

Application of electronic nose technology for the detection of fungal contamination in library paper

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Received 11 December 2003; accepted 13 April 2004

Abstract

Studies were carried out in vitro on a cellulose based agar at two water activities (a_w , 0.975, 0.995) and on three types of paper at two relative humidities (75, 100% RH) for the potential for differentiation of contamination and colonisation by *Aspergillus terreus*, *A. holandicus* and *Eurotium chevalieri*. In vitro studies showed that conducting polymer sensor array gave different responses to each of these species when grown on cellulose agar at both a_w levels. Discriminant function analyses of the data showed differentiation of the controls from the spoilage fungi. Cluster analysis gave a significant ($P = 0.05$) separation of the control and each spoilage fungus. In situ studies on three types of paper showed that using natural substrates the volatile patterns produced by each of these fungi was different from each other and from the control. The results obtained were better at the higher humidity. The three paper types could be successfully differentiated into clusters. For a single paper type, differentiation of controls from spoilage fungal treatments was better at the higher humidity. This study has shown that this technology has potential for the early detection of fungal contamination in library materials and archives for the improved protection of cultural heritage.

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Keywords: Electronic nose; Paper; Spoilage fungi; Microbial volatiles; Differentiation; Cultural heritage; Biodeterioration

1. Introduction

Among the most important factors that influence deterioration of library material are biological, including insects, rodents and microorganisms (Zyska, 1997; Koestler et al., 2000). Fungal colonisation causes significant disfigurement of library materials, especially books and paper. Apart from the undesirable aesthetic aspects, the presence of some microorganisms can lead to the irreversible degradation of the paper if viable cells are not detected early enough resulting in significant loss in value and quality of such material. Thus early tools for the diagnosis of microbial, and more specifically fungal contamination would be very useful for effective management of such valuable material.

The key environmental factors which determine germination and growth on paper-based material are the prevailing

environmental factors of relative humidity (RH), temperature and gas composition (Magan, 1997) and the level of initial contamination of the material. The concentration of viable fungal spores in house dust has been observed to vary from 6×10^3 to 3.2×10^6 CFU (colony forming units) per g of dust (Korpi et al., 1997). To prevent fungal growth, books and archives are commonly stored in controlled environments, with temperature and humidity maintained in a defined range, and periodically cleaned of dust (Pinzari et al., 2003).

Microbial contaminants produce a range of volatile compounds including different alcohols, aldehydes, ketones, aromatic compounds, amines, terpenes, chlorinated hydrocarbons and sulphuric compounds (Korpi et al., 1997). Recently, 1-octen-3-ol was shown to cause genotoxicity effects in the library environment (Fiedler et al., 2001). Many contaminant fungi are also known to produce mycotoxins which can also be present in the spores. Many bacteria and moulds produce a characteristic range of volatiles (Magan and Evans, 2001; Tothill and Magan, 2003). This fingerprint

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could be utilised for early detection of microbial activity on museum materials.

Traditional methods of enumeration of microbial contaminants require a lot of time and labour (Tothill and Magan, 2003). More rapid, early techniques are required for effective management of paper-based museum materials. Recently, the development and commercialisation of sensor-based devices, so-called electronic noses (e-noses), has provided a possible tool for more effective management. Such instruments provide a rapid, simple and non-invasive sampling technique, for the detection and identification of a range of volatile compounds. The electronic nose mimics the human olfaction, and comprises three essential elements: (a) a sampling conditioning unit, which delivers the odour volatiles from the headspace of the sample; (b) a test chamber in which a sensor array is based; and (c) a processing unit, which analyses the sensor responses for pattern analyses. The major limitations include the strong sensitivity to humidity, drift and sensor poisoning. Despite these drawbacks, improvements on sensor technology coupled with more accurate protocols are increasing the potential of the e-nose as an analytical tool that does not require highly trained operators.

Electronic nose technology has been applied to a range of food (Gardner et al., 1992; Magan and Evans, 2001; Van Deventer and Mallikarjunan, 2002), medical (Wang et al., 1997; Pavlou et al., 2000; Gardner et al., 2000; Turner and Magan, 2004) and environmental applications (Gostelow et al., 2001; Bourgeois et al., 2001; Canhoto and Magan, 2003a). Due to the growing interest in environmental and work place monitoring, the detection of analytes such as aromatic and halogenated hydrocarbons is of increasing economic importance (Dickert et al., 1999). However, no studies have previously attempted to examine the potential of using e-nose technology for the early qualitative detection of microbial contamination of museum materials.

