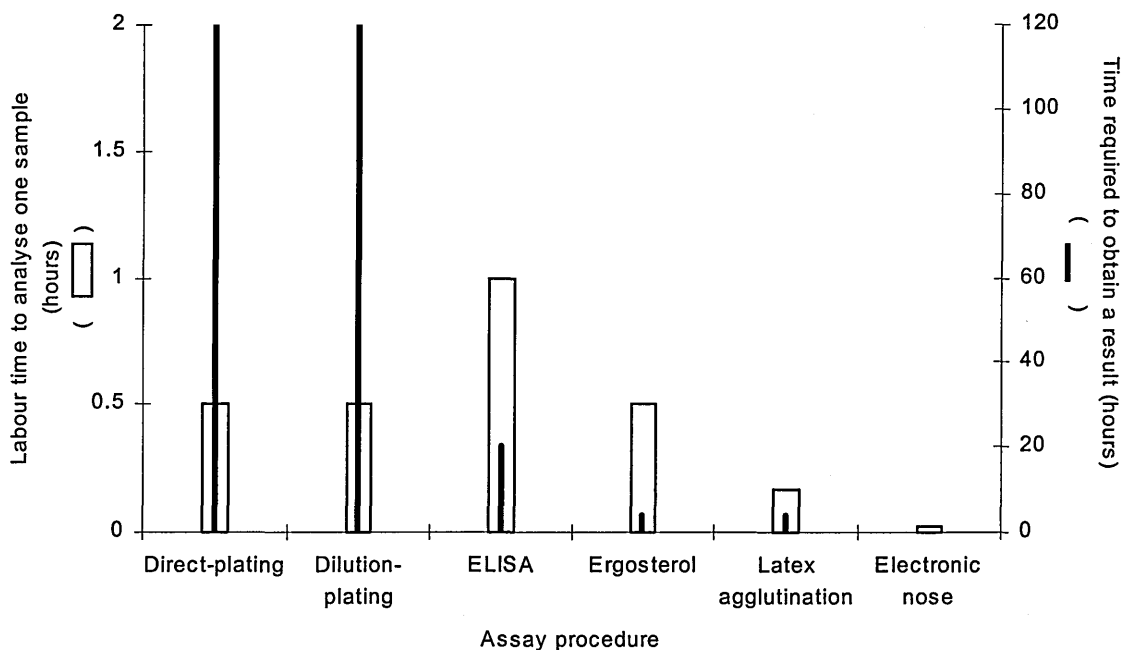
Figure 4.1 Time required to perform the various assays and to obtain results (Twiddy & Phillips, 1995).
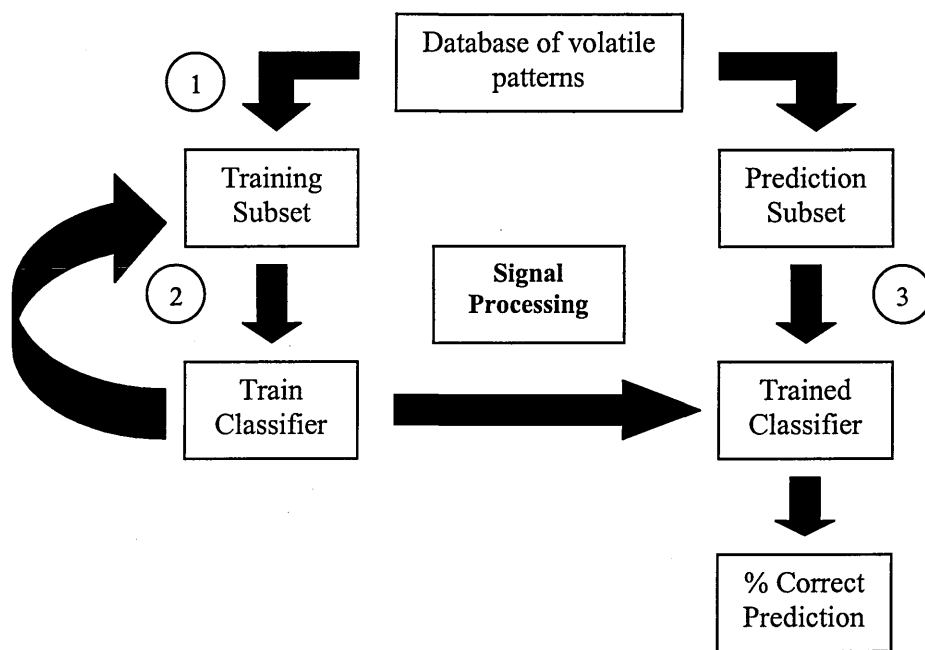
Figure 4.2 A schematic flow diagram of the sensor array pattern recognition.

4.2   EASE OF USE

Table 4.1 summarises some of the features of the available methods. Techniques such as immunological methods require highly trained staff to carry out the procedures. Chemical assays, e.g. ergosterol assay, require lengthy analytical procedures hence the need for a high level of expertise (Twiddy & Phillips, 1995). In contrast, the e-nose systems are user-friendly and do not require highly trained staff for initial raw data collection, provided a protocol is set up. The e-nose combined with artificial neural networks could produce maps of the fungal volatile patterns and control, and log them in a database (Figure 4.2; Zubritsky, 2000; Evans *et al.*, 2000). This database could be used in the training process to train the unit pattern-recognition system thus giving the potential for an online monitoring system for quality control, which may prove to be useful in the bakery industry.

4.3   INTERPRETATION OF RESULTS

For the plating techniques, hypha fragmentation may result in the release of individual spores leading to meaningless numerical data without further identification of the specific types of fungi by trained staff (Twiddy & Phillips, 1995). Biochemical assays such as ergosterol only indicate the absence or presence and the level of general fungal biomass in a sample, with no species differentiation (Table 4.1). Interpretation of ELISA and Latex agglutination data is more difficult. The e-nose data will be easy to interpret when used in combination with a previously trained artificial neural network to recognise specific volatile patterns.

Table 4.1 Comparison of conventional methods of fungal detection with electronic nose (Twiddy & Phillips, 1995).

Attributes	Traditional assays		Immunoassays		Chemical assays	Electronic nose
	Direct-plating	Dilution plating	ELISA	Latex agglutination assay	Ergosterol	
Ease of use	Easy	Moderate	Moderate	Easy	Difficult	Easy
Expertise required to perform the test	Low level	Medium level	High level	Low level	High level	Low level (using an established protocol)
Interpretation of results (degree of difficulty)	Low level for total fungi, high for identification		Moderate	Low	Moderate	Low (using an established protocol)
Availability of reagents	Widely available		Monoclonal antibodies difficult to obtain	Few kits available	Widely available	Widely available
Capital outlay	Autoclave, microscope, incubator		Microtitre plate reader	Centrifuge	HPLC system, rotary evaporator	Electronic nose

4.4   SENSITIVITY

Table 4.2 shows the detection limit of some of the techniques available. For the colony counting the detection limit is 10-100 spores g^{-1} of sample. In contrast, immunological methods tend to be less sensitive and can detect in the order of 10^3-10^5 spores g^{-1} of sample, and for ergosterol the order is in the range of 10^5-10^6 spores g^{-1} of sample (Magan, 1993a; Twiddy, 1994). Mielle *et al.* (2000) showed the detection of ethanol from sample headspace in the concentration range between 10 - 1000 ppm, using metal oxide semiconductor sensors. The conducting polymer based e-nose can detect between 0.1 – 100 ppm (Keller, 1999) if volatiles are taken directly from the sample headspace. Concentrating the volatile sample may increase the sensitivity of the system further (Wilkes *et al.*, 2000).

Table 4.2 Detection limit of some methods used to detect fungi (Twiddy, 1994; Twiddy & Phillips, 1995; de Boer & Beumer, 1999).

Methods	Detection limit $(CFU\ ml^{-1}\ or\ g^{-1})$
Direct-plating	1
Dilution-plating	10-100
Bioluminescence	10^4
Ergosterol	$10^4 - 10^5$
Impedimetry	1
ELISA	$10^3 - 10^5$
Latex agglutination	10^2-10^3
Nucleic acid-based assay	10^3

4.5   SPECIFICITY

Plating methods tend to be non-specific. In contrast, mycological immunoassays have a high degree of specificity and can be species- or group-specific depending on the type of antibodies used. Monoclonal antibodies are often too specific, whereas polyclonal antibodies are generally non-specific. However, the difficulties in raising species-specific antisera to fungi limit the use of this method for detection of specific spoilage fungi in food (Twiddy & Phillips, 1995). The e-nose can differentiate not only the absence or presence of spoilage fungi (provided appropriate controls are used) but also differentiate species and isolates as shown in this thesis. However, the e-nose is qualitative not quantitative.

4.6   COST OF PERFORMING THE ASSAY

Many e-nose systems have been used in research and development in laboratories, although, only a few systems have been commercialised. Commercial limitation includes high cost of systems (in the range £15,000-£40,000). Recently, a comprehensive technical insight report on e-noses published by John Wiley and Sons, Inc. (1999) outlined the importance and the impact of e-noses on the food, chemical and healthcare sector. Table 4.3 lists some of the commercially available e-nose systems.

Table 4.3 Summary of selected commercially available e-noses. It should be noted that the cost price data listed for these systems have been obtained from the companies concerned in the year 2001. Each system relies on a slightly different approach but the basic technique is the same.

Company	Cost price
Alpha M.O.S. http://www.alpha-mos.com/	£40,000
AromaScan (Osmetech) http://www.aromascan.com/	£20,000
Bloodhound Sensors Ltd. http://www.bloodhound.co.uk/bloodhound/	£15,000
Cyrano Sciences http://www.cyranosciences.com/	£4,000
Lennartz Electronic GmbH http://www.lennartz-electronic.de/	£15,000
Nordic Sensor Technologies http://www.nordicsensor.com/	£40,000

Several factors need to be considered when determining a cost comparison of the method. Traditional plating requires reagents, culture media and other consumable items (Petri dishes, microtitre plates, pipettes, tips, membranes etc.) and labour costs (Table 4.1). For example, agar media (500g) and Petri plates (a pack of 500 plates) costs approximately £50 each. The cost of an analysis is also influenced by the nature of the information required. For an immunoassay the material costs are generally high in comparison to the plating techniques.

Initially the e-nose system may prove to be an expensive financial investment. However, in the long term the system can work out cheaper as the labour cost (number of staff hours) required to operate the system would be less than that required for the traditional plating technique. For the plating technique, identification of fungal species by specialised staff adds to the manpower costs.

Capital equipment outlay for the various techniques also differs considerably. The plating technique requires the use of microbiological media for the inoculation and growth of fungi as well as the preparation of these media and their subsequent safe disposal by autoclaving. Incubators are also necessary for the growth of the fungi and a microscope for the subsequent identification as well as electrical costs to run such equipment. Ergosterol assay requires equipment, such as HPLC systems and rotary evaporators, representing a high capital outlay. In contrast, the e-nose does not require culture media preparation or inoculation of plates as the volatile patterns can be taken directly from the sample headspace. The only cost incurred after the purchase of the e-nose system would be the price of the sampling bags, the filters used during headspace sampling and the reagent butan-2-ol, making this method cost-effective (Technical insights report, 1999).

4.7   THE FUTURE

Although the e-nose shows promising results for early detection of moulds, there are still a number of issues that need to be addressed before the technology can be applied to the food industry. For example, the subject of sensor drift as well as the problem of humidity, when using conducting polymer sensor arrays. Currently no

research literature is available on the comparison of the methods used presently in the food industry for detection of fungi versus the e-nose technology. Furthermore, no cross comparison studies have been performed to investigate the different e-nose systems available on the market.

Many manufacturers are looking to miniaturize the e-nose system for the future (D'Amico *et al.*, 2000). Some companies, for example, Cyrano Sciences have already achieved the production of an e-nose little bigger than a handheld calculator and are looking further into making the nose the size of a computer chip. The Bloodhound e-nose is small and portable allowing the possibility of on-line analysis. In addition, companies such as Alpha M.O.S. and Neotronics are now making hybrid e-nose systems containing different types of sensors in an array. For example, the inclusion of both the conducting polymers and the metal oxide sensors in an array improves the discriminating ability of the system as conducting polymers respond better to polar compounds and the metal oxides better to oxidized compounds. Application-specific sensor array and improvement in sensor performance with new sensing materials and refinements in sensor fabrication techniques may reduce the cost of the e-nose system. Reducing the sensor response time will allow for real-time monitoring (Technical insights report, 1999).

Chapter 5

Discussion

5.1 DETECTION OF FUNGAL VOLATILES PROFILES AND DISCRIMINATION BETWEEN SPECIES

The detection of fungi based on their volatile patterns has been demonstrated for the first time using an electronic nose. Four spoilage fungi and the control could be differentiated only after 48h prior to visible growth when using a spore lawn of inoculum. The control media gave some responses which can be regarded as a low background level. Compounds such as straight and branched saturated alkenes have been shown to be derived from the polystyrene Petri dish alone (Larsen & Frisvad, 1994).

Results from this study suggest that it is possible to differentiate between fungal species at a very early stage (48h) prior to any visible signs of growth. This result is in agreement with that of Börjesson *et al.* (1989). The study found measurable amounts of volatiles after 48h, using GC analysis. However, their study used wheat grain as the substrate, at 25% m.c. (= 0.95 a$_w$) over a period of 14 days. Larsen (1997) identified cheese - associated fungi (*Penicillium* spp. and *Aspergillus versicolor*) at even an earlier stage (after 43h) though the medium used was a richer yeast extract sucrose agar. Previously, Börjesson *et al.* (1990; 1992) found differences in fungal volatiles produced by grain fungi (*Penicillium* spp., *A. flavus*, *A. versicolor* and *A. candidus*) using GC. There were fewer differences between grain type and age of cultures than between species. Börjesson *et al.* (1990) found higher amounts of 8-carbon alcohols and 3-methyl-1-butanol for *P. aurantiogriseum* grown on grain than on agar. In their studies fungal cultures were grown for 5 days at a higher water availability than used in the present studies.

5.2 *IN VITRO* STUDY OF SPOILAGE FUNGAL VOLATILE PATTERNS, ENZYME ACTIVITIES AND GROWTH RATES AT DIFFERENT WATER ACTIVITIES

The presence of humidity affects the discriminating abilities of conducting polymers based sensors. Conducting polymers are usually polyaniline and polypyrrole derivatives. The present study investigated the potential of the electronic nose to discriminate and differentiate between spoilage fungal species and the uninoculated control blanks on media of different steady state a_w levels. Better reproducibility of the sensor array to fungal volatiles was observed at higher a_ws of 0.95 and 0.90. Studies by Partridge *et al.* (2000) found the presence of water vapour decreased the conductivity of polypyrroles, whereas Matveeva (1996) observed an increase in conductivity for polyanilines. Work by Swann *et al.* (2000) suggested hydrophobic and hydrophilic volatile compounds adsorbed into regions of the polymer with different solvating environments.

Work by Casalbore-Miceli *et al.* (2000) suggested a mechanism by which ions migrate through a liquid-like solution layer (liquid phase) created inside the polymer film (solid phase) by the absorption of water. These ions dissolve in the surrounding water and the conductivity is due to ion transport in the liquid phase. The study suggested that water molecules may play some part in the volatile compounds-polymer equilibrium. Welle *et al.* (2000) found that the moisture content of the polymer samples influenced the headspace composition of the samples and changed the sensor discrimination. Therefore, the water swells the polymer increasing the diffusion of volatile compounds and reducing the time to reach equilibrium.

In the present study, the clusters for the control blank overlapped in the PCA irrespective of the steady state a_w levels of the wheat meal agar media. Furthermore, the close and overlapped clusters were also observed in the PCA for the fungal species at different a_w levels after 72h growth, indicating the similarities in the fungal volatile patterns. Evans *et al.* (2000) suggested that differentiation was not purely as a result of the moisture content parameters but that the type of fungal volatiles were also significant. The study applied ANN to the conducting polymer sensor array data obtained from different degree of mouldy grain samples. The 20% m.c. wheat samples had a higher CFU count. The work suggested that if the differentiation were due to moisture content then total separate clusters would be observed in the PCA. In contrast, the PCA obtained in their work showed close clusters for 18% and 20% m.c. wheat samples, as both these samples had high *Penicillia* counts.

Numerous studies have tried to address the humidity sensitivity problem by modifications of the sensing material. For example, use of iodine doped poly (2,5-thienylene vinylene) (PTV) sensors (De Wit *et al.*, 1998) and hydrophobic PVC coating over the conducting polymer array (Reddy & Payne, 1999) to reduce the effect of humidity. However, the sensor baseline was observed to change with changes in the humidity of the carrier gas (De Wit *et al.*, 1998).

Recently Clements *et al.* (1998) have shown the use of discotic liquid crystals to overcome the humidity problem. These crystals consist of an aromatic core surrounded by hydrocarbon side chains and have little or no sensitivity to water vapours. Absorption of volatiles on the surface of the sensor causes a change in

sensor resistance (up to 90%), whereas the response to saturated water vapour was found to be negligible.

The current work shows that it was possible to differentiate the spoilage fungi and the control blank at the three a_w levels investigated, prior to visible signs of growth. At 0.90 a_w, *E. rubrum*, *E. herbariorum* and *E. chevalieri* were differentiated, whereas at 0.85 a_w *E. amstelodami* and *E. chevalieri* were only differentiated. Studies by Gervais *et al.* (1988) and Gervais (1990) have shown the affect of a_w on the volatile production by *Trichoderma viride*. Their work found that the 2-heptanone production increased with decreasing a_w of the media from 0.98 to 0.96 over a period of 7 days at 25°C. Furthermore, their study also demonstrated a drastic decrease in heptanone production by *T. viride* when the relative humidity of the air was reduced from 99% to 80% for 10h. The return to 99% relative humidity resulted in an increase in the production of volatiles. Sinha *et al.* (1988) and Tuma *et al.* (1989) also observed highest levels of fungal volatiles in bin-stored wheat at higher moisture contents of 25% (= 0.95 a_w) and 18.2% (= 0.85 a_w) respectively.

The specific activities of seven hydrolytic enzymes were assayed over a period of time. Three predominant enzyme activities were noted to be significant at 0.95 a_w namely β-D-glucosidase, N-Acetyl-β-D glucosaminidase and α-D-galactosidase. *P. chrysogenum* was found to have the former enzyme activity and the *Eurotium* spp. the latter two enzyme activities. However, at lower a_w levels of 0.90 and 0.85, only the latter two enzymes were predominant for the *Eurotium* spp. *P. chrysogenum* was also observed to grow faster at higher a_w of 0.95 in comparison to the *Eurotium* spp., which grew better at lower a_w levels. This is similar to results obtained by Marin *et*

al. (1998) who found these three enzyme activities to be predominant for *Fusarium moniliforme* and *F. proliferatum* colonizing whole maize kernels modified to three a_w levels (0.93, 0.95 and 0.98). In their study, activity of α-D-galactosidase was noted to be the least affected by a_w and the remaining two enzyme activities (β-D-glucosidase and N-acetyl-β-D-glucosaminidase) were higher at 0.98 a_w. However, the lowest a_w investigated in their study was 0.93 not 0.85 a_w. The current study suggests that for *P. chrysogenum*, β-D-glucosidase activity is significant only at the higher a_w of 0.95. In contrast, Magan (1993b) found all these three fungal enzymes to be predominant at lower a_w (0.88 and 0.80) including β-D-glucosidase however, the study examined naturally contaminated wheat grain not inoculated with individual fungi.

Jain *et al.* (1991) and Magan (1993a; 1993b) suggested hydrolytic enzymes as good early indicators for fungal spoilage prior to visible signs of growth. In this study, enzyme activities were noted to be significant after 72h, 120h and 11 days for 0.95, 0.90 and 0.85 a_w respectively. Marin *et al.* (1998) also found the enzymes to be produced at a later time for lower a_w of 0.95 and 0.93. In comparison, the electronic nose was able to differentiated the fungal species and the control blank after 48h growth for the higher a_w and after 5 days growth at 0.85 a_w based on fungal volatile patterns. Hence, the potential use of the electronic nose as a tool for early detection of mould spoilage in food matrices.

5.3   COMPARATIVE   STUDY   OF   VOLATILE   PATTERNS   AND HYDROLYTIC ENZYME PRODUCTION FROM MYCOTOXIGENIC AND NON-MYCOTOXIGENIC FUNGI

Two mycotoxigenic fungal species (*A. flavus* and *W. sebi*) and the control media could be differentiated after 72h whereas, *A. niger*, *A. ochraceus* and *P. chrysogenum* could not. The overlap may be due to similarities in the volatile patterns of these three species. However, GC-MS analysis has to be carried out in order to elucidate if this is the case. Recently, *A. niger* van Tieghem has been shown to produce low concentrations of ochratoxin A. *P. chrysogenum* Thom also produces mycotoxins (Li *et al.*, 2000).

This study used different fungi, however for a true comparison, mycotoxigenic and non-mycotoxigenic strains of the same fungal species need to be examined. Jelen *et al.* (1995) investigated sesquiterpene production of mycotoxigenic and non-mycotoxigenic strains of *Fusarium sambucinum* infecting wheat grain for 5 days at 25°C. Their study showed that the mycotoxigenic strains produced greater amounts of diverse sesquiterpenes compared to the non-mycotoxigenic strains. They also found that the profiles of volatile sesquiterpenes were similar for all mycotoxigenic isolates examined, although the concentration varied in the different strains, whereas for non-mycotoxigenic isolates the volatile profiles were different. This is in contrast to a previous study by Zeringue *et al.* (1993) who examined sesquiterpene fractions of mycotoxigenic and non-mycotoxigenic strains of *A. flavus* grown in liquid culture over 10 days. They observed a correlation between initiation of aflatoxin synthesis and sesquiterpene production. However, the pattern of sesquiterpenes produced was

unique to each strain and not to the entire set of mycotoxigenic isolates. Pasanen *et al.* (1996) also showed differences in volatile production for mycotoxigenic and non-mycotoxigenic strains of *P. verrucosum* over a period of 26 days at 20-23°C using oat grains as the substrate. However, in their study the non-mycotoxigenic spore concentration was lower than that of the mycotoxigenic strain.

Jelen *et al.* (1995) also noted maximum sesquiterpene concentrations to be present in 5-7 day old cultures depending on the strain. However, in the present study differentiation was possible as early as 72h, and after 120h for three spoilage fungi.

All studies have used Tenax TA as the adsorbent polymer to trap volatiles and GC-MS techniques to identify and quantify the volatiles (Zeringue *et al.*, 1993; Jelen *et al.*, 1995; Pasanen *et al.*, 1996). This technique is not only labour intensive but the Tenax polymer has a low adsorptivity for polar and small compounds. Thus, some of the volatiles may be absent from these studies and may not be the complete volatile profile.

After 120h three *Aspergillus* sp. were sporulating, whereas, *P. chrysogenum* and *W. sebi* were not. A number of studies have indicated a relationship between volatile production and sporulation (Börjesson *et al.*, 1993; Larsen & Frisvad, 1994; Nilsson *et al.*, 1996). However, in this study the dendrogram did not show any clustering of sporulating and non-sporulating fungi as was observed by Larsen and Frisvad (1994).

Specific activities of three hydrolytic enzymes were significant after 120h, when visible growth had occurred, which is 48h later than that for the electronic nose. For *P. chrysogenum* and *W. sebi* no increase in enzyme activity was observed for up to 120h. Whereas, detection and differentiation was possible for these species using their volatile patterns. All studies on hydrolytic enzyme production by fungi used grain substrates (Jain *et al.*, 1991; Magan 1993b; Marin *et al.*, 1997; 1998). All these studies found the same predominant enzymes as observed in the present work.

Subsequently studies were made to differentiate between non-mycotoxigenic and mycotoxigenic strains of specific spoilage fungi using general volatile production patterns and electronic nose technology. The study was designed to differentiate strains during very early phases of growth, i.e., up to 96h, *in vitro* at a steady-state water activity (0.95). The cluster analysis of the data suggests that this approach does give some clear separation of a non-mycotoxigenic strain from mycotoxigenic strains, and sometimes of the three mycotoxigenic strains of each species. Interestingly, it was possible to obtain very much earlier differentiation with the volatile patterns (48h) using the electronic nose system than using hydrolytic enzyme production.

Previous GC-MS study of volatile production by mycotoxigenic fungi (*Aspergillus flavus, Pyricularia oryzae, Fusarium macroceros, F. sporotrichiella* and *F. graminearum*) grown on damp rice over a period of 50 days has shown *Fusarium*-specific volatiles to be present in headspace samples (Emokhonov *et al.*, 1994). Furthermore, the *Fusarium* spp. produced qualitatively similar volatiles. However,

the relative concentrations of the volatiles were quantitatively different. It should be noted that the water activity of the substrate was not controlled in their study.

Quantitative studies of microbial volatile organic compounds with a trichothecene-producing strain of *Fusarium sporotrichoides*, and ochratoxigenic and non-ochratoxigenic strains of *Penicillium verrucosum*, found that the production of volatile terpenes appeared to be linked to the formation of trichothecenes by the *Fusarium* spp. and accelerated production of volatile ketones by the ochratoxigenic strain of *P. verrucosum* (Pasanan *et al.*, 1996). Although the water availability of the predominantly grain substrates was not controlled accurately, the results did suggest that specific groups of organic volatiles could be good markers for differentiation between mycotoxigenic and non-mycotoxigenic species. Jelen *et al.* (1997b) found that trichodiene, an intermediate of trichothecene mycotoxin production, was present in increased amounts in harvested grain spikes colonized by *Fusarium* species when harvested a week before harvest and incubated. They suggested that it could be an early indicator for production of these mycotoxins in the grain, although no attempt was made to differentiate between colonization by mycotoxigenic and non-mycotoxigenic strains. For other *Fusarium* species such as *F. sambucinum*, distinctive and characteristic volatiles were produced by non-mycotoxigenic, and mycotoxigenic strains (Jelen *et al.*, 1995). However, all these studies required sophisticated analytical equipment to analyse specific groups of organic volatile compounds. The present study would suggest that recognition of general patterns of volatile production using a sensor array may be a simple and rapid method for detecting and differentiating between strains and species.

The study of specific enzyme activity by germling of these strains and species showed that a number of hydrolytic enzymes are important in the initial colonization of a substrate. However, the relative importance may differ between species. For example, distinctive increases in specific activities of three enzymes were observed for the non-mycotoxigenic strain (6N) of *F. moniliforme*. However, there were fewer differences between strains of *F. proliferatum*. Specific activities of enzymes provide information on the rates of substrate colonization (Marin *et al.*, 1998), and reflects the relative capacity for exploitation of agricultural substrates and occupation of ecological niches (Magan, 1997). However, they were not such good early indicators of fungal activity as the general volatile production patterns.

This study concentrated on very short incubation times. This was done specifically because of the interest in evaluation of the electronic nose system for detection of activity prior to visible growth. Much better volatile patterns and discrimination may occur later when visible growth has occurred. This may be of interest from a purely taxonomic point of view (Magan & Evans 2000), but not for the development of early detection systems.

5.4 EARLY DETECTION OF FUNGAL GROWTH ON MODIFIED BREAD SUBSTRATE BASED ON THE VOLATILE PRODUCTION PATTERNS, ENZYMIC STUDY AND TOTAL FUNGAL POPULATIONS

This study has shown for the first time that early qualitative changes in volatile production patterns by germinating spoilage moulds in bread can be detected using an electronic nose. This can be achieved during the very early phases of establishment, prior to visible growth on a bread substrate. It was demonstrated that differentiation between mould contaminated and uncontaminated samples could be made within 40h of incubation with the initial levels of mould spores at about 10^3 CFUs g^{-1} food substrate. Furthermore, cluster analyses clearly showed that differentiation between spoilage by *P. chrysogenum* (isolated from bread) and xerophilic *Eurotium* spp., could be made from non-moulded controls. The recognition of general qualitative patterns may have some advantages in that information on the status of a sample relative to an unspoiled food product can be obtained rapidly.

Previous work have shown fungal volatile production to be media dependent (Borjesson *et al.*, 1990; 1992; Sunesson *et al.*, 1995). Thus GC-MS may be required to elucidate the similarities or differences in the volatile production by fungi grown on wheat meal agar and bread analogue substrates used in the present study.

Studies have been predominantly in relation to mould contamination of raw product, particularly wheat/barley grain which have recently been reviewed by Schnurer *et al.* (1999) and Magan and Evans (2000). Some studies have recently examined the activity and relative colonisation patterns of cakes by spoilage moulds (Abellana *et al.*, 1999) in relation to environmental regimes, but not for early detection.

Previous studies specifically with bread have used sophisticated analytical methods (GC-MS) showing that inoculation with actinomycetes produced intense musty odours. These were attributed to the presence of 2-methylisoborneol and geosmin, whereas moulds such as *P. roqueforti* and *Botrytis cineria* produced musty/fruity odours caused by these compounds and 8-carbon alcohols and ketones (Harris *et al.*, 1986). However, in these studies whole bread was inoculated and stored for 7-14 days by which time significant visible mould was present. Thus this previous study was mainly aimed at the development of criteria for classifying spoiled product, as opposed to quality assurance and early detection.

It was interesting to note that only three of the seven extracellular hydrolytic enzymes were found to change significantly with mould colonisation of bread, which were good indicators of spoilage. However, changes were significant after 48h. The specific activity of the three individual enzymes also varied with the dominant spoilage mould present. Previous studies have examined the potential for the use of hydrolytic enzymes as indicators of microscopic mould activity in grain prior to visible growth (Jain *et al.*, 1991; Magan, 1993b). N-acetyl-β-D-glucosaminidase, α-D-galactosidase, and β-D-glucosidase were found to significantly change before visible growth was observed in grain. Recently, Marin *et al.* (1998) found that for

mycotoxigenic species of *Fusarium* (*F. verticillioides* and *F. proliferatum*) the same three enzymes were predominantly produced, and indicative of the capacity for invasion of maize. The present study suggests that quantitative changes in these enzymes also occurred in bread substrates due to fungal colonisation. However, changes were later than the qualitative changes in volatile patterns observed.

The populations (CFUs) of each mould species, based on serial dilution of sub-samples, showed an increase with incubation time at the steady state a_w level (0.95) used. This basal method of analysis showed marked increases, particularly of the *Penicillium* species over the incubation period. The increase in CFUs for the *Eurotium* spp., was less because they produce a mixture of both asexual conidia and cleistothecia containing ascospores. Thus, the slower rate of increase in CFUs should be expected. Extensive previous studies of mould contaminants of grain showed that when grain was inoculated with *P. roqueforti* (initial concentration of 10^3 CFUs g^{-1} grain) there was a good correlation between sensor responses with CFUs, and with ergosterol, a biomass marker for activity (Schnurer *et al.*, 1999). Other studies have examined ergosterol as an early indicator of mould activity in grain (Marfleet *et al.*, 1991; Tothill *et al.*, 1992). However, these methods all require long time periods for analysis which are often not available in the food processing industry, particularly intermediate moisture bakery products, which are prone to contamination, and where quality assurance systems need to be more rapid.

5.5 DETERMINATION OF THE THRESHOLD LIMIT OF DETECTION OF FUNGI ON BREAD SUBSTRATE USING THE ELECTRONIC NOSE

This study was aimed at investigating the detection limit of the electronic nose for spoilage fungi over a period of time. It was possible to detect a lower limit of 10^2 spores ml^{-1} after 24h on a bread analogue, prior to visible growth. However, there was some overlapping between 10^2 and 10^4 spores ml^{-1} for both *E. chevalieri* and *P. chrysogenum*. The upper limit of 10^6 spores ml^{-1} used in this study could be clearly differentiated. Further studies are required to investigate the lower limits of detection than that used in the present work. Furthermore, the detection limit may improve if sensor arrays are developed with better sensitivity than that at present available. The technology is moving rapidly and this should occur in the next few years.

Work by Fustier *et al.* (1998) investigated different inoculation techniques on mould growth on the surface of cakes. Their study found that spraying of spores rather than spotting the spores onto cakes may not represent the true inoculum concentration being inoculated onto the cake surface. The present work used a spotting technique to inoculate the bread analogues. In contrast, the study by Harris *et al.* (1986) used the spray method of inoculation on whole wheat bread.

Comparison with other methods used in food mycology suggests that the electronic nose is able to achieve earlier detection and differentiation of spoilage fungi (Twiddy, 1994; Twiddy & Phillips, 1995). Jonsson *et al.* (1997) observed correlation between measured ergosterol content, fungal CFUs and volatile production by fungi colonizing wheat grain using an electronic nose and ANN analysis. Similarly, Olsson *et al.* (2000) also observed that in naturally contaminated maize grain, a stronger

correlation of electronic nose measurements or GC-MS with predicted ergosterol level was obtained than with predicted CFU values. Previously Borjesson *et al.* (1992) also observed strong correlation between volatile production and the ergosterol content by six fungi grown on grain substrates. The correlation between CFUs and volatile production was less strong. An ergosterol detection limit is about 10^4-10^5 CFU g^{-1} (Twiddy, 1994; Twiddy & Phillips, 1995). Whereas, the present study indicates that it is possible to detect 10^4 CFU g^{-1} after 24h incubation. Moreover, the electronic nose does not require complex sample preparations or long incubation periods to obtain results.

The research work on fungal volatile production are all based on *in vitro* cereal-based media and uses the GC-MS technique. The inoculum concentrations used in these studies are in the range of 10^3-10^6 spores ml^{-1} (Borjesson *et al.*, 1990; 1992; 1993; Sunesson *et al.*, 1995). The fungal volatile studies performed on grain substrates do not indicate the initial inoculum concentrations used in their work. Furthermore, no previous studies in the literature were found which investigated inoculum concentration and volatile production.

5.6 EFFECT OF POTASSIUM SORBATE PRESERVATIVE ON VOLATILE PATTEERNS, HYDROLYTIC ENZYMES AND TOTAL POPULATIONS

Potassium sorbate is commonly used as a fungistatic preservative in the bakery industry to retard fungal growth, as the preservative is water soluble and non-toxic to humans (Legan, 1993; Thakur *et al.*, 1994). Inhibitory effects of potassium sorbate are influenced by different factors such as concentration, pH of the media, a_w and temperature (Liewen & Marth, 1985a). Kinderlerer and Hatton (1990) suggested potassium sorbate inhibited growth by interfering with the electrochemical membrane potential across the fungal mitochondrial membranes.

The present work showed the use of the electronic nose in differentiating uninoculated bread analogue and those colonized by spoilage fungi after 48h growth. Furthermore, it was not possible to discriminate the fungal species grown on bread analogue in the presence of potassium sorbate. Volatiles from preservative could affect the sensor array response however, the salts (potassium sorbate and calcium propionate) are not as volatile as the acids (sorbic acid and propionic acid; Thakur *et al.*, 1994). The population (CFUs) of each fungal species was reduced in the presence of potassium sorbate (10^4-10^5 CFU g^{-1} fresh weight bread) than in the absence. No literature was found regarding the effect of potassium sorbate on volatile production by spoilage fungi on bread substrate. Harris *et al.* (1986) studied volatiles produced by spoilage fungi colonizing whole wheat bread, using GC-MS technique however, their study used preservative-free bread.

Most of the literature available is on *Penicillium* spp. isolated from cheeses and tobacco. Studies have shown potassium sorbate to be metabolised as a carbon source by some *Penicillium* species to produce 1,3-pentadiene (a "kerosene" off odour) and suggested this conversion as a form of detoxification (Kinderlerer & Hatton, 1990). Daley *et al.* (1986) isolated and identified *P. roqueforti* from contaminated cheese-based spread. Using GC-MS, the study showed the headspace obtained from the contaminated spread, the headspace above *P. roqueforti* (grown on a medium containing potassium sorbate) and the authentic trans-1,3-pentadiene to be the same. A previous study by Liewen and Marth (1985b) showed that the production of 1,3-pentadiene was accompanied by a decrease in potassium sorbate concentration in cheese and pure fungal cultures. Mutasa and Magan (1990) found that the *Eurotium* spp. (*E. amstelodami*, *E. chevalieri* and *E. rubrum*) from tobacco were not able to metabolise potassium sorbate. Kinderlerer and Hatton (1990) also reported that the *Eurotium* spp. used in their investigation were unable to convert potassium sorbate to 1,3-pentadiene. They also noted variation in potassium sorbate resistance of isolates. For example, a *P. crustosum* isolate from coconut was found to be more resistant to sorbate than that obtained from hazelnut. Further GC-MS analyses of the headspace may be required to clarify the differences in the volatile patterns observed in the present study.

Mutasa and Magan (1990) demonstrated the growth of *P. chrysogenum* (isolated from tobacco) in liquid culture in the presence of 0.1 to 0.3% potassium sorbate at pH 6.0 and 25°C. In addition, increased potassium sorbate concentration stimulated the growth of the *Penicillium* sp.. An inhibition of growth was observed in the presence of 0.4% potassium sorbate. They also observed an increase in pH of the

medium during incubation. At pH 5.0 the growth was found to be inhibited by 0.2-0.3% potassium sorbate at 25°C. Previously, Liewen and Marth (1984) also found the growth of *P. chrysogenum* isolated from cheese, in the presence of 0.05% potassium sorbate at pH 5.5 and 25°C. However, the size of the inocula used in their study was greater than that used in the present study. The pH of the bread analogue used in the present study was in the range 5.78-5.90. Potassium sorbate is more effective at pH 5 than pH 6 because there is a greater proportion of active undissociated ions (pKa 4.76), although potassium sorbate has a maximum pH for activity around 6.0-6.5 (Liewen & Marth, 1985a). Furthermore, sugars can reduce potassium sorbate degradation while glycerol accelerates the degradation (Thakur *et al.*, 1994). In the present study the a_w of the bread analogue was modified to 0.95, using glycerol. However, lowering of a_w using glycerol results in increased losses of potassium sorbate in the food model (Thakur *et al.*, 1994). Moreover, there may be some losses of potassium sorbate from the bread analogue during baking in the present study. However, work by Petro-Turba *et al* (1980) found this loss to be small and in the range of 1.7 to 5.5%.

The incubation temperature modifies the effect of potassium sorbate. The present study was performed at 25°C. A study by Bullerman (1985) found that at 25°C *A. ochraceus* could readily grow in yeast-extract sucrose broth in the presence of 0.15% potassium sorbate and produce equal or greater amounts of ochratoxin than the control. However, at 12°C fungal growth and ochratoxin production was inhibited. In contrast, an unidentifiable *Penicillium* sp. isolated from cheese was inhibited by potassium sorbate at both these temperatures.

Significant differences in specific enzyme activities were noted in the presence of potassium sorbate. Three specific enzyme activities were found to be predominant (α-D-galactosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase) which is in agreement with Magan (1993b) and Marin *et al.* (1997; 1998). However, for the *Eurotium* spp., the latter two enzymes were found to increase 24h later in the presence of potassium sorbate than that observed in the absence of potassium sorbate. Furthermore, for the *Penicillium* sp. the activity of α-D-mannosidase was found to be significant 24h earlier in the presence of potassium sorbate. However, significant N-acetyl-α-D-glucosaminidase activity was noted for both the *Eurotium* spp. and the *Penicillium* sp. in the presence of potassium sorbate. For the remaining enzymes the activity markedly decreased in the presence of potassium sorbate. There are some studies available on fungal enzyme activities on grain substrates (Jain *et al.*, 1991; Magan, 1993b; Marin *et al.*, 1997; 1998) however, no literature was found regarding enzyme activities in the presence of potassium sorbate.

5.7 EFFECT OF CALCIUM PROPIONATE PRESERVATIVE ON FUNGAL VOLATILE PROFILES, ENZYME ACTIVITIES AND TOTAL POPULATIONS

Typically, bakeries use 0.2% calcium propionate based on flour weight, although the maximum permitted level is 0.3% in England and Wales (Legan, 1993). The present work has shown that it was possible to discriminate non-mouldy bread from mouldy bread 24h prior to visible signs of growth. No literature was found on the effect of calcium propionate on fungal volatile production in bakery products.

Interestingly, after 72h growth, it was possible to differentiate the presence and absence of calcium propionate in the uninoculated bread analogue. There was an overlap of the sub-optimal and optimal concentrations of calcium propionate. A previous study also showed that it was possible to differentiate between different preservatives (potassium sorbate and calcium propionate) using the electronic nose (Appendix I). Thus suggesting the possible use of the electronic nose in the screening of preservatives. Nielsen and Rios (2000) studying modified atmosphere packaging (MAP) on fungal growth inhibition on bread substrate observed a concentration effect in the sensory evaluation of the active packaged bread. The investigation found that the cluster for volatiles from rye bread packages containing 2 μl allyl isothiocyanate (AITC, the primary active compound in mustard essential oil) separated out from those from packages containing up to 0-1 μl AITC in the principal component analysis.

Propionate is lost in the form of the free acid during baking. Harvey and Seiler (1982) investigated the loss of propionate from bread during baking. The study found the highest losses from the crust, then the centre of the bread and the lowest from the remainder of the bread. Furthermore, they noted no significant difference between the preservative loss from using different types of propionate (calcium propionate, propionic acid and dipropionate). In the present study, the top crust portion of the bread analogue was removed. However, there may be some preservative losses but this would be small and reasonably consistent throughout the bread analogue used during the investigation.

Like potassium sorbate, the efficacy of calcium propionate is dependent on pH and has optimal activity when 50% is in the undissociated form (pKa 4.87) (Marin *et al.*, 2000). The maximum pH for activity of calcium propionate is around 5.0-5.5 (Liewen & Marth, 1985a). The pH for the bread substrate used in the present study was 5.71.

The current study found three hydrolytic enzyme activities to be predominant and significant after 48h growth. Magan (1993b) and Marin *et al.* (1997; 1998) also found the same enzymes to be predominant however, their studies were mainly on grain substrate in the absence of preservatives. In contrast, the electronic nose could differentiate the mouldy from the non-mouldy bread within 24 growth, when the bread analogue had fungal populations of 10^3-10^4 CFU g^{-1}freash weight bread for individual fungal species

P. chrysogenum was noted to have a higher β-D-glucosidase and N-acetyl-β-D-glucosaminidase activity in the presence of sub-optimal calcium propionate concentration. Furthermore, increases in β-D-fucosidase and N-acetyl-α-D-glucosaminidase activities were only found in the presence of calcium propionate. Whereas, β-D-xylosidase and b-D-glucosidase activities were not significant for *E. chevalieri* and *E. amstelodami* respectively, in the presence of calcium propionate. No previous study is available investigating fungal enzyme activities in the presence of calcium propionate.

Other studies have been carried out on grain substrates. Marin *et al.* (2000) studied influence of propionates on maize mycoflora and the effect on fumonisin accumulation. However, the work used a mixture of propionates not specifically calcium propionate.

5.8   DETECTION AND DIFFERENTIATION OF SPOILAGE FUNGI ON NATURAL BREAD SUBSTRATE USING THE ELECTRONIC NOSE

There is little or no literature available concerning volatile production by spoilage fungi colonizing natural bread substrate. This was the first study aimed at investigating early detection of fungal spoilage of natural bread substrate based on their volatile patterns. This work demonstrated the use of the electronic nose to differentiate the fungal species and the uninoculated bread substrate after 24h incubation, which improved after 48h growth. The total fungal population was found to be 10^4-10^5 CFU g^{-1}. In comparison, the enzyme activities were observed to increase after 48h at the initial microscopic growth stage, thus suggesting the potential of using the electronic nose as a tool for early detection of fungal spoilage.

Harris *et al.* (1986) also studied fungal volatile production on whole wheat bread however, the work did not mention the water activity of the bread or the initial inoculum concentration used in the investigation.

Related studies mainly concern volatiles produced by fungal spoilage of grains (Stetter *et al.*, 1993; Börjesson *et al.*, 1996; Jonsson *et al.*, 1997; Evans *et al.*, 1999; Evans *et al.*, 2000). These studies have applied electronic nose and artificial neural networks to grain classification based on volatile patterns. Borjesson *et al.* (1996) were able to successfully identify 90% of the samples as good or bad grain samples. Whereas, Evans *et al.* (2000) classified 92.3% of the samples with no bad samples misclassification. Jonsson *et al.* (1997) used ANN to predict the degree of mouldiness of grain and correlate with both the measured ergosterol and fungal CFUs. In contrast, Evans *et al.* (2000) suggested the correlation between the ANN predictions and CFUs may be misinterpreted. The study showed that the CFUs from 12.5% and 25% moisture content wheat samples were similar and could not be distinguished. However, these two wheat samples were at the opposite end of the mouldiness scale (good and bad samples) and as such should be well separated. Thus artificial neural network may be applied to the electronic nose data obtained in this current study to classify mouldy and non-mouldy bread. However, this data processing requires large amounts of raw data (a larger number of replicates per treatment) than those collected in this study to train and test the ANN.

In summary, this study shows that the potential exists for exploiting electronic nose technology and systems for rapid qualitative measurement of quality in bakery products. More information is needed to develop artificial neural network systems for the identification of the quality of products as part of such a quality assurance scheme. These types of new ways to detect moulds in food and feed could have widespread applications in the food industry.

Chapter 6

Conclusions and Future work

CONCLUSIONS

1. A method has been developed for the sampling of fungal volatiles using the e-nose both for *in vitro* and model bread substrate work.

2. Good sensor array reproducibility was obtained between replicates of the same species *in vitro* and on modified and natural bread substrates. The reproducibility improved with increasing a_w values. However, reproducibility of the sensor array to volatiles produced by spoilage fungi grown on a bread substrate in the presence of the preservative calcium propionate, was found to be poor.

3. Early detection of fungi was achieved based on their volatile patterns using an e-nose. Initial *in vitro* studies showed it was possible to separate between uninoculated blank plates and the fungal species as early as 48h, prior to visible signs of growth.

4. For 0.95 and 0.90 a_w levels, differentiation between blank control plates and the xerophilic fungi were possible after 48h incubation on 2% wheatmeal agar, prior to any visible signs of fungal growth. Earlier detection and discrimination of the species was possible using an e-nose compared to the enzyme assays.

5. Specific activities of three of the seven fungal enzymes were found to be predominant, i.e. N-acetyl-β-D-glucosaminidase, β-D-glucosidase and α-D-galactosidase.

6. A comparative study of mycotoxigenic and non-mycotoxigenic fungi showed that it was possible to differentiate the uninoculated blank from the fungal species. The differentiation of *A. flavus* and *W. sebi* was possible after 72h incubation. Again earlier detection was achieved using fungal volatile patterns compared to enzyme activities.

7. The e-nose could differentiate between different mycotoxigenic and non-mycotoxigenic strains of *F. moniliforme* and *F. proliferatum* after 48h growth based on their volatile patterns.

8. The non-toxigenic strain was found to have greater enzyme activity than the mycotoxigenic strains especially *F. moniliforme*.

9. Detection and differentiation of mouldy and non-mouldy modified bread substrate was possible as early as 40h after incubation, using an e-nose when compared to enzymatic assays.

10. The threshold limit of detection for both *E. chevalieri* and *P. chrysogenum* with an initial spore concentration of 10^6 spores ml^{-1} treatments could be differentiated, whereas some overlap was observed for 10^4 and 10^2 spores ml^{-1} treatments.

11. The work on bread analogues containing potassium sorbate preservative showed differentiation between the uninoculated bread analogue, and those colonised by *E. chevalieri* and *P. chrysogenum* after 48h incubation in the absence and presence of potassium sorbate.

12. Specific enzyme activity decreased for *Eurotium* spp. and increased for the *Penicillium* sp., with increasing levels of potassium sorbate.

13. Specific activity of enzymes was affected by the presence of potassium sorbate. For example, N-acetyl-α-D-glucosaminidase was found to increase significantly whereas, β-D-fucosidase activity was insignificant.

14. The mouldy and non-mouldy bread substrate could be differentiated after 24h growth in the absence and presence of the preservative calcium propionate, prior to visible signs of growth.

15. The presence of calcium propionate affected the activities of the hydrolytic enzymes. Specific activity of enzymes such as N-acetyl-α-D-glucosaminidase was found to significantly increase in the presence of calcium propionate.

16. Using the e-nose, it was possible to differentiate between mouldy and non-mouldy natural bread substrate after 24h growth, prior to any signs of visible growth. Earlier detection was achieved using the e-nose in comparison to that for enzyme assays.

SUGGESTIONS FOR FUTURE WORK

1. Quantification of volatile patterns produced by other spoilage fungi both *in vitro* and on modified bread substrate.

2. Further studies of fungal volatile patterns produced by pure fungal cultures colonizing bread analogues, using the e-nose. These include bread analogues at different a_w levels, pH and preservative concentrations. Correlation of the above volatile patterns with enzyme activity and fungal population changes for early detection of (i) germination and (ii) growth. Repetition of these studies using mixed rather than single populations.

3. Correlation between fungal volatile patterns using an e-nose system and concentration of volatiles by GC-MS.

4. Application and development of artificial neural networks to the sensor array raw data in response to fungal volatiles.

5. Development of sampling procedure to improve reproducibility. Cross comparison investigation of different e- nose systems and use of new sensor arrays.

6. On-line testing and comparison with traditional methods in bakery product production lines.

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Appendices

APPENDIX A

Volatile compounds found in cultures of fungi grown on grain substrate (Kaminski & Wasowicz 1991).