This study investigated the potential of using electronic nose technology (12 or 14 conducting polymer sensor-based array systems) for in vitro discrimination of three xerophilic *Aspergillus/Eurotium* species on cellulose-based media, and in situ detection and differentiation of these species on different types of paper samples.

2. Materials and methods

2.1. In vitro studies

2.1.1. Cultural media and incubation

Three fungal strains, *Aspergillus terreus* Thom (ATCC 10690); *Aspergillus hollandicus* (Anam.: *Eurotium amstelodami* (Mangin) Thom and Church) and *Eurotium chevalieri* L.Mangin, from culture collection of Instituto Centrale di Patologia del Libro, were isolated from deteriorated paper materials (Pinzari et al., 2003).

A growth medium of 1% cellulose (Oxoid), 1% malt extract (Lab M) and 2% technical agar (Oxoid), modified

to 0.995 and 0.975 water activity (a_w) with glycerol, were inoculated with a 0.2 ml suspension (10^6 spores ml^{-1}) of each species, and spread-plated over the surface. The plates were enclosed in polyethylene bags and incubated at 25°C. Four replicates per strain were destructively sampled after 20 and 40 h. Un-inoculated medium served as controls at each sampling time (Keshri and Magan, 2000).

2.1.2. Gas sampling and sensing procedure

Single replicate Petri plate cultures were placed in sampling bags (500 ml capacity), with the lid carefully removed, filled with filter-sterilised air and sealed. The bags were incubated for 1 h at 25°C, to equilibrate the headspace. The headspace from each bag was subsequently sampled through an air-filter system, which consisted of a bio-filter (0.45 μm , PTFE Whatman, Hepa-Vent) and an activated carbon filter (Whatman), to ensure clean airflow. An electronic nose system (BH114, Bloodhound Sensors, UK) that uses an array of 14 conducting polymer sensors was used in this study. Samples were analysed randomly, including the controls. The data was collected and analysed by the e-nose software package system. For each sensor response a set of data includes absorption, desorption, divergence (maximum response), area (of sensor response) and ratio (adsorption/desorption) is plotted. Normalised data were analysed using xlStat programme (Microsoft Excel add-in). Principal component analysis (PCA), discriminant function analysis (DFA) and cluster analysis (CA) techniques were applied to discriminate between the treatments. PCA is a non-supervised technique which means that the different classes or groups are not defined and the technique finds any hidden relationship between samples. PCA is commonly used to summarise the pattern of correlation among the observed variables (e.g., sensor responses). DFA is a supervised technique, the different classes are defined in the analysis. Linear function analysis involves finding one or more linear combinations that maximises the between-group differences, with 95% confidence. CA uses the Mahalanobi's squared distance between groups from the DFA to construct the dendrogram.

2.2. In situ studies

The same fungal species used for in vitro studies were tested in situ: *A. hollandicus*, *A. terreus* and *E. chevalieri*.

2.2.1. Paper types

Three types of paper with different compositions, based on the origin of the fibres and the chemical treatments undergone during the manufacturing process, were used in this study. A minimum of three types of paper were required to verify if the sensors could detect differences in fungal growth:

- (a) Paper type A (Whatman 1CHR), made of pure cellulose;

- (b) Paper type B (produced by Fedrigoni Mills, Italy), called “Freelife vellum”, this being a long-life type. It is made with 80% recycled fibres, 15% cellulose chlorine free and 5% cotton fibres;
- (c) Paper type C (produced by Fedrigoni Mills, Italy), called “Old mill” ivory, this being uncoated long-life type. It is made with 100% cellulose fibres.

The paper samples were cut into 2×6 cm strips. Both sides of the strips were exposed to UV light for 45 min, in order to sterilise the surface from airborne fungal and bacterial cells.

2.2.2. Inoculation of paper treatments

Mycelial cultures were inoculated on Malt Extract Agar (MEA) and prepared according to Pitt and Hocking (1985). The cultures were kept in the dark at 25°C before their use for the inoculation of paper strips. A spore suspension was prepared in a 0.02% Tween 80 (Merck) and distilled water. Spores were then filtered through a sterile cotton cloth to remove impurities. The spore density was obtained for each strain by spore-counting in a Thoma Chamber and optical microscopy. A defined volume of each spore suspension was diluted with nutritive broth (Sabouraud Broth, DIFCO), in order to inoculate the paper strips.