Key to reference a, Börjesson *et al.*, (1989); b, Wasowicz (1988); c, Wilkins & Scholl (1989); d, Kaminski *et al.*, (1972, 1974); e, Harris *et al.*, (1986); f, Tuma *et al.*, (1989)

Volatile Compound	Grain medium	Fungal strain	Reference
ALCOHOLS			
Ethanol	Wheat	*Aspergillus amstelodami,* *A. flavus, Fusarium culmorum,* *Penicillium cyclopium*	a
1-Butanol	Wheat & Corn	*A. flavus, A. parasiticus* *P. chrysogenum, Alternaria sp.*	b
2-Methyl-1-propanol	Wheat	*A. flavus, A. amstelodami,* *P. verrucosum, F. culmorum*	a
1-Pentanol	Barley	*P. viridicatum*	c
2-Methyl-1-butanol	Wheat	*A. amstelodami*	a
3-Methyl-1-butanol	Wheat	*A. flavus, A. niger, A. ochraceus* *A. oryzae, A. parasiticus,* *A. nidulans P. chrysogenum, P. raistricki,* *P. viridicatum, Alternaria sp.,* *Cephalosporium sp., Fusarium sp.*	d
	Whole wheat bread	*A. flavus*	e
	Wheat	*Alternaria alternata, A. repens, A. flavus,* *A. versicolor, P. chrysogenum, P. cyclopium,* *F. moniliforme, F. semitectum*	f
	Wheat	*A. flavus, P. cyclopium*	a
	Barley	*P. aurantiogriseum, P. verrucosum* *P. viridicatum, P. coprophilium*	c
	Corn	*A. flavus, A. parasiticus,* *P. chrysogenum, Alternaria sp.*	b

APPENDIX A (Continued)

Volatile Compound	Grain medium	Fungal strain	Reference
1-Octanol	Wheat	*A. flavus, A. ochraceus, A. oryzae, A. parasiticus, A. nidulans, P. citrinum P. viridicatum*	d
3-Octanol	Wheat	*A. flavus, A. niger, A. ochraceus A. oryzae, A. parasiticus, A. nidulans, P. chrysogenum P. citrinum, P. viridicatum, Cephalosporium sp.*	d
1-Octen-3-ol	Wheat	*A. flavus, A. niger. A. ochraceus, A. oryzae, A. parasiticus, A. nidulans, P. chrysogenum, P.viridicatum, Alternaria sp., Cephalosporium sp. Fusarium sp.*	d
	Whole wheat bread	*P. roqueforti, A. flavus, A. niger*	e
	Wheat	*Alternaria alternata, A. repens A. flavus, A. versicolor, P. chrysogenum, P. cyclopium*	f
	Wheat	*A. amstelodami, P. cyclopium*	a
	Barley	*P. aurantiogriseum, P.verrucosum P. viridicatum*	c
	Corn	*A. flavus, A. parasiticus, P. chrysogenum, Alternaria sp.*	b
2-Octen-1-ol	Wheat	*A. flavus, A. niger, A. ochraceus, A. oryzae, A. parasiticus, P. chrysogenum, P. citrinum, P. funiculosum, P. raistricki, P. viridicatum, Alternaria sp., Fusarium sp.*	d
1,5-Octadien-3-ol	Whole wheat bread	*P. roquefortii*	d

Key to reference a, Börjesson *et al.*, (1989); b, Wasowicz (1988); c, Wilkins & Scholl (1989); d, Kaminski *et al.*, (1972, 1974); e, Harris *et al.*, (1986); f, Tuma *et al.*, (1989)

APPENDIX A (Continued)

Volatile Compound	Grain medium	Fungal strain	Reference
CARBONYLS			
Acetaldehyde	Wheat & Corn	*A. flavus, A. parasiticuss, P.chrysogenum, Alternaria sp.*	b
2-Pentanone	Wheat	*A. amstelodami*	a
Hexanal	Wheat & Corn	*A. flavus, A parasiticus, P. chrysogenum, Alternaria sp.*	b
Octanal	Whole wheat bread	*A. flavus, A niger*	e
3-Octanone	Wheat	*A. flavus, A. niger, A. ochraceus, A. oryzae, A. parasiticus, A. nidulans, P. chrysogenum, P. citrinum, P. funiculosum, P. raistricki, P. viridicatum, Alternaria sp. Cephalosporium sp., Fusarium sp.*	d
	Whole wheat bread	*P. roqueforti, A. flavus, A. niger*	e
	Corn	*A. flavus, A. parasiticus, P. chrysogenum, Alternaria sp.*	b
	Wheat	*A. repens, P. cyclopium*	f
Nonanal	Whole wheat bread	*A. flavus, A. niger*	e
2-Methyloacetophenone	Barley	*P. coprophilum*	c
HYDROCARBONS			
Dimethylbenzene	Whole wheat bread	*A. niger, A. flavus*	e
Trimethylhexene	Whole wheat bread	*A. roqueforti*	e
2,4-Dimethylhexan	Wheat	*F. culmorum*	a

Key to reference a, Börjesson *et al.*, (1989); b, Wasowicz (1988); c, Wilkins & Scholl (1989); d, Kaminski *et al.*, (1972, 1974); e, Harris *et al.*, (1986); f, Tuma *et al.*, (1989)

APPENDIX A (Continued)

Volatile Compound	Grain medium	Fungal strain	Reference
2,3,5-Trimethylhexan	Wheat	*F. culmorum* *P.chrysogenum, Alternaria sp.*	a
Styrene	Barley	*P. aurantiogriseum, P. verrucosum* *P. viridicatum, P. coprophilum*	c
Naphtalene	Whole wheat	*A. niger*	e
MISCELLANEOUS			
Ethyl acetate	Wheat	*F. culmorum*	a
2-Methylfuran	Wheat	*A. amstelodami A. flavus,* *P. cyclopium*	a
2-(1-Pentyl)-furan	Barley	*P. aurantiogriseum, P. verrucosum,* *P. viridicatum, P. coprophilum*	c
2-(2-Furyl)-pentanal	Barley	*P. aurantiogriseum, P. verrucosum,* *P. viridicatum, P. coprophilum*	c
2-ethyl-5-methylphenol	Barley	*P. aurantiogriseum, P. verrucosum,* *P. viridicatum, P. coprophilum*	c
3-Methylanisole	Barley	*P. aurantiogriseum, P. coprophilum*	c
Monoterpenes	Wheat	*F. culmorum*	a
2-Methyl-isoborneol	Whole wheat bread	*P. roqueforti*	e
Damascenone	Whole wheat bread	*P. roqueforti*	e

Key to reference a, Börjesson *et al.*, (1989); b, Wasowicz (1988); c, Wilkins & Scholl (1989); d, Kaminski *et al.*, (1972, 1974); e, Harris *et al.*, (1986); f, Tuma *et al.*, (1989)

APPENDIX B

A table showing the normal mode settings for Prima home bakery kit for natural bread substrate.

	Time (mins)
Kneading	10
Resting	20
Kneading	15
Resting	30
Rising	40
Baking	45

APPENDIX C

Calculations of total and specific enzyme activity

(i) Calculation of total enzyme activity

1. Sample absorbance reading – control absorbance reading = actual absorbance reading of sample

2. Calculate the p-nitrophenol concentration (μmol ml^{-1}) present in the sample from the p-nitrophenol calibration curve.

$$\text{p-nitrophenol concentration} = \frac{\text{Actual absorbance reading of sample}}{\text{gradient of standard p-nitrophenol curve}}$$

3. $$\text{Total activity} = \frac{[\text{Conc. of p-nitrophenol (μmol ml}^{-1}) \text{ x Dilution factor}] \times \text{Volume of extract (ml)}}{\text{Time (min)}}$$

The dilution factor used was 2.625. Total activity is expressed as μmol p-nitrophenol released min^{-1}.

(ii) Calculation of specific enzyme activity

1. Calculate the total protein present in the sample using the BSA standard curve.

Total protein = Absorbance of sample/gradient of BSA standard curve

2. $$\text{Specific activity} = \frac{\text{Total activity (μmol p-nitrophenol released min}^{-1}) \times 1000}{\text{Total protein (μg)}}$$

Specific activity is expressed as nmol p-nitrophenol min^{-1} μg^{-1} protein.

APPENDIX D

CFUs calculation

1. Determine the mean colony count per plate at a particular dilution.

2. Calculate the CFU g^{-1} of that particular dilution by dividing by the volume (ml) of liquid transferred to each plate.

3. Calculate the CFU g^{-1} of sample by multiplying by the reciprocal of dilution.

4. CFU g^{-1} = $\dfrac{\text{Mean colony count}}{\text{Volume of liquid x dilution}}$

Total fungal population is expressed as CFU g^{-1}.

APPENDIX E

The Table below shows 14 sensor types present in the Bloodhound BH114 electronic nose sensor array. The actual materials of the sensor type are unknown due to company confidentiality.

Sensor position	Sensor type used in Section 2.2.1 *in vitro* study	Sensor type used in the remaining studies
1	01	01
2	04	04
3	12	12
4	15	12
5	16	01
6	19	19
7	2E	19
8	1E	1E
9	1A	1A
10	1B	1B
11	1C	1B
12	1F	04
13	2E	1E
14	22	22

APPENDIX F

Principal component analysis showing the differentiation of 2% butan-2-ol from the control (blank agar plates) and the fungal species after 72h incubation at 25°C on a wheat meal agar. Key to treatment: f1-f5, *Aspergillus flavus*; n1-n5, *A. niger*; o1-o5, *A. ochraceus*; p1-p5, *Penicillium chrysogenum*; s1-s5, *Wallemia sebi*; c1-c5, control blank agar plates and b1-b9, 2% butan-2-ol.
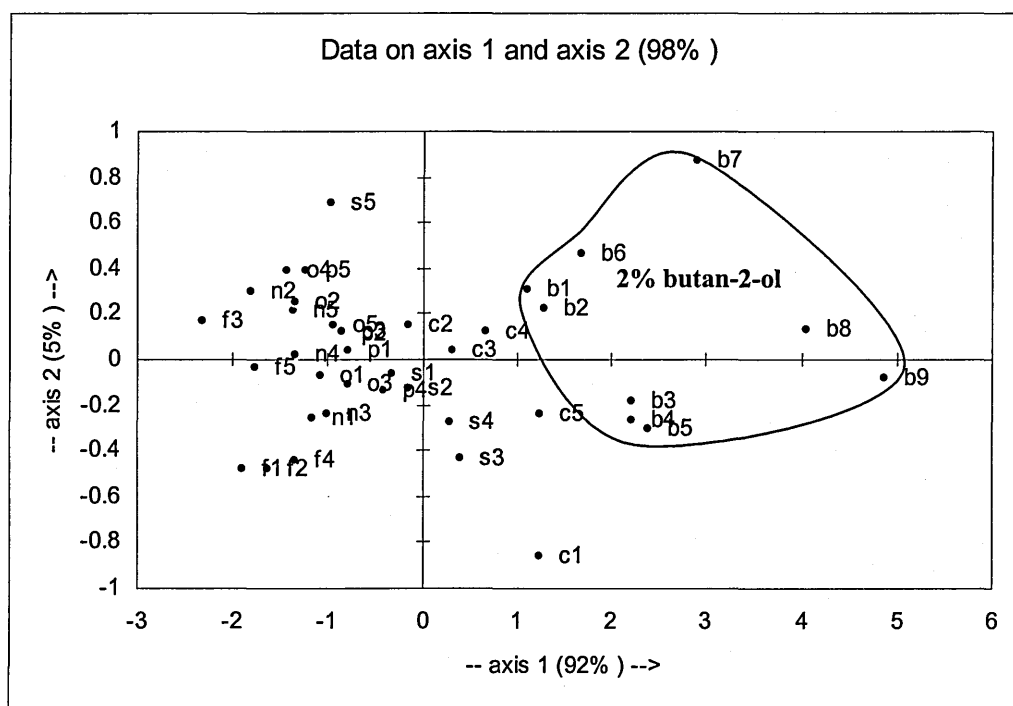
APPENDIX G

Specific activity of hydrolytic enzymes (N-acetyl-α-D-glucosaminidase, α-D-mannosidase, β-D-fucosidase and β-D-xylosidase).

(1) *In vitro* study of spoilage fungal volatile patterns, enzyme activities and growth rates at different water activities. Key to treatments: blank, blank agar plate; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri*; Eh, *E. herbariorum*; Er, *E. rubrum*; Pen, *Penicillium chrysogenum*.

(i) 0.95 a_w

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)						L.S.D.
		Blank	Ea	Ech	Eh	Er	Pen	
N-acetyl-α-D-glucosaminidase	48	0	0	0	0	0.084	0.316	0.385
	72	0	0	0	0.100	0	0.466	0.479
	96	0.044	1.000	0.041	0.059	0	1.964	0.560
α-D-mannosidase	48	0	0	0	0	0	0	0
	72	0	0	0	0	0	0	0
	96	0	0.270	0.051	0.282	0	0.346	0.557
β-D-fucosidase	48	0	0	0	0	0	0	0
	72	0	0	0	0	0	0	0
	96	0	0	0	0	0	0.467	0.335
β-D-xylosidase	48	0	0	0	0	0	0	0
	72	0	0	0	0	0	0.043	0
	96	0	0.093	0	0	0	0.645	0.399

(ii) 0.90 a$_w$

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)						L.S.D.
		Blank	Ea	Ech	Eh	Er	Pen	
N-acetyl-α-D–glucos aminidase	48	0.046	0.090	0	0.354	0.205	0.190	0.476
	72	0.023	0.343	0.258	0.182	0.138	0.114	0.366
	96	0.091	0	0	0.096	0.098	0.256	0.305
	120	0	0	0	0	0	0.138	0.147
	144	0.094	0	0	0	0.256	0.349	0.237
α-D-mannosidase	48	0.068	0.118	0.133	0.086	0.043	0.142	0.108
	72	0.020	0.075	0.072	0.034	0	0	0.136
	96	0.062	0	0	0.206	0	0	0.260
	120	0.019	0.126	0.088	0	0	0	0.150
	144	0.058	0.199	0.062	0	0.175	0	0.273
β-D-fucosidase	48	0.006	0.025	0	0	0	0	0.023
	72	0	0.059	0	0	0	0	0.069
	96	0	0	0	0	0	0	0
	120	0	0	0	0	0	0	0
	144	0	0	0	0	0	0	0
β-D-xylosidase	48	0.065	0.372	0.319	0.347	0.391	0.444	0.387
	72	0.040	0.379	0.337	0.425	0.364	0.487	0.460
	96	0.031	0.670	0.447	0.251	0	0.285	0.699
	120	0.035	0.648	0.210	0.260	0	0.479	0.661
	144	0	0	0	0	0	0	0

Key to treatments: blank, blank agar plate; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri*; Eh, *E. herbariorum*; Er, *E. rubrum*; Pen, *Penicillium chrysogenum*.

(iii) 0.85 a$_w$

Enzyme	Time (days)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)						L.S.D.
		Blank	Ea	Ech	Eh	Er	Pen	
N-acetyl-α-D-glucosaminidase	3	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
	11	0	0	0	0	0	0	0
	14	0	0	0	0.489	0.017	0.235	0.563
α-D-mannosidase	3	0	0	0	0	0	0	0
	5	0	0.039	0	0	0	0	0.047
	7	0	0	0	0	0.008	0	0.023
	11	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0
β-D-fucosidase	3	0	0	0.194	0	0.146	0.047	0.234
	5	0	0	0	0	0	0	0
	7	0.055	0	0.490	0	0	0	0.448
	11	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0
β-D-xylosidase	3	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
	11	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0

Key to treatments: blank, blank agar plate; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri*; Eh, *E. herbariorum*; Er, *E. rubrum*; Pen, *Penicillium chrysogenum*.

(2) Comparative study of volatile patterns and hydrolytic enzyme production from mycotoxigenic and non-mycotoxigenic fungi. Key to treatments: blank, blank agar plates; Pen, *Penicillium chrysogenum*; AN, *Aspergillus niger*; AO, *A. ochraceus*; AF, *A. flavus* and WS, *Wallemia sebi*.

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)						L.S.D.
		Blank	Pen	AN	AO	AF	WS	
N-acetyl-α-D-glucosaminidase	72	0	0	0	0.104	0.041	0	0.164
	120	0	0	0.657	0.166	0.087	0.135	0.744
α-D-mannosidase	72	0	0.145	0.092	0.136	0	0	0.223
	120	0	0.308	0.377	0.163	0.298	0	0.408
β-D-fucosidase	72	0	0.091	0	0.062	0	0	0.160
	120	0.030	0.026	0	0.157	0.172	0	0.196
β-D-xylosidase	72	0.013	0.510	0	0.058	0.106	0	0.508
	120	0.066	1.000	0.498	0.144	0.489	0.233	0.951

(3) Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of two *Fusarium* spp. using volatile profiles and enzyme activities.

(i) *F. moniliforme*

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)					L.S.D.
		Blank	25N	6N	150N	48N	
N-acetyl-α-D-glucosaminidase	48	0	0	0	0	0	0
	72	0	0.061	0	0	0	0.073
	96	0	0	0	0	0	0
α-D-mannosidase	48	0	0	0	0	0	0
	72	0	0.036	0.035	0	0	0.045
	96	0	0	0	0	0	0
β-D-fucosidase	48	0	0	0	0	0	0
	72	0	0.039	0.039	0.039	0.038	0.041
	96	0.039	0.037	0.038	0.037	0.037	0.001
β-D-xylosidase	48	0	0	0	0	0	0
	72	0	0	0	0	0	0
	96	0.037	0.033	0	0	0.038	0.010

(ii) *F. proliferatum*

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)					L.S.D.
		Blank	20N	58N	55N	112N	
N-acetyl-α-D-glucosaminidase	48	0	0	0	0	0	0
	72	0	0	0	0	0	0
	96	0	0	0	0	0	0
α-D-mannosidase	48	0	0	0	0	0	0
	72	0	0.017	0.023	0.023	0.024	0.028
	96	0.021	0.020	0.023	0.025	0.021	0.010
β-D-fucosidase	48	0	0.023	0.022	0.023	0.023	0.027
	72	0.024	0.018	0.024	0.025	0.026	0.010
	96	0	0	0	0	0	0
β-D-xylosidase	48	0	0	0	0	0	0
	72	0.024	0.019	0.023	0.024	0.026	0.010
	96	0	0	0	0	0	0

(4) Early detection of fungal growth on modified bread substrate based on the volatile production patterns, enzymic study and total fungal populations. Key to treatments: blank, uninoculated bread analogue; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri* and Pen, *Penicillium chrysogenum*.

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	26	0.302	0.123	0	0	0.380
	40	0.344	0.235	0.127	0.032	0.445
	48	0.134	0.361	0.173	0.268	0.241
	72	0.288	0.906	0.331	0.816	0.246
α-D-mannosidase	26	0	0	0	0	0
	40	0	0	0	0	0
	48	0	0.086	0	0	0.132
	72	0.040	0.349	0.078	0.0830	0.170
β-D-fucosidase	26	0	0	0	0	0
	40	0	0	0	0	0
	48	0.010	0.059	0.059	0.005	0.127
	72	0	0.092	0.078	0.363	0.121
β-D-xylosidase	26	0	0	0	0	0
	40	0	0	0	0	0
	48	0.006	0.174	0.037	0.153	0.121
	72	0.010	0.228	0.148	0.538	0.119

(5) Effect of potassium sorbate preservative on fungal volatile patterns, hydrolytic enzymes and total populations. Key to treatments: Blank, uninoculated bread analogue; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri* and Pen, *Penicillium chrysogenum*.

(i) in the absence of potassium sorbate

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	24	0	0	0	0	0
	48	0	0.043	0.082	0.094	0.101
	72	0	0	0.585	0	0.318
	96	0	0.033	0.333	0	0.242
α-D-mannosidase	24	0	0	0	0	0
	48	0	0	0	0	0.193
	72	0.042	0.207	0.247	0.294	0.199
	96	0.087	0.292	0.533	0.507	0.205
β-D-fucosidase	24	0	0	0	0	0
	48	0	0	0.135	0	0.155
	72	0	0	0.229	0.365	0.412
	96	0	0.008	0.472	0.660	0.171
β-D-xylosidase	24	0	0	0	0	0
	48	0	0	0	0.412	0.120
	72	0	0	0.368	0.562	0.197
	96	0	0	0.190	0.916	0.130

(ii) in the presence of 0.15% potassium sorbate

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} μg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	24	0	0	0	0	0
	48	0	0	0	0	0.101
	72	0.057	0.022	0.346	0.884	0.318
	96	0	0.140	0.562	0	0.242
α-D-mannosidase	24	0	0	0	0	0
	48	0	0	0	0	0.193
	72	0.030	0.117	0.187	0.470	0.199
	96	0.064	0.326	0.325	0.214	0.205
β-D-fucosidase	24	0	0	0	0	0
	48	0	0	0	0	0.155
	72	0.014	0	0.046	0.005	0.412
	96	0.384	0.081	0.090	0.601	0.171
β-D-xylosidase	24	0	0	0	0	0
	48	0	0	0.007	0	0.120
	72	0.060	0	0.019	1.202	0.197
	96	0.057	0.103	0.185	0.537	0.130

Key to treatments: Blank, uninoculated bread analogue; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri* and Pen, *Penicillium chrysogenum*.

(iii) in the presence of 0.3% potassium sorbate

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	24	0	0	0	0	0
	48	0	0	0	0	0.101
	72	0	0	0	0.416	0.318
	96	0	0.383	0	0.206	0.242
α-D-mannosidase	24	0	0	0	0	0
	48	0	0	0.171	0	0.193
	72	0	0	0.131	0.345	0.199
	96	0	0.288	0.027	0.285	0.205
β-D-fucosidase	24	0	0	0	0	0
	48	0	0	0	0	0.155
	72	0	0	0	0.303	0.412
	96	0	0	0	0.266	0.171
β-D-xylosidase	24	0.002	0	0	0	0
	48	0	0.017	0	0	0.120
	72	0	0	0	0.469	0.197
	96	0	0	0	0.594	0.130

Key to treatments: Blank, uninoculated bread analogue; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri* and Pen, *Penicillium chrysogenum*.

(6) Effect of calcium propionate preservative on fungal volatile profiles, enzyme activities and total populations. Key to treatments: Blank, uninoculated bread analogue; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri* and Pen, *Penicillium chrysogenum*.

(i) in the absence of calcium propionate

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} μg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	24	0	0	0	0	0.139
	48	0	0.030	0.048	0	0.295
	72	0	0.069	0.008	0	0.221
α-D-mannosidase	24	0	0.129	0.308	0	0.277
	48	0.749	0.307	0.428	0.006	0.433
	72	0.918	0.730	0.312	0.479	0.202
β-D-fucosidase	24	0.010	0.006	0.050	1.017	0.136
	48	0.411	0.237	0.124	0.042	0.237
	72	0.515	0.121	0.196	0.325	0.100
β-D-xylosidase	24	0.168	0.142	0.137	0.079	0.115
	48	0.189	0.133	0.131	0.081	0.138
	72	0.010	0.116	0.232	0.308	0.168

(ii) in the presence of 0.15% calcium propionate

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} μg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	24	0.216	0.063	0.186	0	0.139
	48	0.537	0.322	0.164	0	0.295
	72	0.379	0.361	0.172	0.191	0.221
α-D-mannosidase	24	0.169	0.113	0.279	0.216	0.277
	48	0.117	0.421	0.335	0.268	0.433
	72	0.353	0.645	0.244	0.225	0.202
β-D-fucosidase	24	0.118	0.173	0.164	0.102	0.136
	48	0.248	0.235	0.180	0.158	0.237
	72	0.195	0.291	0.090	0.273	0.100
β-D-xylosidase	24	0.159	0.233	0.226	0.157	0.115
	48	0.331	0.344	0.387	0.359	0.138
	72	0.286	0.384	0.360	0.991	0.168

(iii) in the presence of 0.3% calcium propionate

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	24	0.357	0.586	0.253	0.493	0.139
	48	0.448	0.639	0.636	1.161	0.295
	72	0.742	0.973	0.587	0.969	0.221
α-D-mannosidase	24	0.301	0.074	0.219	0.230	0.277
	48	0.357	0.510	0.499	0.471	0.433
	72	0.293	0.377	0.370	0.395	0.202
β-D-fucosidase	24	0.246	0.187	0.248	0.108	0.136
	48	0.209	0.299	0.242	0.388	0.237
	72	0.208	0.188	0.377	0.392	0.100
β-D-xylosidase	24	0.322	0.328	0.362	0.314	0.115
	48	0.414	0.366	0.395	0.371	0.138
	72	0.640	0.607	0.574	0.942	0.168

Key to treatments: Blank, uninoculated bread analogue; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri* and Pen, *Penicillium chrysogenum*.

(7) Detection and differentiation of spoilage fungi on natural bread substrate using the electronic nose. Key to treatments: Blank, uninoculated bread analogue; Ea, *Eurotium amstelodami*; Ech, *E. chevalieri* and Pen, *Penicillium chrysogenum*.

Enzyme	Time (h)	Specific activity (nmol p-nitrophenol min^{-1} µg^{-1} protein)				L.S.D.
		Blank	Ea	Ech	Pen	
N-acetyl-α-D-glucosaminidase	24	0	0	0	0	0
	48	0	0	0.055	0.068	0.245
	72	0.097	0.280	0.310	0.245	0.100
α-D-mannosidase	24	0	0.004	0.046	0.039	0.064
	48	0.073	0.103	0	0.052	0.058
	72	0.061	0.187	0.116	0.269	0.193
β-D-fucosidase	24	0.034	0.011	0.003	0	0.120
	48	0.010	0.074	0.061	0.066	0.079
	72	0	0.144	0.137	0.256	0.803
β-D-xylosidase	24	0.097	0.126	0.086	0.086	0.073
	48	0.088	0.047	0.086	0.003	0.077
	72	0.027	0.097	0.231	6.202	0.122

APPENDIX H

0.95 a_w

y = 2.3172x - 1.9027
R^2 = 0.9899

0.90 a_w

y = 1.9407x - 1.2605
R^2 = 0.9943

0.85 a_w

y = 1.1218x - 2.173
R^2 = 0.9955
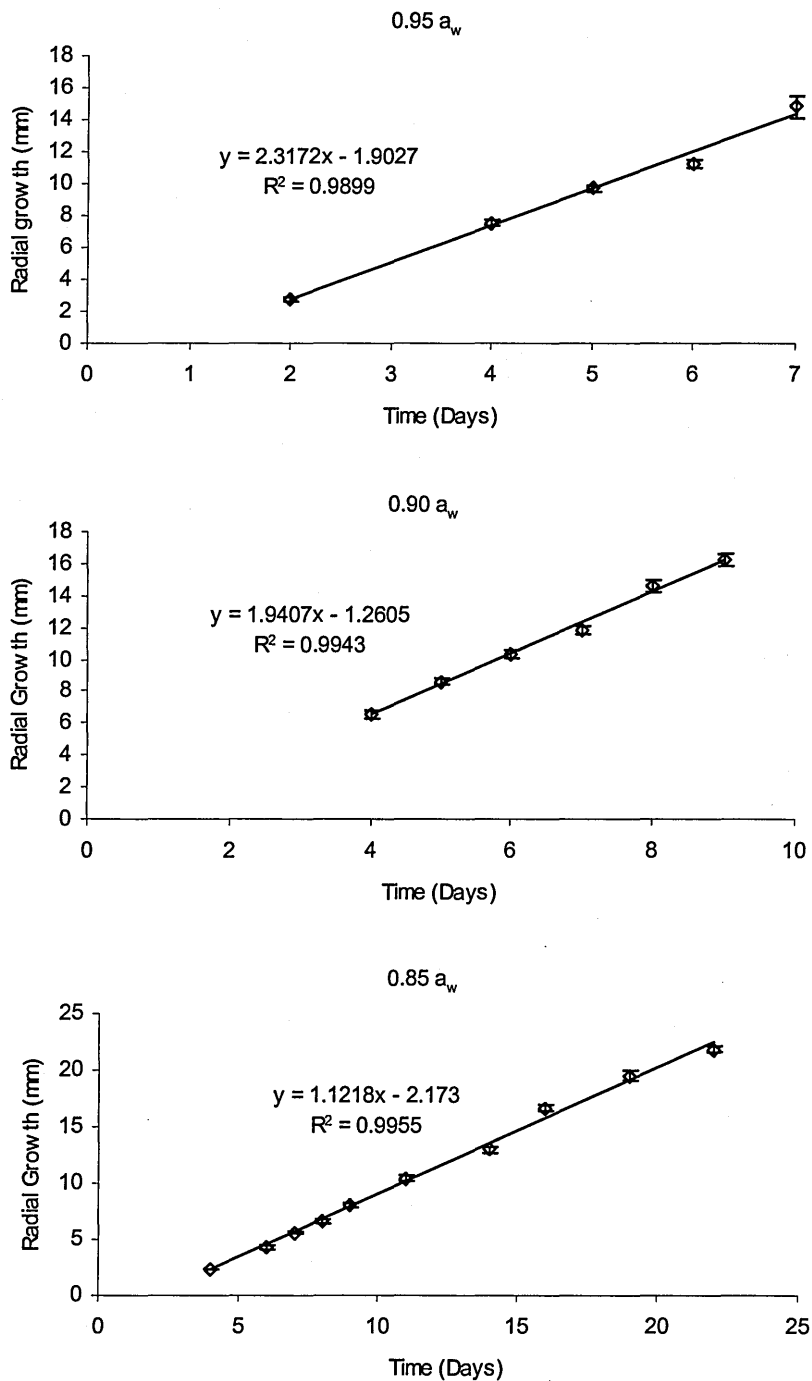
Growth of *Eurotium chevalieri* at different water activities: (a) 0.95 a_w; (b) 0.90 a_w and (c) 0.85 a_w on 2% wheatmeal agar at 25°C. Each data point is a mean of ten replicates and the bar indicates the error bar. Note the different axes scales.
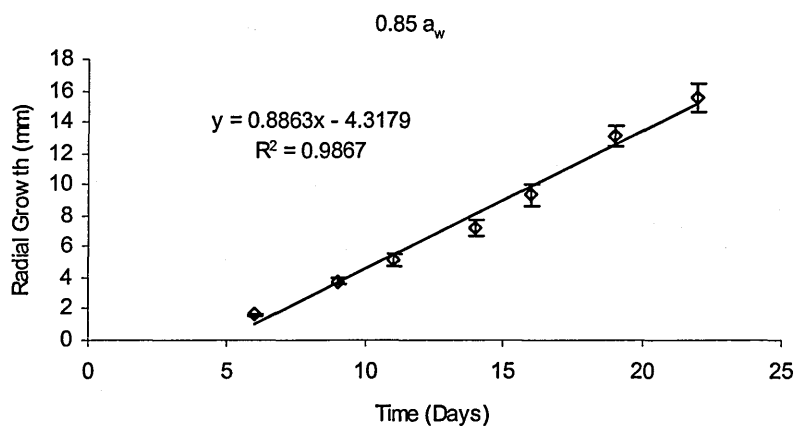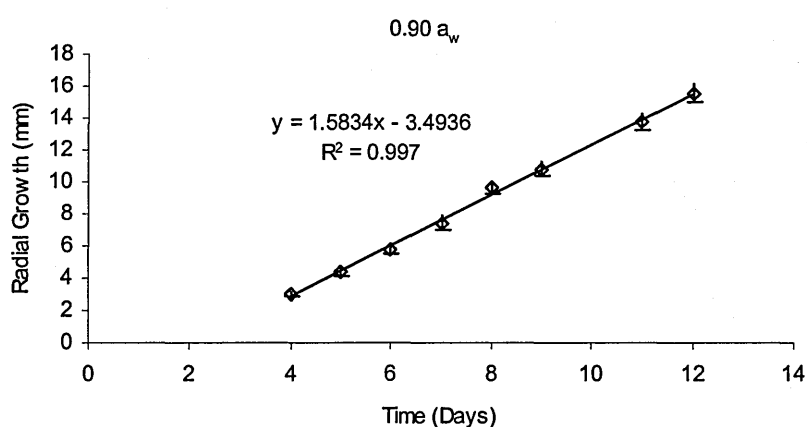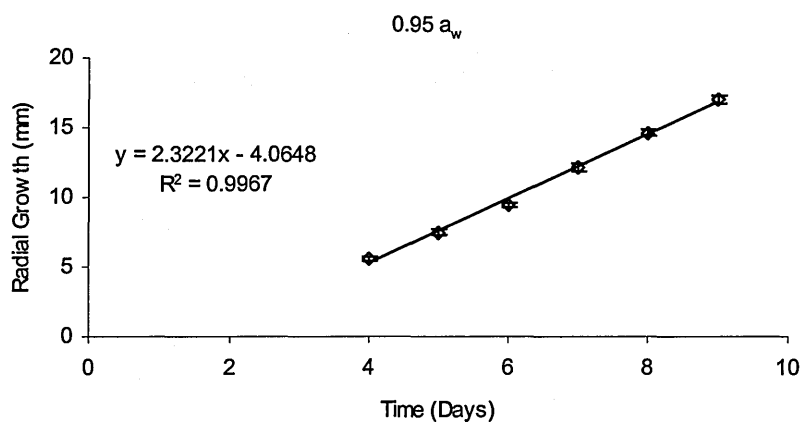
Growth of *Eurotium amstelodami* at different water activities: (a) 0.95 a_w; (b) 0.90 a_w and (c) 0.85 a_w on 2% wheatmeal agar media at 25°C. Each data point is a mean of ten replicates and the bar indicates the error bar. Note the different axes scales.
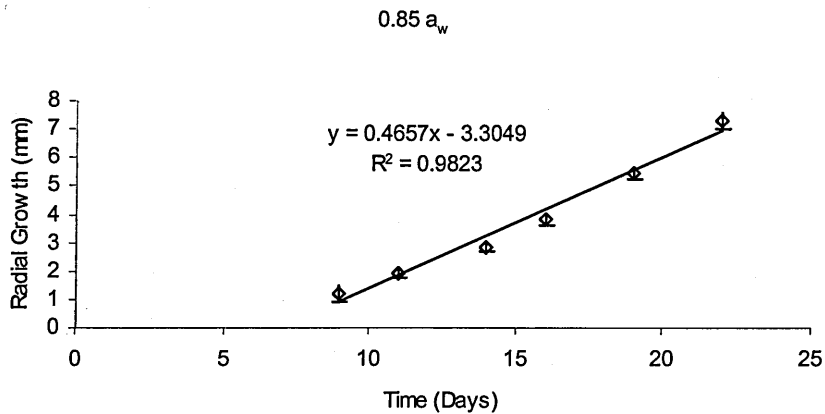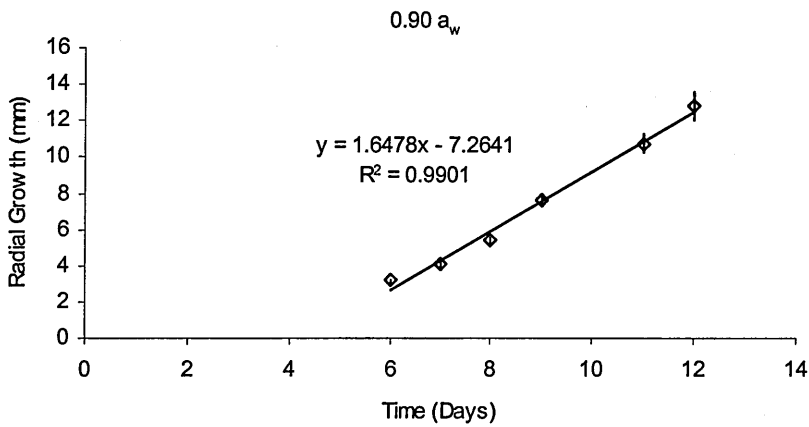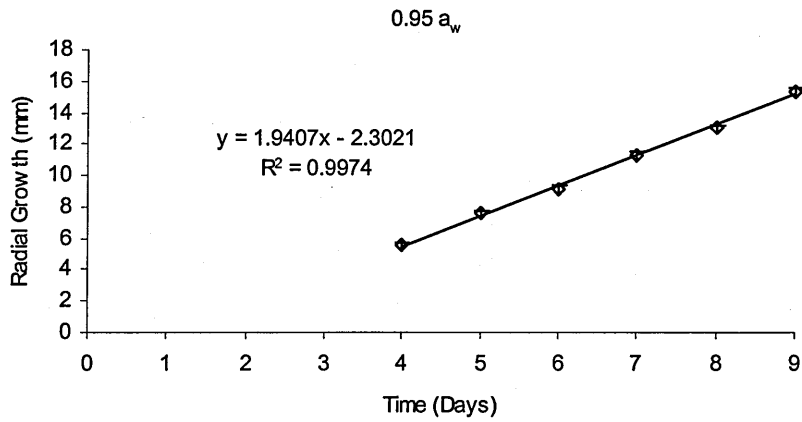
Growth of *Eurotium herbariorum* at different water activities: (a) 0.95 a_w; (b) 0.90 a_w and (c) 0.85 a_w on 2% wheatmeal agar media at 25°C. Each data point is a mean of ten replicates and the bar indicates the error bar. Note the different axes scales.
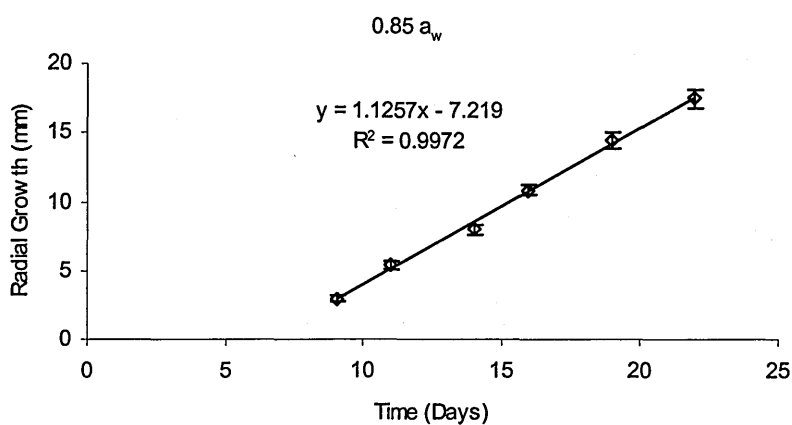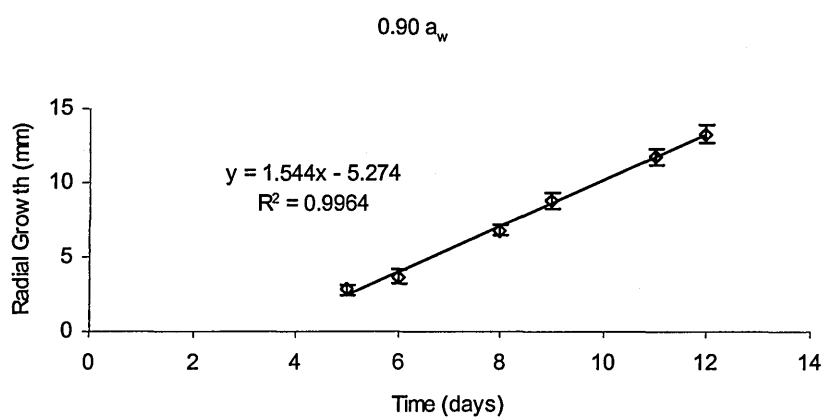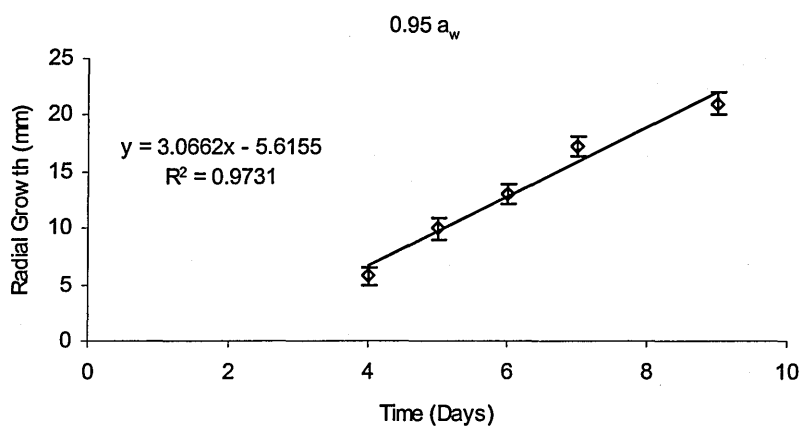
Growth of *Eurotium rubrum* at different water activities: (a) 0.95 a_w; (b) 0.90 a_w and (c) 0.85 a_w on 2% wheatmeal agar media at 25°C. Each data point is a mean of ten replicates and the bar indicates the error bar. Note the different axes scales.
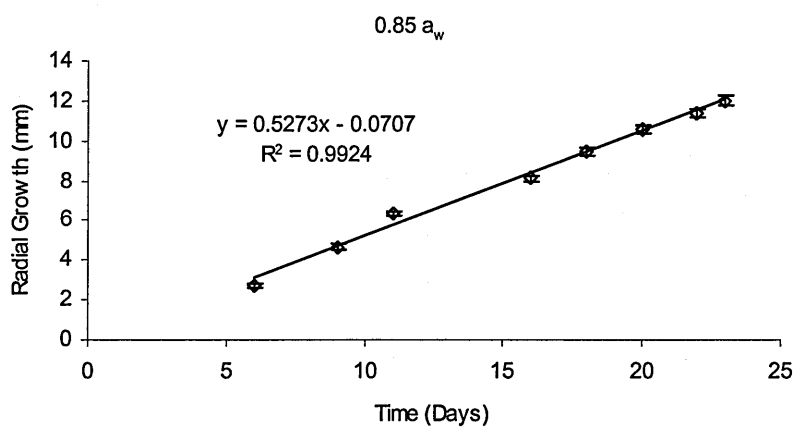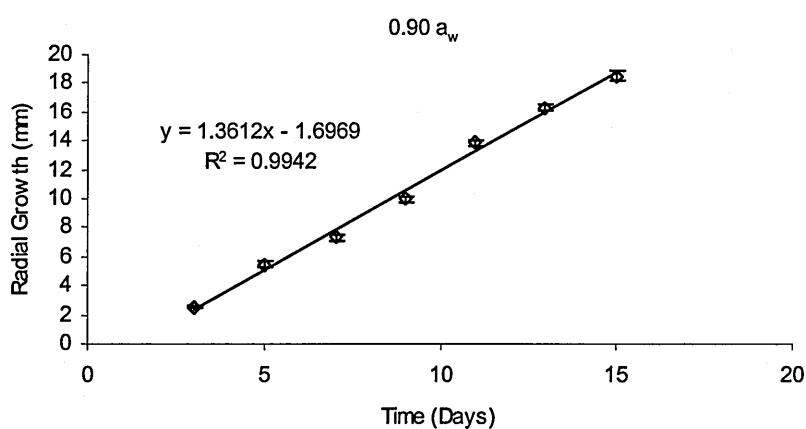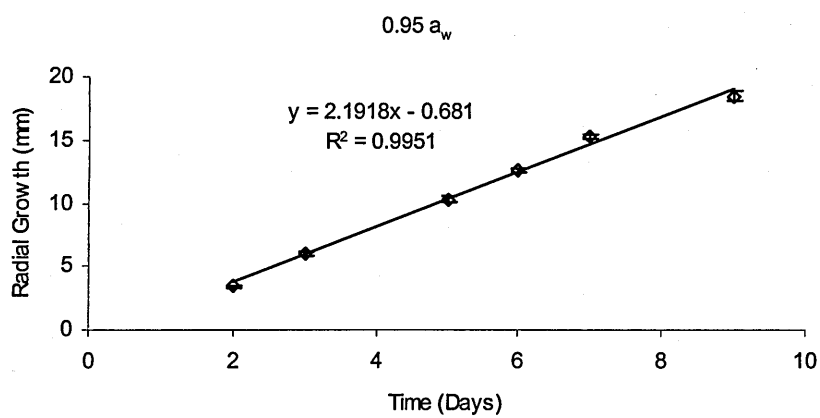
Growth of *Penicillium chrysogenum* at different water activities: (a) 0.95 a_w; (b) 0.90 a_w and (c) 0.85 a_w on 2% wheatmeal agar media at 25°C. Each data point is a mean of ten replicates and the bar indicates the error bar. Note the different axes scales.

APPENDIX I

Principal component analysis showing the differentiation of bread analogue in the (a) absence of preservative and in the presence of (b) potassium sorbate and (c) calcium propionate. Key to treatments: a1-a4, 0.15% calcium propionate; b1-b4, 0.3% calcium propionate; d1-d4, 0.15% potassium sorbate; e1-e4, 0.3% potassium sorbate and c1-c4, control blank bread analogue with no preservative.
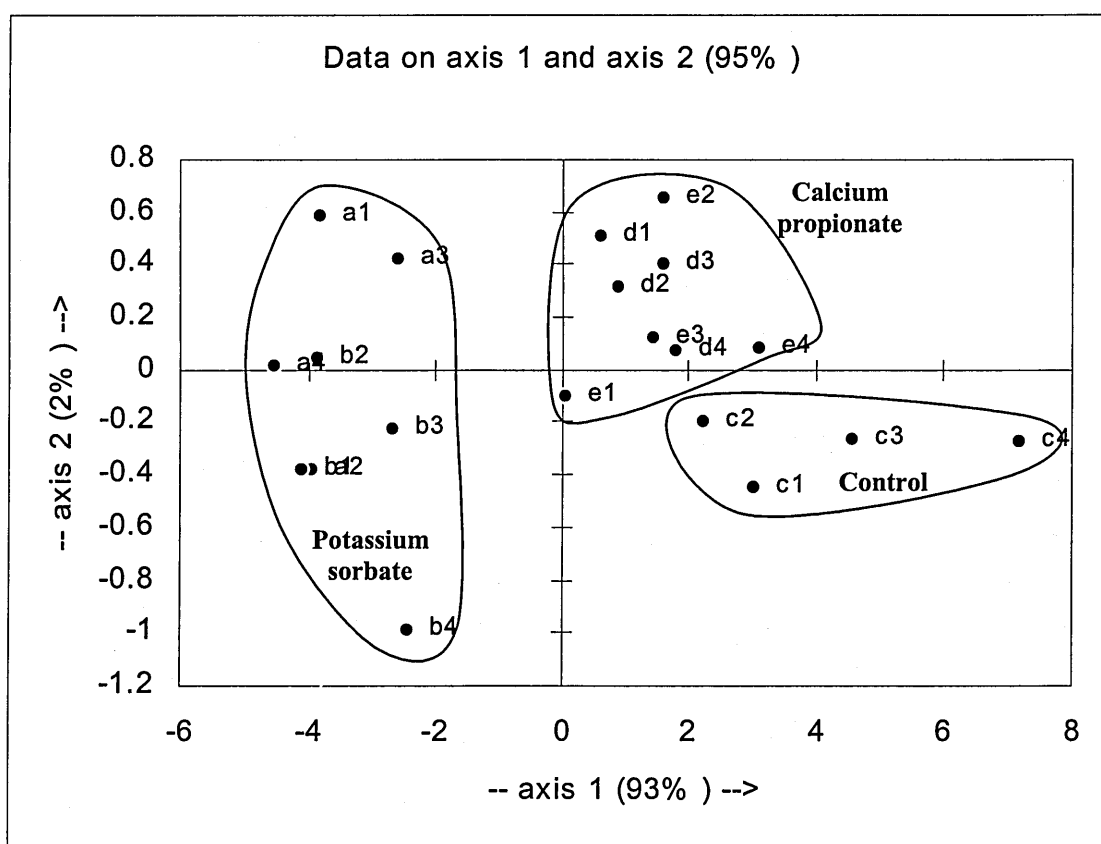
APPENDIX J

Published papers

Keshri, G., Magan, N. & Voysey, P. (1998). Use of an electronic nose for the early detection and differentiation between spoilage fungi. *Letters in Applied Microbiology.* **27**, 261-264.

Magan, N., Pavlou, A. & Keshri, G. (1998). Detection of fungal volatiles using an electronic nose. *In* Proceedings of The Fifth World Congress on Biosensors, Berlin, Germany, 3-5 June 1998, Elseviers Science, pp. 363.

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

Keshri, G. & Magan, N. (2000). Detection of mould spoilage in bread analogues using electronic nose technology. *In* the Proceedings of ISOEN 2000, Brighton, U. K., 20-24 July 2000. Eds. Persaud, K. C. & Gardner, J. W. pp.197-198.

Keshri, G., Voysey, P. & Magan, N. (2001). Early detection of spoilage moulds in bread using volatile production patterns and quantitative enzyme assays. *Journal of Applied Microbiology.* **91, In Press.**

Use of an electronic nose for the early detection and differentiation between spoilage fungi

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G. KESHRI, N. MAGAN AND P. VOYSEY. 1998. Six spoilage fungi (four *Eurotium* species, a *Penicillium* sp. and *Wallemia sebi*) were grown as spore lawn surface cultures at 0·95 water activity and 25 °C. Prior to and during visible growth (24 and 48, and 72 h), single cultures were enclosed in polyethylene bags, the head space was sampled with an electronic nose unit, consisting of 14 polymer sensors, and the data analysed. There was good replication between volatile patterns of the same species and using principal component, discriminant function and cluster analyses it was possible to differentiate between the agar blanks, three *Eurotium* spp., the *Penicillium* sp. and *W. sebi* during microscopic growth for the first time. This suggests that there is potential for the early detection of the activity of spoilage fungi in general, as well as possible differentiation between related xerophilic spoilage fungi, by detection of the patterns of volatile odours produced using an electronic nose system.

INTRODUCTION

Traditionally, analyses of fungal contamination of grain and other raw materials for the food industry have depended on culture methods which require long periods of time for enumeration and quantification. Other methods which have been examined as indicators of the activity of spoilage fungi include effects on degradation of grain components (Magan 1993), fungal enzyme activity (Jain *et al.* 1991; Marin *et al.* 1998), biochemical markers such as ergosterol and ATP (Williams 1989; Tothill *et al.* 1992), and the respiratory activity of moulds (Lacey *et al.* 1994). Recently, there has been interest in the potential of using the dominant odour volatiles produced by fungi, particularly 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone, as indicators of spoilage in stored grain (Kaminsky *et al.* 1985; Tuma *et al.* 1989; Borjesson *et al.* 1990). Indeed, Borjesson *et al.* (1990, 1992) found good correlation between odour volatiles detected by gas chromatography and CO_2 production and ergosterol, but not with colony forming units (cfu) for some *Penicillium* and *Aspergillus* species. Recently, Jonsson *et al.* (1997) reported on the classification of grain quality using an electronic nose in combination with an artificial neural network. However,

no attempt has been made to examine the potential of using the patterns of volatile production and an electronic nose to detect early activity of spoilage fungi, prior to visible growth, and the potential for distinguishing between fungal species.

The objective of this study was to evaluate an electronic nose system for the detection and differentiation of six different xerophilic spoilage fungi, four related *Eurotium* spp., a *Penicillium* sp. and *Wallemia sebi*, based on the patterns of volatile organic compounds produced.

MATERIALS AND METHODS

Fungal species

The species used in this study were *Eurotium amstelodami, E. chevalieri, E. herbariorum, E. rubrum*, a *Penicillium* species and *Wallemia sebi*. All were isolated from cereal grain except for the *Penicillium* sp. which was isolated from mouldy bread.

Media and incubation

A 2% milled wheat agar (2% technical agar No. 2) was modified to 0·95 water activity using glycerol and inoculated with 0·2 ml of a spore suspension ($1\cdot5 \times 10^6$ ml^{-1}) and spread-plated to obtain an even covering of the agar surface. Approx-

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imately 12 replicates per species were incubated at 25 °C for 72 h.

Sampling procedure

After 24, 48 h (prior to visible growth) and 72 h (visible growth), four replicate single Petri plates of each species were placed in sample bags (500 ml capacity) which were then inflated with filtered air and sealed. The bags were allowed to equilibrate for 1 h at 25 °C and species and replicates then sampled in a random fashion using the electronic nose. Blank uninoculated 2% milled wheat agar plates were used as controls. The bags were sampled with a needle + filter apparatus which drew the head space volatiles over the sensors. This was controlled using a computer software package.

Electronic nose

A Bloodhound (Bloodhound Sensors Ltd, Leeds, UK) electronic nose system incorporating 14 polymer sensors was used in these experiments, enabling the profiles produced by each mould species to be compared for each of the sensors.

Data analyses and treatment

Normalized data were analysed using the program xISTAT Principal Component Analysis (PCA) and Discriminant Function Analysis (DFA) (CA, USA), and cluster analysis techniques were employed to classify the fungal species.

RESULTS AND DISCUSSION

Generally, it was difficult to separate the germinating spores of different species after 24 h growth. However, after 48 h, there was very good reproducibility between replicates of the same species in relation to the response of the 14 sensor array, as shown for the *Penicillium* species (Fig. 1). Sensor drift was small over the experimental period ($<8\%$). Similar data for all the species, including the controls (2% milled wheat agar), were used in DFA to try to differentiate between the fungal species and the milled wheat agar controls. This showed that for all six fungal species and the agar blanks, quite good separation was possible (93%), with some overlap between *E. amstelodami* and *E. herbariorum*. However, excluding the latter species gave very good separation (97%) using this
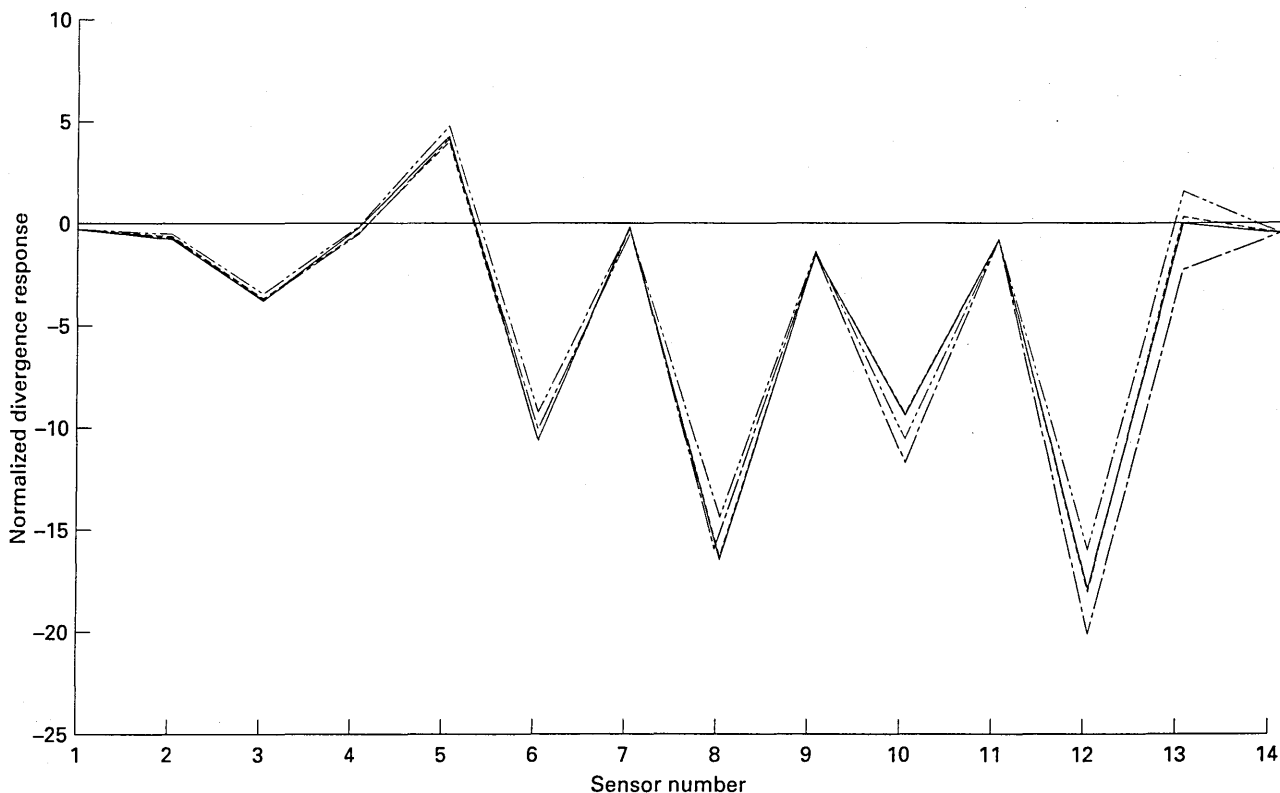
Fig. 1 Reproducibility of the response of the sensor array to volatiles produced by a *Penicillium* species grown *in vitro* as a spore lawn on a 2% milled wheat agar at 25 °C for 48 h. Lines are four replicates of the same treatment

technique after a 48 h experimental period (Fig. 2). All the data were used to produce a dendrogram of the response of the sensor array to the six spoilage fungi, based on the profile of volatiles (Fig. 3). This suggests that five of the species could be differentiated using this approach, while the related species of *E. amstelodami* and *E. herbariorum* could not, at

such an early stage of growth (48 h). Better detection patterns were observed after 72 h incubation when growth was visible, but this is too late a stage for the use of an electronic nose for the early detection of mould activity in agrofood substrates.

Previously, Borjesson *et al.* (1990, 1992) had found that there were differences in the fungal volatiles produced by
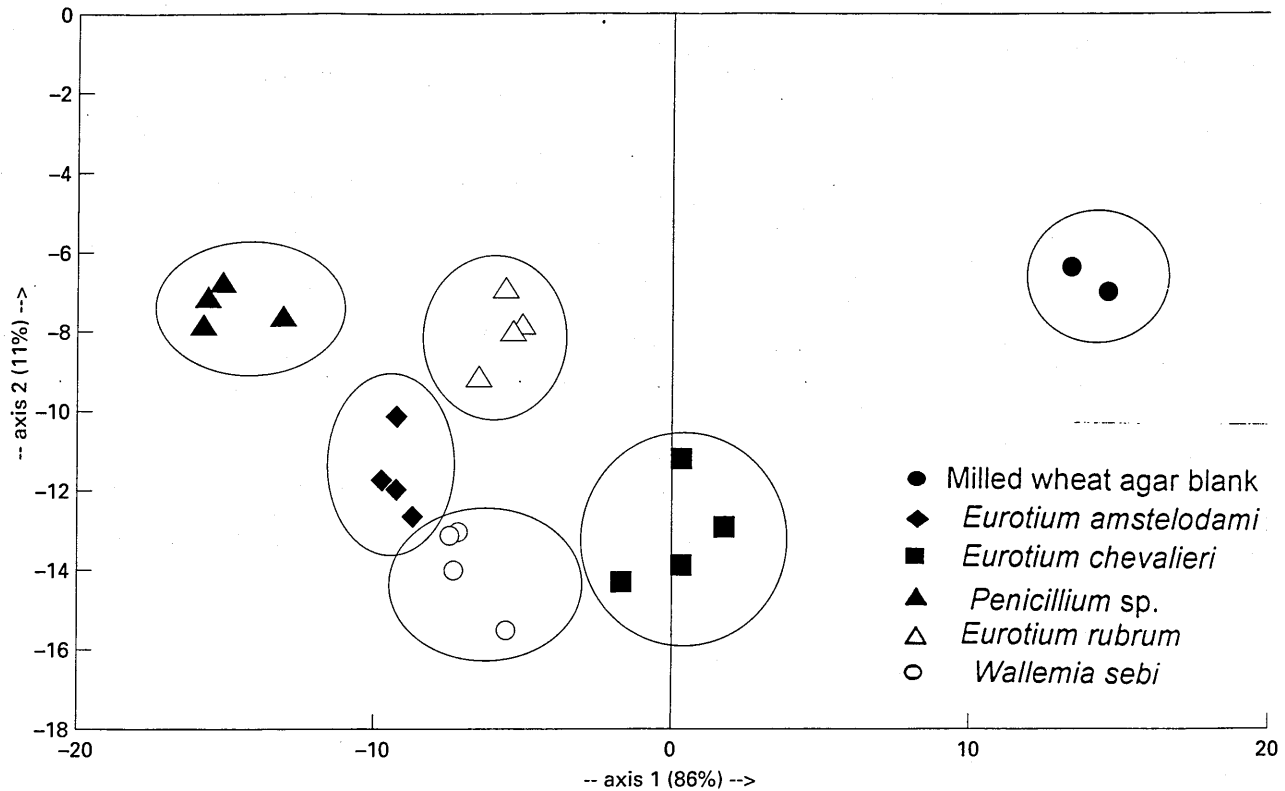
Fig. 2 Results of the discriminant function analyses and classification of five of the spoilage fungi and the controls (blank agar plates) based on volatile production on a 2% milled wheat agar after 48 h at 25 °C (data on axis 1 and 2 account for 97% discrimination)
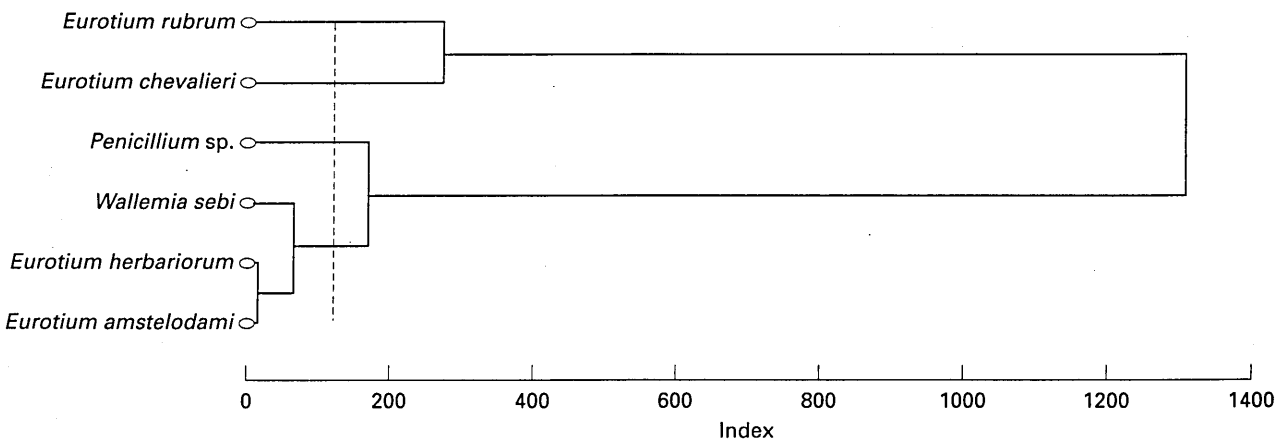
Fig. 3 A dendrogram of the response of the sensor array to the six spoilage fungal species based on the profile of volatiles

grain fungi (*Penicillium* species, *Aspergillus flavus*, *A. versicolor* and *A. candidus*) using gas chromatography. Interestingly, there were fewer differences between grain substrate type and age of cultures than between species. Borjesson *et al.* (1990) also found that with *P. aurantiogriseum*, the production of 8-carbon alcohols and 3-methyl-1-butanol were higher in grain than on agar substrates. In their studies, fungal cultures were grown for 5 d at a higher water availability than used in the present studies. Interestingly, Pasanen *et al.* (1996) have suggested that volatile organic compounds, particularly ketones, may also be useful markers of the activity of mycotoxigenic species such as *Fusarium sporotrichoides* and *Penicillium verrucosum*. Thus, this approach could also be a powerful tool for the rapid and early detection of the activity of mycotoxigenic species.

More detailed information is required on whether modifications in water availability and storage temperature affect the patterns of volatiles produced, and those detected by the electronic nose. Studies are in progress to compare volatile profiles with fungal enzymatic assays and conventional methods for the rapid and early detection of fungal spoilage in cereal grains, and in intermediate moisture bakery products.

ACKNOWLEDGEMENTS

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The Fifth World
Congress on Biosensors

Inter-Continental Hotel, Berlin,
Germany
3-5 June 1998

REFEREED

ABSTRACTS

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Detection of fungal volatiles using an electronic nose

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Moulds are responsible for significant spoilage of intermediate moisture bakery products. Often growth is associated with the production of characteristic mixtures of volatiles and toxic secondary metabolites (mycotoxins). There is a need for the rapid ans early detection of spoilage activity. Potential exists for using the production of volatile compounds as an indicator of product quality.

A range of spoilage moulds have been compared for their odour production under different steady state and fluctuating environmental conditions including temperature and water availability. Studies of head space analyses have shown that it is possible to discriminate between closely related mycotoxigenic *Fusarium* spp. (5 different species) as well as xerotolerant and xerophilic *Aspergillus* and *Penicillium* spp. in in vitro studies using an electronic nose employing an array of conducting polymer sensors with good reproducibility. Studies are now in progress to examine temporal effects and culture age on odour production and discrimination of spoilage moulds by their volatile profiles using bakery product analogues to correlate with spoilage indices and product shelf-life.

Key words: volatiles, electronic nose, spoilage moulds, food quality

Detection of fungal volatiles using an electronic nose

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Introduction

♦ In recent years environmental and consumer pressure has resulted in a reduction in the use of preservatives in intermediate moisture food products.

♦ Reduction in preservatives in bakery products could result in increased problems due to the activity of spoilage moulds, with some able to produce mycotoxins.

♦ There is a need to be able to rapidly evaluate the potential for initiation of such spoilage to enable quality and shelf-life to be optimised (Magan, 1993).

♦ Early detection of characteristic volatiles by spoilage fungi could provide a method for rapid quantification of product quality.

♦ This poster presents initial data on the potential for using an electronic nose to differentiate between important fungal spoilage organisms when growing *in vitro* on a cereal-based medium.

Materials and Methods

Fungal species: The xerophilic/xerotolerant species used were *Eurotium amstelodami, E. chevalieri, E. rubrum, E. herbariorum,* a *Penicillium* species, and *Wallemia sebi*.

In vitro **medium**: A 2% milled wheat agar was modified to 0.95 water activity (= 95% r.h.) and inoculated with a spore suspension (10^5 ml^{-1}) and spread-plated to obtain an even covering. Up to 10 replicate plates per species were incubated at 25°C for 3 days.

Dry cereals: Seven dry grain samples (25 g) including maize, wheat, oats, barley, soya, rapeseed and linseed were also examined.

Sampling procedure: Single plates were placed in sample bags (500 ml) which were inflated with clean filtered air and sealed. The bags were allowed to equilibrate for 1 hr at 25°C and then sampled using the electronic nose. Blank wheat agar plates were also sampled, and deionised water used as a control.

Electronic nose: A Bloodhound electronic nose system incorporating 14 polymer sensors was used in these experiments enabling the profiles produced by each mould species and grain to be compared.

Data treatment: Normalised data were analysed using the program xlStat Priniciple Component Analysis (PCA), Discriminant Function Analysis (DFA), and Cluster Analysis techniques were employed to classify the fungal species. Artificial Neural Network (ANN) was also applied and the back propagation of errors used as a training algorithm.

Results
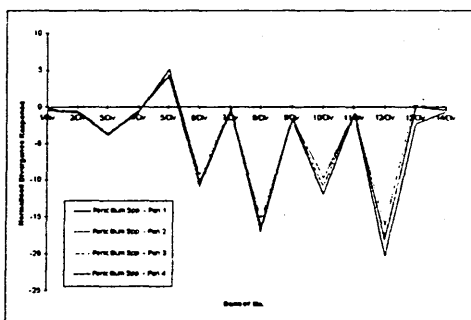
Figure 1. Reproducibility of the response of the sensor array to volatiles produced by a *Penicillium* sp. grown *in vitro* on a 2% wheat agar at 0.95 water activity and 25°C.
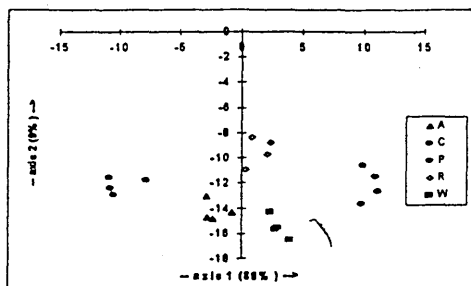
Figure 2. Results of discriminant function analyses and classification of five spoilage fungi based on volatile production on a 2% wheat agar: A, *Eurotium amstelodami*; C, *E.chevalieri*; R, *E.rubrum*; P, *Penicillium* sp.; W, *Wallemia sebi*.
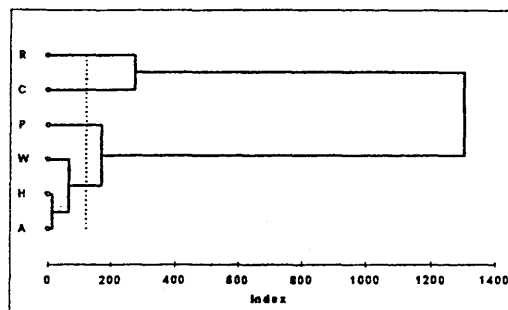
Figure 3. A dendogram of the response of the sensor array to six spoilage fungal species based on the profile of volatiles: A, *Eurotium amstelodami*; C, *E.chevalieri*; R, *E.rubrum*; H, *E.herbariorum*; P, *Penicillium* sp.; W, *Wallemia sebi*.
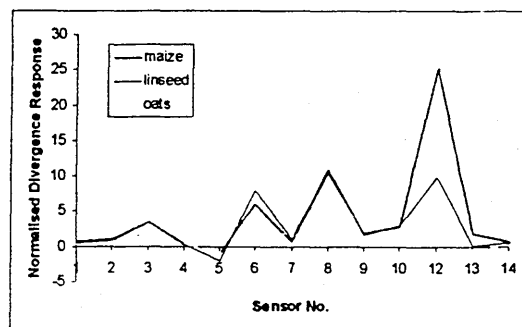
Figure 4. Comparison of the profile of the divergence responses of three different grain types.

Conclusions

♦ The sensor array showed a good reproducibility in response to volatiles produced by fungi *in vitro* on cereal-based media.

♦ The classification rate for all six fungal species was 87%, and for five of the six species was 97%.

♦ *Eurotium rubrum; E.chevalieri,* and the *Penicillium* sp. were correctly identified.

♦ Neural network classification rate for all six spoilage fungi was 90%.

♦ PCA showed good discrimination between maize and soya bean but there was overlap between temperate cereals.

♦ Studies are in progress to compare volatile profiles with enzymatic and conventional microbial methods for the rapid and early detection of fungal spoilage in cereal grains, and in intermediate moisture bakery products.

Acknowledgements

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Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of two *Fusarium* spp. using volatile production profiles and hydrolytic enzymes

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G. KESHRI AND N. MAGAN. 2000. Volatile profiles and hydrolytic enzyme production by one non-mycotoxigenic and three mycotoxigenic strains of *Fusarium moniliforme* and *F. proliferatum*, grown *in vitro* for up to 96 h on a grain medium at 25 °C/0·95 water activity, were examined for differentiation of isolates. After spore lawn inoculation, measurements were made after 48, 72 and 96 h by sampling the head space above cultures with an electronic nose system using a 14 sensor surface polymer array, and by extraction and quantification of hydrolytic enzymes. There was good reproducibility of volatile patterns between replicates of the same treatment. Principal component analysis indicated that discrimination could be achieved between the uninoculated controls, the non-mycotoxigenic strain and the mycotoxin-producing strains for both species after 48 h. The total and specific activity of three out of seven enzymes (β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase) were found to increase significantly in the non-mycotoxigenic when compared with the toxigenic strains of both species after 72 h. Activities of the others (β-D-fucosidase, α-D-mannosidase, β-D-xylosidase and N-acetyl-α-D-glucosaminidase) were not significantly different between strains. The study has shown for the first time that it is possible to differentiate between mycotoxigenic and non-mycotoxigenic strains of such spoilage fungi based on their volatile production patterns using an electronic nose system. These results have significance in the development of methods for the early detection of toxin-producing spoilage moulds in the food industry.

INTRODUCTION

Cereals are colonized by a range of fungal species which produce mycotoxins, including *Aspergillus flavus* (aflatoxins), *A. ochraceus* (ochratoxins), *Penicillium verrucosum* (ochratoxins) and *Fusarium moniliforme* and *F. proliferatum* (fumonisins) (Chelkowski 1993; Samson *et al.* 1995). The detection of these mycotoxigenic species and their mycotoxins in agricultural raw materials, and in processed cereal-based foods, has become important to evaluate and identify the risks of contamination and prevention of entry into the human and animal food chain.

It is known that the spoilage fungi producing mycotoxins often use different biosynthetic pathways, depending on the end secondary metabolite. For example, aflatoxin production by *Aspergillus flavus* and *A. parasiticus* follows the

general scheme of acetate → polyketide → anthraquinones → xanthones → aflatoxins. Studies by Zeringue *et al.* (1993) examined the head-space volatiles from aflatoxigenic and non-toxigenic strains of *A. flavus* in submerged culture in an attempt to differentiate between the strains. They found that *A. flavus* produced several distinct compounds (α-gurjunene, trans-caryophyllene and cadinene) which were present in aflatoxigenic strains only. There appeared to be some correlation between release of these compounds and biosynthesis of aflatoxins. Subsequent studies on *Fusarium sambucinum* strains with the ability to produce trichothecene mycotoxins again suggested that there were different sesquiterpenes produced by toxigenic and non-toxigenic strains (Jelen *et al.* 1995, 1997a, 1998).

Recently, Keshri *et al.* (1998) demonstrated that it was possible to differentiate between five different spoilage fungi, within 48 h of spore germination *in vitro*, by comparing the volatile compound profiles using an electronic nose system, which uses an array of 14 surface polymer sensors

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sensitive to different groups of volatiles produced by micro-organisms. Grain volatiles have been shown to be good early indicators of spoilage (Jelen and Wasowicz 1998; Schnurer et al. 1999; Evans et al. 2000; Magan and Evans 2000). However, no studies have been carried out to evaluate whether such systems could rapidly differentiate between fungal volatile patterns produced by non-toxic and toxic strains of important species from agricultural raw materials.

Previously, quantitative enzyme assays for specific hydrolytic enzymes produced by spoilage fungi, including *Fusarium* spp., had been shown to be good early indicators of growth prior to visible moulding (Jain et al. 1991; Magan 1993; Marin et al. 1998). However, no studies have evaluated whether extracellular enzyme production may be used for the differentiation between non-toxic and toxigenic strains of individual species.

The objectives of this study were thus (i) to examine the potential for detection and discrimination between toxigenic and non-toxigenic strains of *Fusarium moniliforme* and *F. proliferatum* from volatile production patterns using an electronic nose system and (ii) to compare these with hydrolytic enzyme production profiles as early indicators of growth by non-toxic and toxigenic strains.

MATERIALS AND METHODS

Fungal strains

Four strains of *Fusarium moniliforme* (6N, non-producer; 25N, fumonisin producer; 150N, zearalenone and fumonisin producer; and 48N, trichothecene producer) and of *F. proliferatum* (20N, non-producer; 112N, fumonisin producer; 58N, trichothecene producer; 55N, trichothecene and fumonisin producer) isolated from Spanish cereals were used in this study. The isolates are all held in the culture collection of the Food Technology Department, University of Lleida, Spain. The mycotoxin profiles of the strains have previously been reported (Sala 1993; Sala et al. 1994).

Fungal growth and volatile production

Wheat meal (2%) agar (2%, Lab M), modified to 0·95 water activity with glycerol, were inoculated with a 0·2 ml suspension (10^6 spores ml^{-1}) of each strain and species, and spread-plated over the surface using a surface-sterilized bent Pasteur pipette. The plates (16 replicates per treatment) were enclosed in polyethylene bags and incubated at 25 °C. Four replicates per strain were destructively sampled after 48, 72 and 96 h. Sub-samples from replicates were used for enzyme analyses and for volatile detection. Uninoculated agar medium served as controls at each sampling time.

Volatile detection

Single replicate Petri plate cultures were placed in sampling bags (500 ml capacity), with the lid carefully removed, filled with 300 ml filter-sterilized air and sealed (Keshri et al. 1998). The bags were subsequently equilibrated for 1 h at 25 °C. The head space from each bag was subsequently sampled in a 25 °C constant temperature room using an electronic nose system (BH114, Bloodhound Sensors Ltd, Leeds, UK), which uses a 14 surface-responsive sensor array (Pavlou et al. 2000). The response of each sensor is logged as a change in conductance and gives data which enable qualitative differences between samples to be compared.

The normalized divergence and absorption data were analysed using the program xlSTAT (Microsoft Excel add-in). Principal Component Analysis (PCA), Discriminant Function Analysis and Cluster Analysis were applied to classify the fungal species. To carry out analyses, the response data for each of the 14 sensors and each of the replicates were used. The groupings obtained using the PCA are based on unsupervized evaluation of these parameters for each replicate. Where responses are similar, the PCA will identify overlap between groups. Cluster analysis is based on the Malahanobi's squared distance between groups obtained in the discriminant analysis.

Quantification of enzyme production

Extraction of enzymes. Using a cork borer, three discs of agar were removed from the treatment plates and placed in 4 ml potassium phosphate extraction buffer (10 mmol l^{-1}; pH 7·2). The bottles were shaken on a wrist-action shaker for 1 h at 4 °C. The washings were decanted into 1 ml plastic Eppendorf tubes and centrifuged in a bench microfuge for 15 min. The supernatant fluid was removed and stored at −20 °C for total and specific enzyme activity determination (Marin et al. 1998).

Total enzyme activity determination. Seven hydrolytic enzyme activities were assayed using p-nitrophenyl substrates (Sigma). Table 1 shows the substrate concentration and buffer used for each enzyme assay. Enzyme extract (40 μl), substrate solution (40 μl) and the appropriate buffer (20 μl) were pipetted into the wells of the microtitre plate and incubated at 37 °C for 1 h along with the appropriate controls.

The reaction was stopped by the addition of 5 μl 1 mol l^{-1} sodium carbonate solution and left for 3 min. The enzyme activity was measured, using an MRX multiscan plate reader (Dynex Technologies Ltd, Billinghurst, West Sussex, UK), by the increase in optical density at 405 nm caused by the liberation of p-nitrophenol by enzymatic

Table 1 Summary of the enzyme assay, their substrates, concentrations, buffer and pH used

Enzymes	Substrate	Substrate concentration (mm)	Buffer	PH
β-D-fucosidase	4-nitrophenyl-β-D-fucopyranoside	2·0	25 mmol l^{-1} acetate	5·0
α-D-galactosidase	4-nitrophenyl-α-D-galactopyranoside	4·0	25 mmol l^{-1} acetate	5·0
β-D-glucosidase	4-nitrophenyl-β-D-glucopyranoside	2·0	25 mmol l^{-1} acetate	5·0
α-D-mannosidase	4-nitrophenyl-α-D-mannopyranoside	4·0	25 mmol l^{-1} acetate	5·0
β-D-xylosidase	4-nitrophenyl-β-D-xylopyranoside	2·0	25 mmol l^{-1} acetate	5·0
N-acetyl-α-D-glucosaminidase	p-nitrophenyl-N-acetyl-α-D-glucosaminide	2·0	25 mmol l^{-1} acetate	4·2
N-acetyl-β-D glucosaminidase	p-nitrophenyl-N-acetyl-β-D-glucosaminide	2·0	25 mmol l^{-1} acetate	4·2

hydrolysis of the substrate. Total enzyme activity was calculated from the calibration curve of absorbance at 405 nm against p-nitrophenol concentration and expressed as μmol p-nitrophenol released min^{-1}.

Specific enzyme activity determination. Protein concentration determination was carried out using the Bicinchoninic acid protein assay kit (Sigma). This kit consisted of bicinchoninic acid solution, copper (II) sulphate pentahydrate 4% solution and albumin standard (containing bovine serum albumin (BSA) at a concentration of 1·0 mg ml^{-1}). Protein reduces alkaline Cu (II) to Cu (I), which forms a purple complex with bicinchoninic acid (a highly specific chromogenic reagent). The resultant absorbance at 550 nm is directly proportional to the protein concentration.

The working reagent was obtained by the addition of 1 part copper (II) sulphate solution to 50 parts bicinchoninic acid solution. The reagent is stable for one day provided it is stored in a closed container at room temperature. Aliquots (10 μl) of each standard or enzyme extract were placed in the appropriate microtitre plate wells. Potassium phosphate extraction buffer 10 mmol l^{-1} pH 7·2 (10 μl) was pipetted into the blank wells. The working reagent (200 μl) was added to each well. After shaking, the plates were incubated at 37 °C for 30 min. The plates were allowed to cool to room temperature before measuring the absorbance at 550 nm using a Dynex MRX multiscan plate reader. The protein concentrations in the enzyme extracts were obtained from the calibration curve of absorbance at 550 nm against BSA concentration. These values were used to calculate the specific activity of the enzymes in nmol p-nitrophenol released min^{-1} μg^{-1} protein. Analysis of variance (ANOVA) was used to compare the total and specific enzyme activity of treatments for each enzyme and at each sampling time.

RESULTS

Volatile profiles

Figure 1 shows an example of the normalized divergence responses for the sensor array for four replicate samples of germinating spore lawn cultures of one *F. moniliforme* strain (48N), which were very reproducible with only a small standard error, in most cases < 10%.

Principal component analyses were used in an attempt to separate the non-producer isolates from the other three toxigenic strains after 48, 72 and 96 h of incubation. Figure 2 shows that for strains of *F. moniliforme*, it was possible to use 99% and 93% of the data for separation of the control blanks from the non-toxigenic strain (6N), and also to obtain some discrimination between the others. The separation between strains was better after 72 and 96 h incubation when visible mycelial growth had occurred on the agar plates. For *F. proliferatum*, separation between
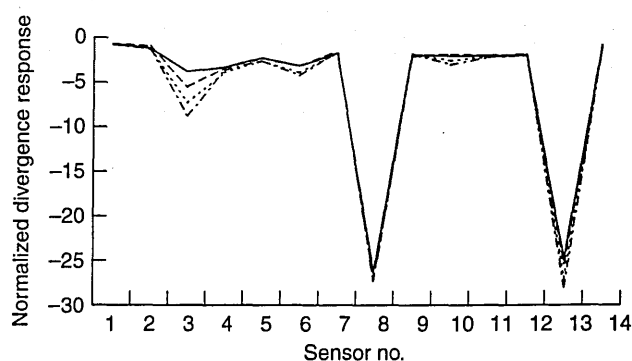
Fig. 1 Normalized divergence responses showing reproducibility of the sensor array to fungal volatiles produced by four replicates of germinating spore cultures of *Fusarium moniliforme* after 48 h of incubation at 25 °C on a wheat meal agar medium

strains was also achieved and could account for 83, 93 and 96% after 48, 72 and 96 h, respectively (Fig. 3). The non-toxigenic strain (20N) could be differentiated from the toxigenic strains, and from the uninoculated controls.

Figure 4 shows that for both *F. moniliforme* and *F. proliferatum*, it was possible to use cluster analyses to separate some of the strains. After 48 h, the dendrograms show that

for both species it was possible to distinguish the non-toxigenic strains from the others. For *F. moniliforme*, there was some overlap between strains 25N (fumonisin producer) and 150N (zearelenone and fumonisin producer). For *F. proliferatum*, there was some overlap between strains 55N (trichothecene and fumonisin producer) and 112N (fumonisin producer).
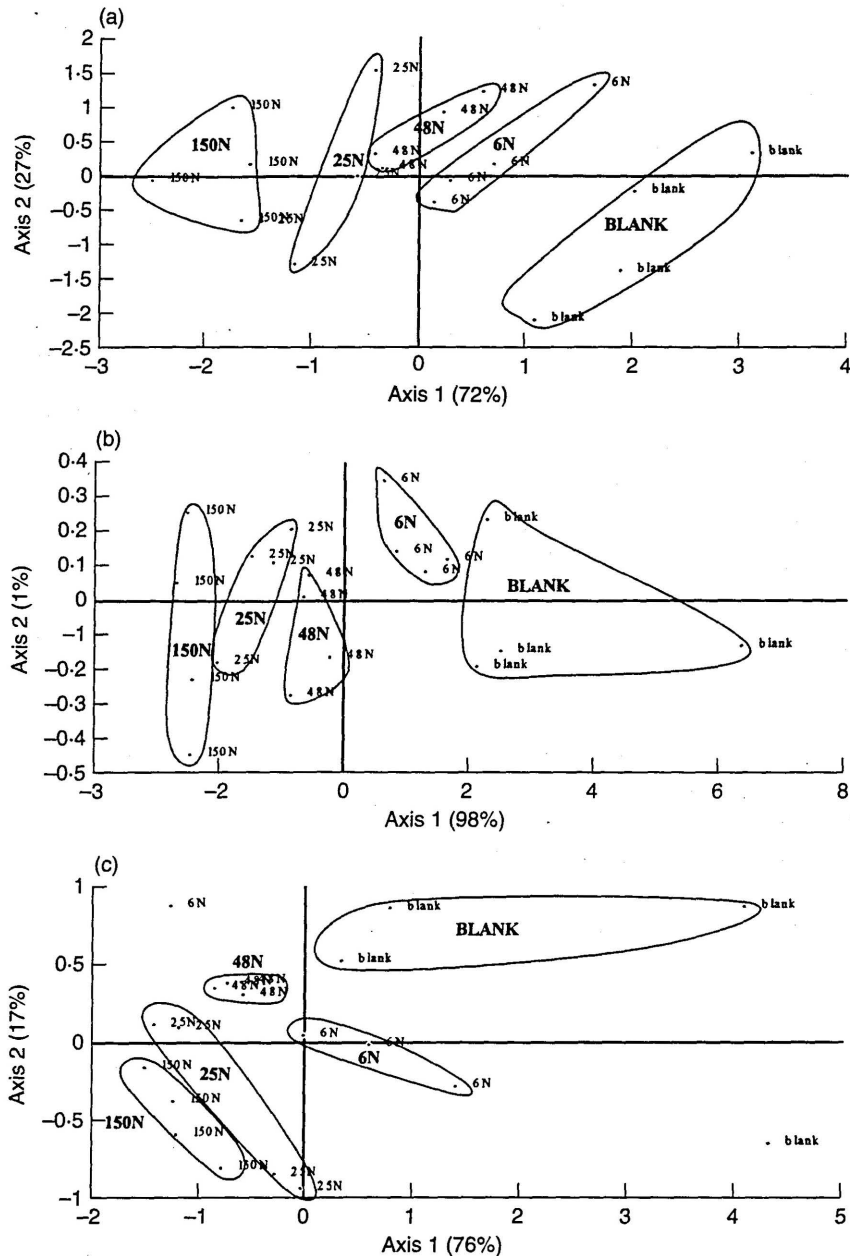
Fig. 2 Principal component analysis of volatiles produced by four isolates of *Fusarium moniliforme* and control (blank agar plates) after (a) 48 h (b) 72 h and (c) 96 h growth at 25 °C on a wheat meal agar medium
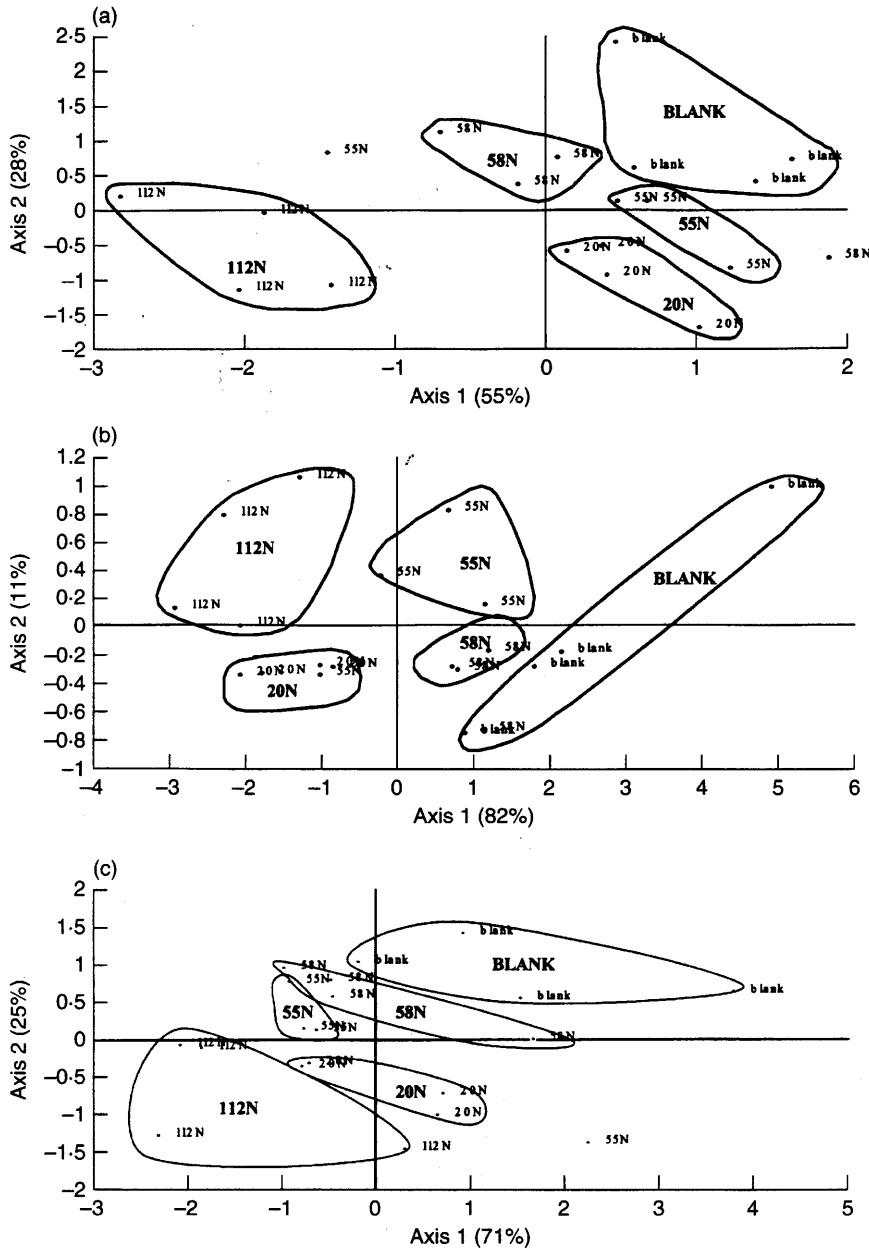
Fig. 3 Principal component analysis of volatiles produced by four isolates of *Fusarium proliferatum* and control agar blanks after (a) 48 h (b) 72 h and (c) 96 h growth at 25 °C on a wheat meal agar medium

Hydrolytic enzyme activity

The total hydrolytic enzymes produced by germlings and by growing mycelium were quantified after 48, 72 and 96 h; statistical analyses showed that the activities of β-D-glucosidase, α-D-galactosidase and N-acetyl-β-glucosaminidase significantly increased over time in all isolates, while that of

the other four enzymes were unaffected or changed little during the experiments. Table 2 statistically compares the temporal changes in total enzyme activity of the four strains of each species of *F. moniliforme* and *F. proliferatum*.

Figure 5 compares the temporal changes in total activity of three enzymes produced by each of the four isolates of *F. moniliforme*. It shows that significantly more β-D-gluco-
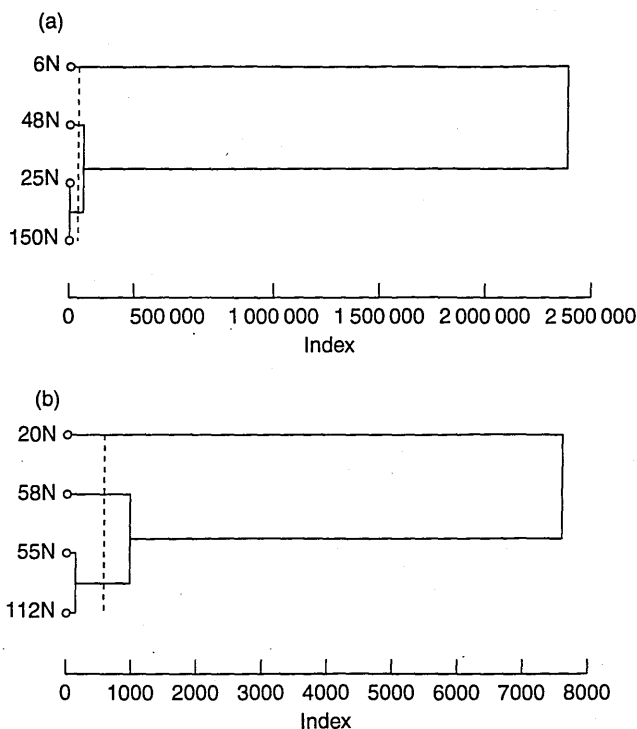
Fig. 4 Cluster analysis of sensor array responses to volatiles produced by (a) *Fusarium moniliforme* and (b) *F. proliferatum* after 48 h. The agar blanks have been excluded from the analysis

sidase and N-acetyl-β-glucosaminidase enzymes were produced by the non-toxigenic strain 6N than by the other three mycotoxigenic strains after 48, 72 and 96 h. For α-D-galactosidase, there was only a significant difference after 96 h of growth. This is further confirmed by quantification of the specific activity of the enzymes, where significantly greater activity of these three enzymes was obtained in strain 6N than in the others (Fig. 6).

For *F. proliferatum*, differences were less clear based on the total enzyme activity, although there was a significant increase in activity of α-D-galactosidase after 72 h and of N-acetyl-β-glucosaminidase after 96 h (Fig. 7). In terms of specific activity, the non-toxigenic strain 20N had a higher activity of N-acetyl-β-glucosaminidase than the other strains, but this was again only significant after 96 h (Fig. 8).

DISCUSSION

This is the first study to attempt to differentiate between non-toxic and mycotoxigenic strains of spoilage fungi using general volatile production patterns and electronic nose technology. The present study was designed to differentiate strains during very early phases of growth, i.e., up to 96 h, *in vitro* at a steady-state water activity (0·95). The cluster analysis of the data suggests that this approach does give

Table 2 Statistical comparisons of the total activity of seven different hydrolytic enzymes by strains of (a) *Fusarium proliferatum* and (b) *Fusarium moniliforme* after 48, 72 and 96 h. Key to significant differences between strains: $a = P < 0.01$; $b = P < 0.05$

|	df	48 h SS ($\times 10^{-7}$)	F	72 h SS ($\times 10^{-7}$)	F	96 h SS ($\times 10^{-7}$)	F
β-D-fucosidase	4	14·2	4·58[b]	23·8	7·97[a]	9·8	2·57
α-D-galactosidase	4	19·2	8·95[a]	737	65·91[a]	845	4·84[b]
β-D-glucosidase	4	284	9·89[a]	1686	15·82[a]	8458	64·66[a]
α-D-mannosidase	4	60	3·37[b]	22	8·38[a]	3·48	1·28
β-D-xylosidase	4	53·7	10·85[a]	22·9	4·52[b]	22·3	0·67
N-acetyl-α-D-glucosaminidase	4	47·6	9·59[a]	43·8	15·47[a]	20·1	2·86
N-acetyl-β-D-glucosaminidase	4	248	2·70	2268	116·63[a]	9667	72·16[a]

	df	48 h SS ($\times 10^{-7}$)	F	72 h SS ($\times 10^{-7}$)	F	96 h SS ($\times 10^{-7}$)	F
β-D-fucosidase	4	4·46	1·03	0·996	0·48	14·4	2·57
α-D-galactosidase	4	83·5	3·97[b]	10·7	3·79[b]	9510	2·26
β-D-glucosidase	4	477	35·87[a]	948	20·24[a]	8635	17·03[a]
α-D-mannosidase	4	97·8	2·25	1·22	0·57	3·49	1·06
β-D-xylosidase	4	4·2	2·51	0·364	0·35	15·3	2·58
N-acetyl-α-D-glucosaminidase	4	3·55	0·47	48	1·34	19·1	2·90
N-acetyl-β-D-glucosaminidase	4	1079	19·00[a]	632	9·37[a]	3077	10·59[a]

Fig. 5 Total enzyme activities (μmol 4-nitrophenyl min^{-1}) of three enzymes produced by *Fusarium moniliforme*. The bars indicate the L.S.D. ($P = 0.05$) between the four isolates examined at each sampling time. (a) β-D-glucosidase; (b) α-D-galactosidase; (c) N-acetyl-β-glucosaminidase. (●), 25N; (□), 6N; (△), agar; (○), 150N; (▲), 48N

Fig. 6 Specific enzyme activities (nmol 4-nitrophenyl min^{-1} μg^{-1} protein) of three enzymes produced by *Fusariun moniliforme*. The bars indicate the L.S.D. ($P = 0.05$) between isolates at each sampling time. (a) β-D-glucosidase; (b) α-D-galactosidase; (c) N-acetyl-β-glucosaminidase. (●), 25N; (□), 6N; (△), agar 2; (○), 150N; (▲), 48N

some clear separation of the one non-mycotoxigenic strain, and sometimes of the three toxigenic strains of each species. Interestingly, it was possible to obtain very much earlier differentiation with the volatile patterns (48 h) using the electronic nose system than using hydrolytic enzyme roduction.

Previous quantitative studies of microbial volatile organic ompounds with a trichothecene-producing strain of *usarium sporotrichoides*, and ochratoxigenic and non-ochra-oxigenic strains of *Penicillium verrucosum*, found that the

production of volatile terpenes appeared to be linked to the formation of trichothecenes by the *Fusarium* spp. and accel-erated production of volatile ketones by the ochratoxigenic strain of *P. verrucosum* (Pasanen *et al.* 1996). Although the water availability of the predominantly grain substrates was not controlled accurately, the results did suggest that speci-fic groups of organic volatiles could be good markers for differentiation between toxic and non-toxic species. Jelen *et al.* (1997b) found that trichodiene, an intermediate of tri-chothecene mycotoxin production, was present in increased
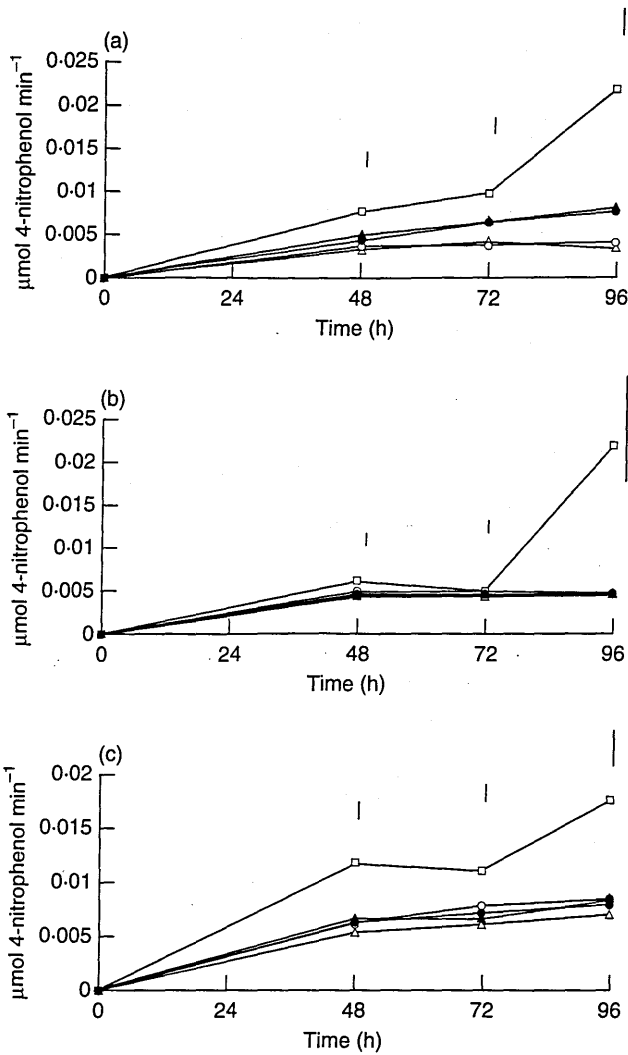
Fig. 7 Total enzyme activities (μmol 4-nitrophenyl min^{-1}) of three enzymes produced by *Fusarium proliferatum*. The bars indicate the L.S.D. ($P = 0.05$) between the four isolates examined at each sampling time. (a) β-D-glucosidase; (b) α-D-galactosidase; (c) N-acetyl-β-glucosaminidase. (●), 20N; (□), 58N; (△), agar 1; (○), 55N; (▲), 112N

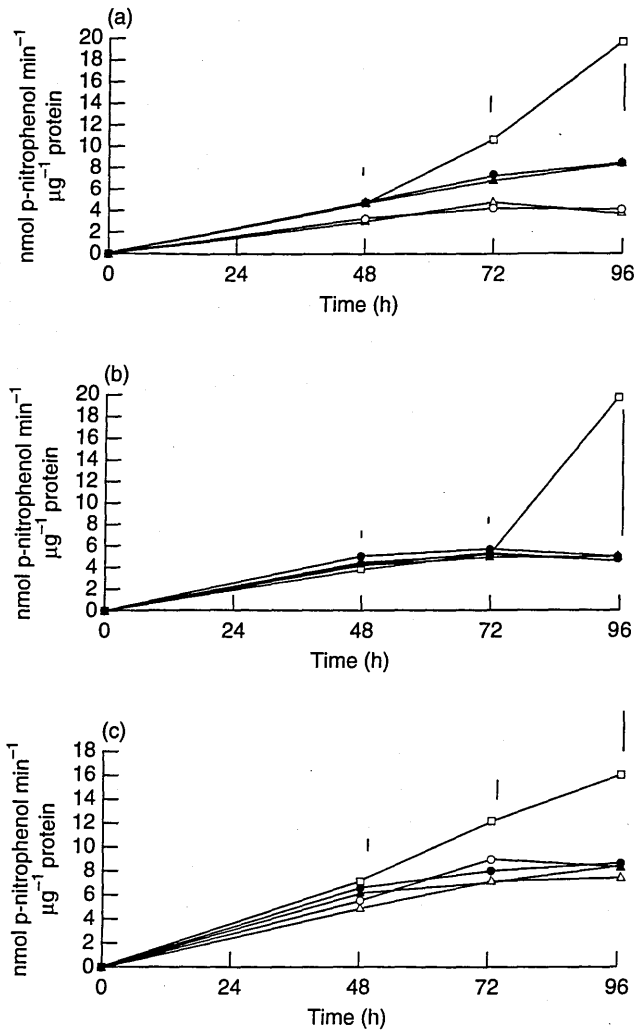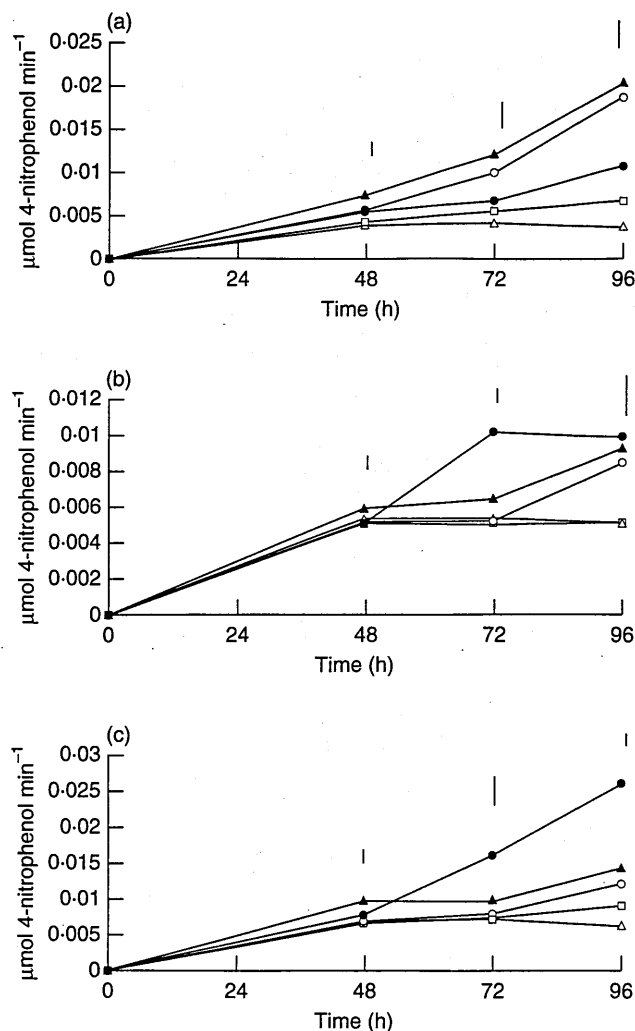**Fig. 8** Specific enzyme activities (nmol 4-nitrophenyl min^{-1} μg^{-1} protein) of three enzymes produced by *Fusarium proliferatum*. The bars indicate the L.S.D. ($P = 0.05$) between isolates at each sampling time. (a) β-D-glucosidase; (b) α-D-galactosidase; (c) N-acetyl-β-glucosaminidase. (●), 20N; (□), 58N; (△), agar 1; (○), 55N; (▲), 112N

amounts in harvested grain spikes colonized by *Fusarium* species when harvested a week before harvest and incubated. They suggested that it could be an early indicator for production of these mycotoxins in the grain, although no attempt was made to differentiate between colonization by toxigenic and non-toxigenic strains. For other *Fusarium* species such as *F. sambucinum*, distinctive and characteristic volatiles were produced by non-toxic, and toxic strains (Jelen *et al.* 1995). However, all these studies required sophisticated analytical equipment to analyse specific

groups of organic volatile compounds. The present study would suggest that recognition of general patterns of volatile production using a sensor array may be a simple and rapid method for detecting and differentiating between strains and species. Previous studies with the electronic

nose system suggested that the general volatile patterns produced by an ochratoxigenic strain of *A. ochraceus* and *A. niger* were very different and distinctive (Keshri and Magan, unpublished data).

The studies of both total and specific enzyme activity by germlings of these strains and species showed that a number of hydrolytic enzymes are important in the initial colonization of a substrate. However, the relative importance may differ between species. For example, distinctive increases in both total and specific activities of three enzymes were observed for the non-toxigenic strain (6N) of *F. moniliforme*. However, there were fewer differences between strains of *F. proliferatum*. Both total and specific activities of enzymes provide complimentary information on rates of substrate colonization (Marin *et al.* 1998), and reflects the relative capacity for exp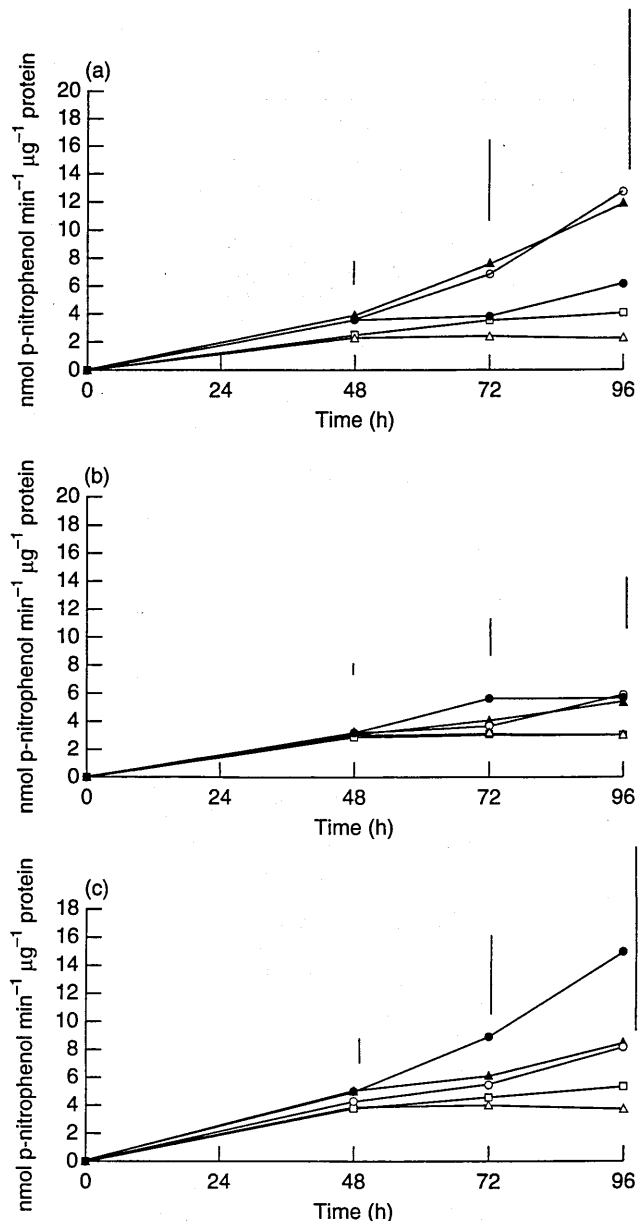loitation of agricultural substrates and occupation of ecological niches (Magan 1997). However, they were not such good early indicators of fungal activity as the general volatile production patterns.

This study concentrated on very short incubation times. This was done specifically because of the interest in evaluation of the electronic nose system for detection of activity prior to visible growth. Much better volatile patterns and discrimination may occur later when visible growth has occurred. This may be of interest from a purely taxonomic point of view (see Magan and Evans 2000), but not for the development of early detection systems.

ACKNOWLEDGEMENTS

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ABSTRACTS
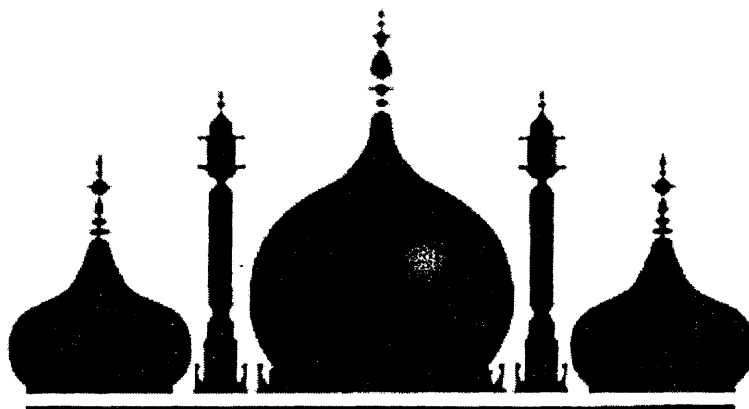
Detection of mould spoilage in bread analogues using Electronic Nose technology

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Abstract. This study examines the potential of using patterns of fungal volatile production as an early indicator of spoilage using electronic nose technology, when compared to traditional methods used in the food industry. Slices of bread analogues were inoculated with spoilage fungi and incubated at 25°C for up to 72 hours. Replicates of the colonised substrate were sampled destructively using the electronic nose system. Subsamples were used for enzyme assays and cfus. The study showed earlier discrimination of mouldy and non-mouldy substrates based on the volatile patterns compared to enzyme activity and fungal populations.

1. Introduction

A number of methods are available for detection of spoilage moulds in food substrates [1], however many of these have drawbacks. Recently with the development of electronic nose technology, interest has been focused on the potential for using characteristic volatile patterns produced by spoilage moulds as an early indicator of spoilage. The main objectives of this study were firstly, to determine the potential for using an electronic nose system for early detection of spoilage moulds in model intermediate moisture food products, and secondly, to compare with other measures of fungal activity such as enzyme assays and populations (c.f.u.s).

2. Experimental

Bread analogue slices modified to 0.95 water activity, were inoculated with conidia (10^6 spores ml^{-1}) of three individual fungi (*Eurotium amstelodami*, *E. chevalieri* and a *Penicillium* spp.) and incubated at 25°C for up to 72 hours. Uninoculated bread analogues were used as controls. After 26, 40, 48 and 72 hours volatile patterns were measured using a Bloodhound electronic nose [2]. Subsamples were used for enzyme assays [3] and c.f.u.s.

3.   Results

Figure 1. PCA of the response of the sensor array to three spoilage fungi based on their volatile profiles after 26 hours growth: b, control bread analogue; a, *Eurotium amstelodami*; c, *E. chevalieri*; p, a *Penicillium* spp.. Axes 1 and 2 represent 99% of data.
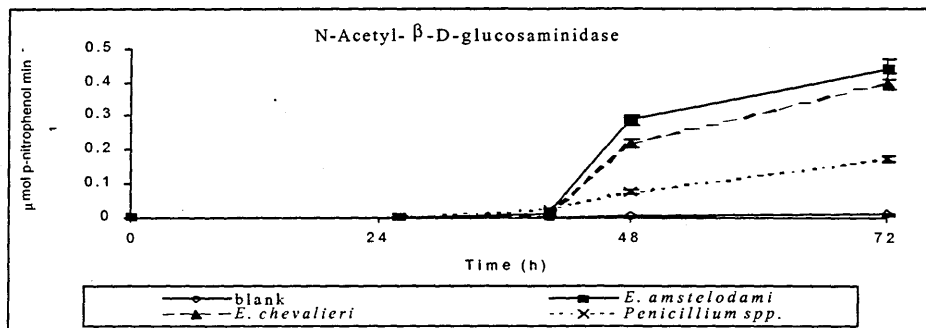
Figure 2. Total enzyme activity of N-Acetyl-β-D-glucosaminidase for *Eurotium amstelodami, E. chevalieri* and a *Penicillium* spp. inoculated on bread analogue (0.95$_{aw}$).

4.   Discussion

The sensor array was found to show good reproducibility to the fungal volatile patterns produced on the bread analogue treatments. Principal component analysis of the sensor responses showed discrimination of non-mouldy and mouldy bread analogues. Classification of the fungal species was possible as early as 26 hours, prior to visible growth. Increase in the fungal populations (c.f.u.s) and total enzyme activity (β-D-glucosidase; α-D-galactosidase and N-acetyl-β-D-glucosaminidase) was observed to be significant after 40 and 48 hours, respectively. This study suggests that fungal volatile patterns measured using an electronic nose may be useful for earlier detection of fungal spoilage when compared to enzyme activities and cfus measurements.

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Detection of mould spoilage in bread analogues using electronic nose technology
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Introduction

- A number of methods are available for detection of spoilage moulds in food substrates (Magan, 1993) however many of these have drawbacks.

- Recently with the development of electronic nose technology, interest has been focused on the potential for using characteristic volatile pattern produced by spoilage moulds as an early indicator of spoilage.

- The main objectives of this study were firstly to determine the potential for using an electronic nose system for early detection of spoilage moulds in model intermediate moisture food products and secondly to compare with other measures of fungal activity such as enzyme assays and populations (c.f.u.s).

Materials and Methods

- Fungal species: *Eurotium amstelodami, E. chevalieri* and a *Penicillium* spp.
- Slices of bread analogues (modified to 0.95$_{aw}$ using glycerol) inoculated with 100µl of spore suspension (10^6 spores ml^{-1}).
- Uninoculated bread analogues used as control blank.
- Slices (24 replicates per treatment) incubated at 25°C in a humidity chamber at 0.95$_{aw}$ for up to 72h.

Six replicates per fungal species sampled after 26, 40, 48 and 72h using electronic nose. Subsamples used for enzyme assays and C.F.U. measurements.

C.F.U.s
C.F.U.s measurements carried out at times 0, 26, 40, 48 and 72h on malt extract agar media (0.95$_{aw}$) at 25°C.

Extraction of enzyme
Enzyme extraction carried out in potassium phosphate buffer (10mM, pH 7.2) (Marin et al., 1998).

Sampling procedure
- Single plates placed in sampling bags (500ml), inflated with filtered air and equilibrated for 2h at 25°C.
- Headspace sampled with Bloodhound Electronic nose (Keshri et al., 1998).

Enzyme activity determination
- Total activity: Seven hydrolytic enzymes assayed using p-nitrophenyl substrates (Marin et al., 1998). Incubated at 37°C for 1h and absorbance read at 405nm.
- Specific activity: Bicinchoninic acid (BCA) kit used (Sigma Chemical Co.). Incubated at 37°C for 30 minutes and absorbance read at 550nm.

Data treatment
The following data analyses performed using the program xlSTAT:
- Principal component analysis (PCA).
- Discriminant Analysis (DA).
- Cluster Analysis (CA).

Results

Figure 1. Reproducibility of the sensor array to volatiles produced by six replicates of *E. chevalieri* after 26h growth on bread analogue at 0.95$_{aw}$.
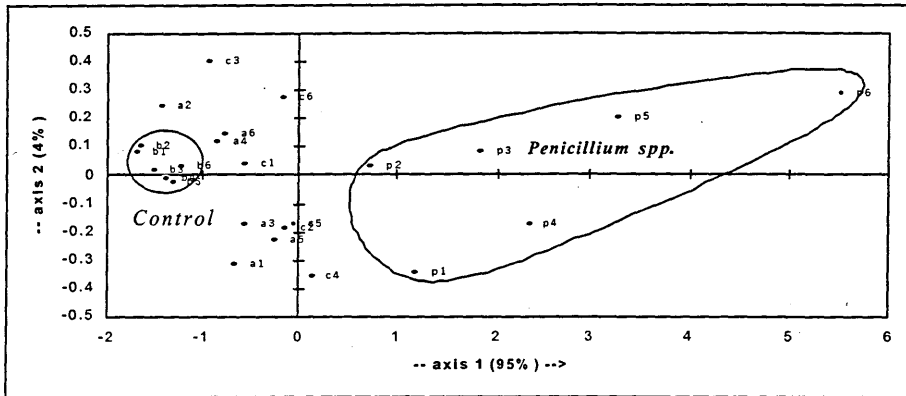

Figure 2. Principal component analysis of the response of the sensor array to three spoilage fungi based on their volatile profiles after 26h growth: b, control bread analogue; a, *Eurotium amstelodami*; c, *E. chevalieri*; p, a *Penicillium* spp.. Axes 1 and 2 represent 99% of data.
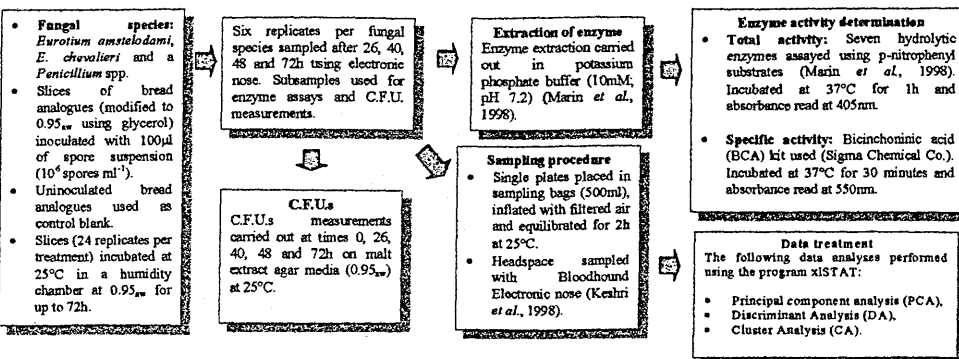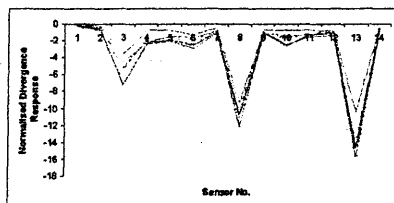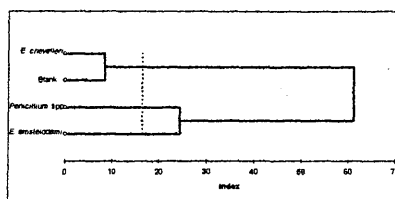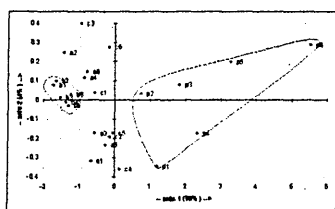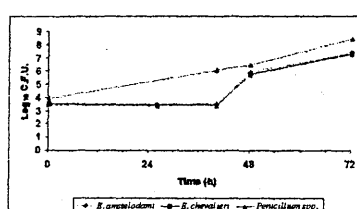

Figure 3. A dendrogram showing classification of three spoilage fungi and control bread analogue based on the volatile profiles after 26h growth.

Figure 4. Growth of fungal population inoculated on bread analogue at 0.95$_{aw}$.
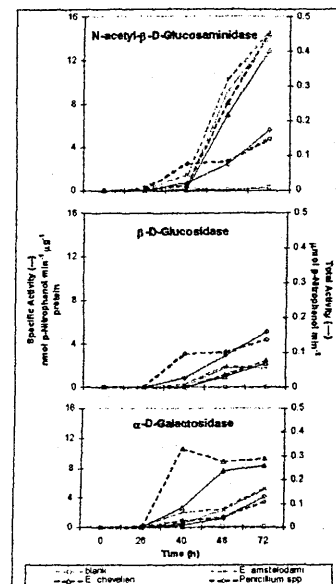
Figure 5. Total and specific enzyme activities of three fungal enzymes for *Eurotium amstelodami, E. chevalieri* and a *Penicillium* spp. inoculated on bread analogue (0.95$_{aw}$).

Conclusions

- Good reproducibility of volatile profiles of fungi grown on bread analogue achieved using the sensor array.
- The PCA shows that discrimination was possible between non-mouldy bread analogues and those colonized by fungi after 26h growth.
- Classification was possible for all three fungi especially *Penicillium* spp. after 26h, prior to visible growth.
- For all three fungal species an increase in C.F.U.s was observed after 40h growth.
- Total and specific activities of three enzymes: β-D-glucosidase, α-D-galactosidase and N-acetyl-β-D-glucosaminidase were found to be significant after 48h growth.
- It was possible to achieve early discrimination of fungi based on their volatile profiles using the electronic nose compared to methods such as enzyme assays and C.F.U. measurements.

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Marin, S., Sanchis, V., Ramos, A. & Magan, N. (1998). Effect of water activity on hydrolytic enzyme production by *Fusarium moniliforme* and *Fusarium proliferatum* during colonisation of maize. *International Journal of Food Microbiology* 42, 185-194.

Early detection of spoilage moulds in bread using volatile production patterns and quantitative enzyme assays

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G. KESHRI, P. VOYSEY AND N. MAGAN. 2001.

Aims: Early detection of spoilage fungi (two *Eurotium* spp., a *Penicillium chrysogenum* species) in bread analogues over periods of 72 h at 25°C and 0·95 water activity was evaluated using volatile production patterns, hydrolytic enzyme production, and changes in fungal populations.

Methods and Results: Using an electronic nose system it was possible to differentiate between uninoculated controls and samples contaminated with *P. chrysogenum* within 28 h. After 40–48 h it was possible to differentiate between the *Eurotium* spp., *P. chrysogenum* and the control using Principal Component Analysis (PCA). Cluster analyses could differentiate between the control, *P. chrysogenum* and the *Eurotium* spp. after 40 h. Of seven hydrolytic enzymes examined after 48 h, the specific activities of three were significantly different from uninoculated control bread. There were also differences between the mould species in production of three enzymes. *Penicillium chrysogenum* populations increased fastest, from about 10^3 cfu g^{-1} to 10^6–10^7 cfu g^{-1} after 72 h. For the *Eurotium* spp. this increase was slower.

Conclusion: Overall, this study suggests, for the first time, that an electronic nose system using a surface polymer sensor array is able to detect qualitative changes in volatile production patterns for the early detection of the activity of spoilage moulds in bakery products.

Significance and Impact of the Study: Potential exists for application of such systems for microbial quality assurance in intermediate moisture food products.

INTRODUCTION

Early detection of spoilage moulds in foods is important because of legislative and consumer pressure to reduce the use of preservatives, particularly those based on organic acids, in intermediate moisture bakery products. However, as such preservatives are fungistats and not fungicides, reduced concentrations can result in a stimulation of mould growth (Magan and Lacey 1986; Lacey and Magan 1991; Marin *et al.* 1999).

In the last decade, the rapid advances made in the development of electronic nose technology has attracted much interest in applications for the detection of spoilage micro-organisms (Stetter *et al.* 1993; Dickinson *et al.* 1998; Magan and Evans 2000). While electronic nose systems are qualitative, not quantitative, volatile production patterns can

be specific for different species. Bacterial species can be differentiated based on their volatile production patterns (Gibson *et al.* 1997), although much less information is available on food spoilage moulds (Keshri *et al.* 1998; Magan *et al.* 2000). Some elegant studies have been carried out on the detection of spoilage fungi in grain (Borjesson *et al.* 1990, 1992, 1993, 1996) and recently, both Schnurer *et al.* (1999) and Magan and Evans (2000) reviewed this work. Correlations have been found for fungal contamination of grain with the level of ergosterol and with colony-forming units (cfu), and Magan (1993) hypothesized that the specific activity of hydrolytic enzymes was a good early indicator of the activity of spoilage moulds. Keshri *et al.* (1998) in *in vitro* studies could detect the growth of germlings of *Wallemia sebi*, *Eurotium* spp. and a *Penicillium* species after 48 h, prior to any visible growth. They could also distinguish between control blanks, the xerophilic *Wallemia sebi*, *Eurotium* spp. and a *Penicillium* sp. However, no studies have attempted to examine the early detection of spoilage moulds in bakery product substrates. Harris *et al.* (1986),

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using GC-MS, found that musty aroma compounds were produced when some actinomycetes, *Penicillium* and *Aspergillus* spp. were grown on whole wheat bread. They suggested that seven musty aroma-type categories could be used to define musty taints produced by micro-organisms in food and feedstuffs. However, the potential for early detection of spoilage moulds was not considered.

The objectives of this study were: (i) to evaluate the use of an electronic nose system for the early detection of volatile production patterns by two *Eurotium* spp. and *P. chrysogenum* over 72 h in bread analogues; (ii) to quantify the hydrolytic enzyme production patterns; and (iii) to quantify the fungal populations. The results were compared to determine whether volatile production patterns could be used as an early detection method for spoilage moulds in bakery products, prior to visible growth.

MATERIALS AND METHODS

Fungal isolates used in this study

The spoilage moulds used in this study were *Eurotium amstelodami*, *Eurotium chevalieri* and *Penicillium chrysogenum* isolated from mouldy brown bread. The former are held in the culture collection of the Food Technology Department, University of Lleida, Spain, and the latter by the Technical University of Denmark.

Bread analogue preparation and inoculation

The bread dough was prepared by mixing flour (200 g), margarine (10 g), salt (1 g), yeast (1 g) and sucrose (1 g) with water (126 ml)/glycerol (14 g) mixtures to obtain 0·95 water activity (Patterson and Damoglou 1986). The dough was placed in a baking tin lined with greaseproof paper, sealed with tape and autoclaved for 15 min. When cooled, the bread analogue (pH 5·7) was sliced into 3 mm slices (2 × 2 cm, 5 g) and placed in sterile Petri plates. The water activity (a_w) of the bread analogue was measured with a Humidat ICII (NovaSina, Switzerland) and found to be 0·95.

Slices of bread analogue were inoculated with the spore suspensions of individual spoilage moulds (10^6 spores ml^{-1}). Aliquots (100 µl) of the spore suspension were pipetted randomly onto slices of the bread analogue (Fustier *et al.* 1998). Uninoculated slices were used as controls. Petri plates containing the slices of bread analogues were placed in humidity chambers with a water/glycerol solution (400 ml) to maintain the equilibrium relative humidity at the same level (0·95) as the bread, and incubated at 25°C. A total of 20 replicates were set up per treatment, with four replicates destructively sampled after 0, 26, 40, 48 and 72 h (data not shown). These were used for the detection of the

volatile production profiles, and subsamples were immediately taken for subsequent enzyme assays (2 g) and fungal populations (cfu, 1 g). The experiment was carried out twice.

Volatile detection using the electronic nose

Bread analogue slices were placed in sampling bags (500 ml capacity), filled with 300 ml filter-sterilized air and sealed (Keshri *et al.* 1998). The bags were allowed to equilibrate at 25°C for 2 h. The headspace of each bag was subsequently sampled in a 25°C constant temperature room using the electronic nose system (BH114 electronic nose, Bloodhound Sensors Ltd, Leeds, UK). The system employs 14 conducting surface polymer sensors. The interaction of the volatile compounds and the conducting polymer surface produces a change in resistance which can be amplified and analysed through the electronic nose software package system. Qualitative differences between samples can be compared. The flow rate over the sensors was fixed at 4 ml min^{-1} to generate the sensor baselines. Four sensor parameters can be studied with this system: adsorption (maximum rate of change of resistance), desorption (maximum negative rate of change of resistance), divergence (maximum step response) and area (area under the actual sensor curve). The divergence response was primarily used in this study, and the sampling profile was set at 15 s of adsorption and 22 s of desorption.

Normalized electronic nose data were analysed using the programme xlSTAT (Microsoft Excel add-in programme). Principal component analysis (PCA), discriminant function analysis (DA) and cluster analysis (CA) techniques were applied to differentiate and classify the fungal species. To carry out analyses, the response data for each of the 14 sensors and each of the replicates were used. The groupings obtained using the PCA are based on unsupervised evaluation of these parameters for each replicate. Where responses are similar, the PCA will identify overlap between groups. Cluster analysis is based on the Malahanobi's squared distance between groups obtained in the discriminant analysis at the 95% confidence limit. The treatments are grouped according to the relative distance between them based on the cluster analysis index. Analysis of variance (ANOVA) was used to compare the specific enzyme activity of treatments for each enzyme and at each sampling time.

Quantitative enzyme assay

For enzyme extraction, 2 g subsamples of bread analogue were placed in 4 ml potassium phosphate extraction buffer (10 mmol l^{-1}; pH 7·2). The bottles were shaken on a wrist action shaker for 1 h at 4°C. The washings were decanted

into 1·5 ml plastic Eppendorf tubes and centrifuged in a bench microfuge for 15 min. The supernatant fluid was decanted and stored in aliquots at −20°C for total and specific enzyme activity determinations (Marin *et al.* 1998). The total activity of seven hydrolytic enzyme activities was assayed using p-nitrophenyl substrates (Sigma). Table 1 shows the substrate concentration and buffer used for each enzyme assay. Enzyme extract (40 μl), substrate solution (40 μl) and the appropriate buffer (20 μl) were pipetted into the wells of the microtitre plate and incubated at 37°C for 1 h along with the appropriate controls. The reaction was stopped by the addition of 5 μl sodium carbonate (1 mol l⁻¹) solution and left for 3 min. The enzyme activity was measured, using a MRX multiscan plate reader (Dynex Technologies Ltd, Billinghurst, UK), by the increase in optical density at 405 nm caused by the liberation of p-nitrophenol by enzymatic hydrolysis of the substrate. Total enzyme activity was calculated from a calibration

curve of absorbance at 405 nm against p-nitrophenol concentration and expressed as micromoles of p-nitrophenol released per minute.

For specific activity determinations, the protein concentration was obtained using a bicinchoninic acid protein assay kit (Sigma Chemical Co., UK). This kit consists of bicinchoninic acid solution, copper (II) sulphate pentahydrate 4% solution and albumin standard (containing bovine serum albumin (BSA) at a concentration of 1·0 mg ml⁻¹). Protein reduces alkaline Cu (II) to Cu (I), which forms a purple complex with bicinchoninic acid (a highly specific chromogenic reagent). The resultant absorbance at 550 nm is directly proportional to the protein concentration.

The working reagent was obtained by the addition of 1 part copper (II) sulphate solution to 50 parts bicinchoninic acid solution. The reagent is stable for 1 day provided it is stored in a closed container at room temperature. Aliquots

Table 1 Summary of the enzyme assay, their substrates, concentrations, buffer and pH used

| Enzymes	Substrate	Substrate concn (mmol l⁻¹)	Buffer (mmol l⁻¹ acetate)	pH
β-D-fucosidase	4-nitrophenyl-β-D-fucopyranoside	2·0	25	5·0
α-D-galactosidase	4-nitrophenyl-α-D-galactopyranoside	4·0	25	5·0
β-D-glucosidase	4-nitrophenyl-β-D-glucopyranoside	2·0	25	5·0
α-D-mannosidase	4-nitrophenyl-α-D-mannopyranoside	4·0	25	5·0
β-D-xylosidase	4-nitrophenyl-β-D-xylopyranoside	2·0	25	5·0
N-acetyl-α-D-glucosaminidase	p-nitrophenyl-N-acetyl-α-D-glucosaminide	2·0	25	4·2
N-acetyl-β-D glucosaminidase	p-nitrophenyl-N-acetyl-β-D-glucosaminide	2·0	25	4·2
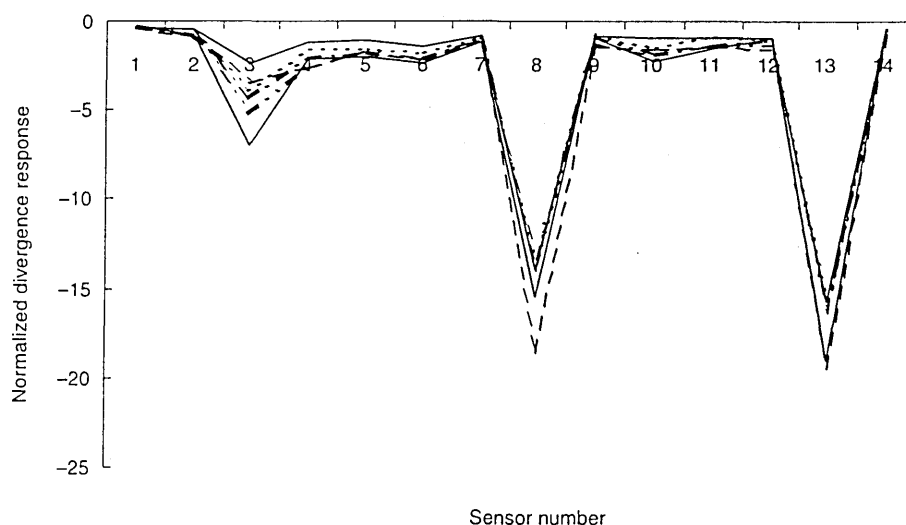
Fig. 1 Reproducibility of a 14 sensor array for six replicate samples of *Eurotium amstelodami* after 26 h incubation
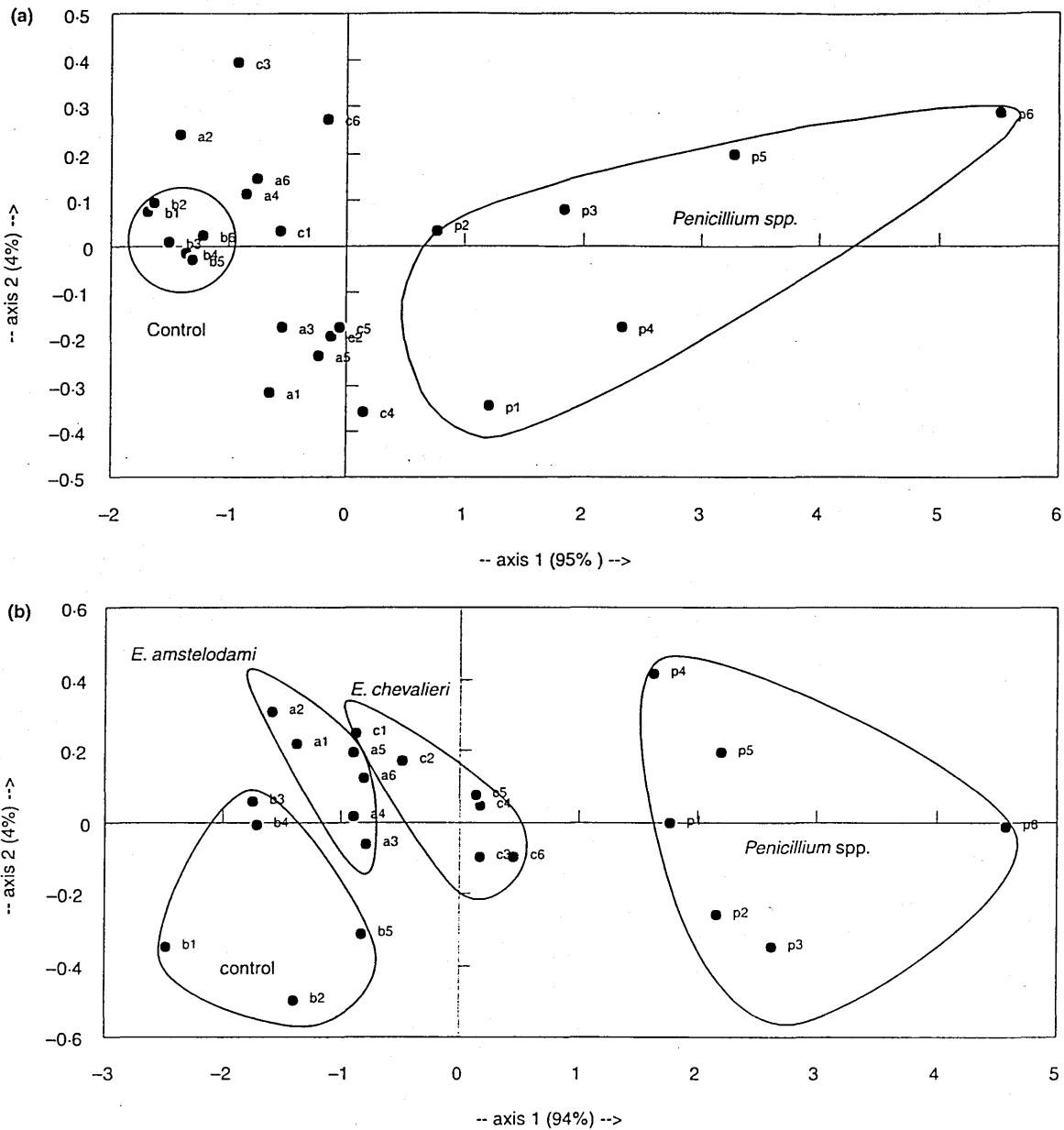
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Fig. 2 (a) Principal component analysis (PCA) of the data after 26 h incubation, showing the grouping for the control blanks (b1–b6) and *Penicillium chrysogenum* species (p1–p6). For the *Eurotium* spp. (a1–a6; c1–c6), there was a wider variation. (b) PCA after 40 h showing the discrimination between different treatments. Key to treatments: b1–b6, control bread samples; *E. amstelodami*, a1–a6; *E. chevalieri*, c1–c6; *P. chrysogenum*, p1–p6

(10 μl) of each standard or enzyme extracts were placed in the appropriate microtitre plate wells. Potassium phosphate extraction buffer 10 mmol l⁻¹ pH 7·2 (10 μl) was pipetted into the blank wells. The working reagent (200 μl) was added to each well. After shaking, the plates were incubated at 37°C for 30 min. The plates were allowed to cool to room temperature before measuring the absorb-

ance at 550 nm using a MRX multiscan plate reader. The protein concentrations in the enzyme extracts were obtained from the calibration curve of absorbance at 550 nm against BSA concentration. These values were used to calculate the specific activity of the enzymes in nanomoles of p-nitrophenol released per minute per microgram of protein.
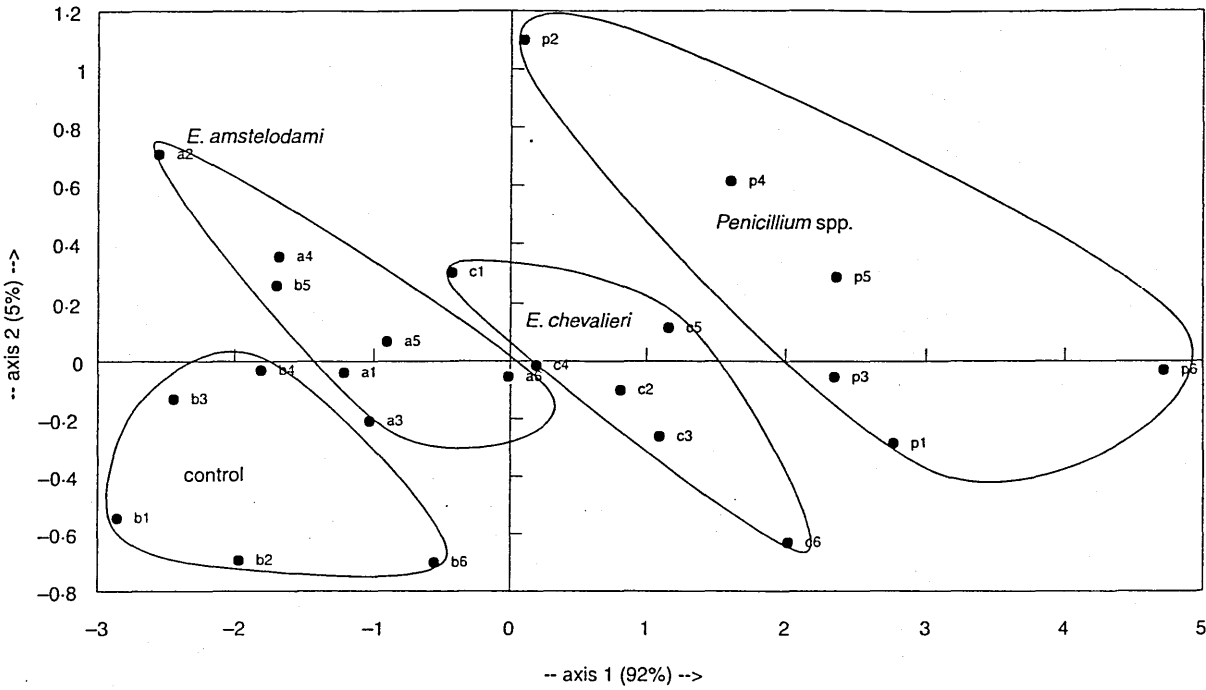
Fig. 3 Principal components analysis after 48 h showing the discrimination between different treatments when inoculated on slices of bread and incubated at 25°C. Key to treatments: b1–b6, control bread samples; *Eurotium amstelodami*, a1–a6; *E. chevalieri*, c1–c6; *Penicillium chrysogenum*, p1–p6
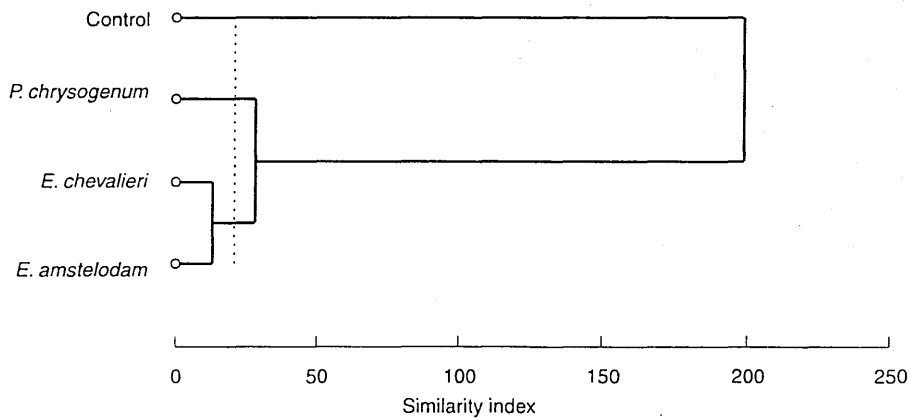
Fig. 4 Cluster analysis of the data 40 h after inoculation showing discrimination between the untreated control bread samples and *Penicillium chrysogenum*, and the similarity between the two *Eurotium* spp. The Index indicates the relative distance between treatments

Fungal populations

Bread samples (1 g) were placed in 10 ml of a glycerol/water diluent solution + 0·01% Tween 80 (0·95 a_w), macerated and serially diluted, with 200 μl of each dilution spread on malt extract agar (2%) modified to 0·95 a_w with glycerol. Two replicates were set up for each treatment at each dilution, and the plates incubated at 25°C for up to 7 days. The number of colonies per plate was counted and expressed as cfu g^{-1} bread substrate.

RESULTS.

Discrimination based on volatile production patterns

Figure 1 shows an example of the divergence of four replicates of the same treatment given by the 14 sensors of the electronic nose system used in this study. There was only a small variation in these patterns between replicates of the same treatment. The PCA analysis of the data after 26 h growth shows that there was a close cluster for the control untreated bread, and a clear difference for the *P. chrysogenum* species. However, for the *Eurotium* spp., there was less discrimination. After 40 h, discrimination between the growth of the *Eurotium* spp., the *P. chrysogenum* on the bread substrate and the control was obtained (Fig. 2). After 48 h, this was even more distinct (Fig. 3).

From a cluster analysis (95% confidence level) of the data, which groups treatments according to the relative distances between each treatment, it was found that after 40 h it was possible to differentiate between the control untreated bread, the *P. chrysogenum*, but not the *Eurotium* spp., which grouped together (Fig. 4).

Discrimination based on specific enzyme activity

Of the seven enzymes assayed, only three changed significantly due to fungal growth. These were N-acetyl-β-D-glucosaminidase, α-D-galactosidase and β-D-glucosidase. Figure 5 shows that for all three enzymes, the background levels in the control were very low. An increase in enzyme activity was first detected after 48 h. Generally, the activity of the first enzyme was significantly higher for the *Eurotium* spp., while for the latter two enzymes the activity was significantly higher in bread colonized by *E. chevalieri* and *P. chrysogenum*, respectively.

Fungal population changes

The changes in fungal populations over a period of 72 h is shown in Fig. 6. Generally, the increase in populations of the *Eurotium* spp. was slower than that of the *P. chrysogenum*. Some microscopically observable growth was present after 48 h in the *P. chrysogenum* isolate treatments, but not in the *Eurotium* inoculated bread. However, over the incubation period, by 72 h at 25°C the inoculated bread treatments all carried populations $> 10^6$ cfu g^{-1} fresh weight bread.

DISCUSSION

This study has shown for the first time that early qualitative changes in volatile production patterns by germinating

spoilage moulds in bread can be detected using an electronic nose or odour mapping system. This can be achieved during the very early phases of establishment, prior to visible growth on a bread substrate. It was also demonstrated that differentiation between mould contaminated and uncontaminated samples can be made within 40 h of incubation

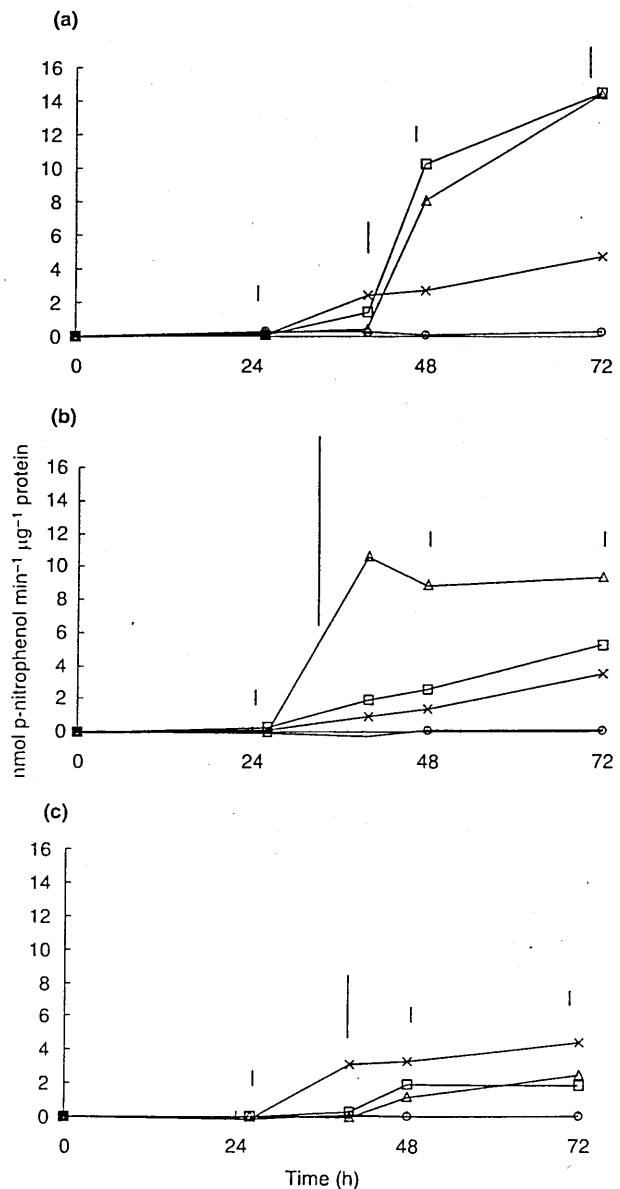**Fig. 5** Comparison of the temporal changes in specific activity of three enzymes in inoculated bread slices with each of the treatments. The bars at each time interval indicates the least significant difference ranges. (a) N-acetyl-β-D-glucosaminidase; (b) α-D-galactosidase; (c) β-D-glucosidase. (O), blank; (×), *Penicillium chrysogenum*; ($\triangle$), *Eurotium chevalieri*; ($\square$), *E. amstelodami*
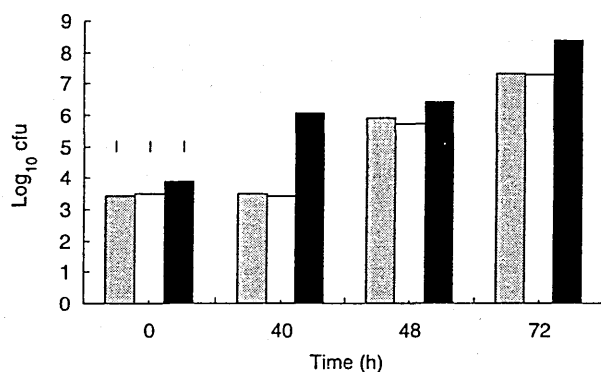
Fig. 6 Temporal changes in $\log_{10}$ colony forming units (cfu) over the experimental period. Bars indicate least significant differences for each treatment over time. (▨), *Eurotium amstelodami*; (□), *E. chevalieri*; (■), *Penicillium chrysogenum*

with initial levels of mould spores at about 10^3 cfu g^{-1} food substrate. Furthermore, cluster analysis clearly showed that differentiation between spoilage by *P. chrysogenum* isolated from bread and xerophilic *Eurotium* spp. could be made from non-mouldy controls. The recognition of general qualitative patterns may have some advantages in that information on the status of a sample relative to an unspoiled food product can be obtained rapidly.

Few similar studies have been carried out on mould spoilage in food products. Keshri *et al.* (1998) demonstrated differentiation between the xerophilic spoilage fungi *Wallemia sebi* and *Eurotium* spp. *in vitro*, and Magan *et al.* (2000) demonstrated the potential differentiation between bacterial and yeast contamination of a milk medium using electronic nose technology. Other studies, predominantly in relation to mould contamination of raw food materials, particularly wheat/barley grain, have recently been reviewed by Schnurer *et al.* (1999) and Magan and Evans (2000). Some studies have recently examined the activity and relative colonization patterns of cakes by spoilage moulds (Abellana *et al.* 1999) in relation to environmental regimes, but not for early detection. Recently, it was also shown that shifts in volatile production patterns for *Fusarium* spp. can occur between mycotoxigenic and non-mycotoxigenic strains of the same species, and used as a tools for their differentiation (Keshri and Magan 2000). Previous studies specifically with bread have used sophisticated analytical methods (GC-MS) to show that inoculation with actinomycetes produces intense musty odours. These are attributed to the presence of 2-methylisoborneol and geosmin, whereas moulds such as *Penicillium roqueforti* and *Botrytis cinerea* produced musty/fruity odours caused by these compounds and 8-carbon alcohols and ketones (Harris *et al.* 1986). However, in these studies whole wheat bread was inoculated and stored for 7–14 days, by which time significant visible mould was

present. Thus, this previous study was mainly aimed at the development of criteria for classifying spoiled product, as opposed to quality assurance and early detection.

It was interesting to note that only three of the seven extracellular hydrolytic enzymes were found to change significantly with mould colonization of bread, and were good indicators of spoilage. However, changes were significant after 48 h. The specific activity of the three individual enzymes also varied with the dominant spoilage mould present. Previous studies have examined the potential for the use of hydrolytic enzymes as indicators of microscopic mould activity in grain prior to visible growth (Jain *et al.* 1991; Magan 1993). N-Acetyl-β-D-glucosaminidase, α-D-galactosidase and β-D-glucosidase were found to change significantly before visible growth was observed in grain. Recently, Marin *et al.* (1998) found that for mycotoxigenic species of *Fusarium* (*F. verticillioides* and *F. proliferatum*), the same three enzymes were predominantly produced, indicative of the capacity for invasion of maize. The present study suggests that quantitative changes in these three enzymes also occurred in bread substrates due to fungal colonization. However, changes were later than the qualitative changes in volatile patterns observed.

The populations (cfu) of each mould species, based on serial dilution of subsamples, showed an increase with incubation time at the steady-state a_w level (0·95) used. This basal method of analysis showed marked increases, particularly of *P. chrysogenum*, over the incubation period. The increase in cfu for the *Eurotium* spp. was lower and may have been partially due to fragments of developing mycelium and a mixture of both asexual conidia and cleistothecia containing ascospores. Thus, the slower rate of increase in cfu by *Eurotium* spp. should be expected. Extensive previous studies of mould contaminants of grain showed that when grain was inoculated with *P. roqueforti* (initial concentration of 10^3 cfu g^{-1} grain), there was a good correlation between sensor responses with cfu and with ergosterol, a biomass marker for activity (Schnurer *et al.* 1999). Other studies have examined ergosterol as an early indicator of mould activity in grain (Marfleet *et al.* 1991; Tothill *et al.* 1992). However, these methods all require long time periods for analysis often unavailable in the food processing industry, particularly of intermediate moisture bakery products which are prone to contamination and where quality assurance systems need to be more rapid.

In summary, this study shows that potential exists for exploiting electronic nose technology and systems for rapid qualitative measurement of quality in bakery products. More information is needed to develop neural network systems for the identification of the quality of products as part of such a quality assurance scheme. These new ways to detect moulds in food and feed could have widespread application in the food industry.

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