Each paper strip was inoculated with 100 μ l of spore suspension (10^3 spores ml^{-1}). For control samples, paper strips were inoculated with 100 μ l of nutritive broth. Four replicates (a–d) for each treatment were used. The paper strips were placed in a 50 ml vial and closed with ventilated caps. Two values of RH were considered: 100% RH, obtained with distilled water; and 75% RH, obtained with saturated solutions of NaCl. Vials were kept in glass containers in a thermostatically controlled incubator at 27°C for 7 days. A sensor (Hygrolog-D Rotronic AY, Swiss) to register the internal RH, was placed in each container throughout the incubation time.

2.2.3. Headspace analyses

Before analysis, samples were placed at 25°C for approximately 30 min to equilibrate volatiles in the headspace. Samples were placed in a 50 ml vial and were inserted in a glass sampling vessel on the sampling stage, and allowed to equilibrate at 30°C for 15 min. The headspace from the sampling system were analysed using an eNOSE 4000 sensor array module (Marconi Applied Technologies, UK). This instrument consists of an array of 12 conducting polymer sensors in a temperature-controlled sensor chamber. Filtered nitrogen was also used to purge the sensors between sample acquisition. As previously described for in vitro studies, data were analysed using the xlStat program and the same statistical techniques. Previous studies have shown that results obtained with the two conducting polymer electronic nose systems are very similar (Canhoto and Magan, 2003b). The only difference is the sensor arrays are slightly different in fabrication and number. Apart from this, the eNOSE

4000 flushes the sensor chamber with filtrated nitrogen while BH-114 uses filtrated air.

3. Results

3.1. In vitro studies

For in vitro studies, two aspects were taken into consideration, the water availability ($0.995a_w$ and $0.975a_w$) and the incubation time (20 and 40 h). Fig. 1 shows the response of the different sensors in the array to volatiles produced by the fungi on cellulose agar. This confirms that the fungi produce a slightly different mixture of volatiles in the headspace resulting in significant differences in the sensor responses. Fig. 2 shows the PCA for the results obtained for the fungal cultures incubated at (A) $0.995a_w$ and (B) $0.975a_w$ for 20 h at 25°C. In both cases, there was discrimination of the control samples from the other treatments after this period. Discriminant function analysis showed that 98% of the data could be accounted for with clear differentiation between the controls and the spoilage fungi after 20 h (Fig. 3). Similar results were obtained at both a_w levels.

Cluster analysis, based on the Mahalanobi's squared distance between groups obtained in the discriminant analysis at the 95% confidence limit showed that increasing the incubation time to 40 h resulted in a better separation between the control and fungal treatments (Fig. 4).

3.2. In situ studies

Fig. 5 shows a comparison between the response profiles for the 12 conducting polymer sensors of the e-nose employed in this study. This shows that, for each of the treatment fungi, growing on type C paper, each sensor has a different response to the volatiles produced in the headspace. Using the cluster analysis to represent the sensor responses (Fig. 6), it was possible to obtain a clear separation between

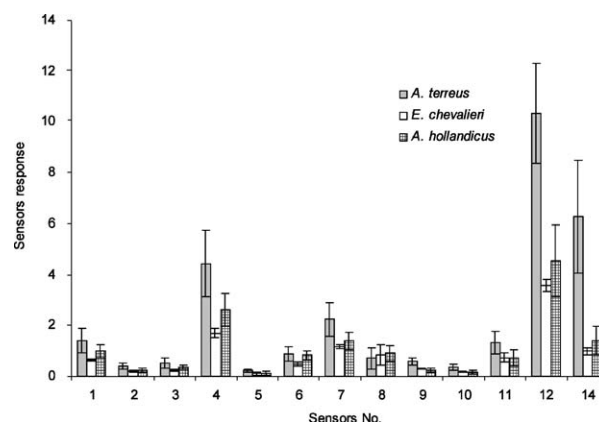


Fig. 1. Sensor responses to three fungal species (*A. hollandicus*, *E. chevalieri* and *A. terreus*) inoculated in vitro on a cellulose agar at $0.995a_w$ for 20 h.

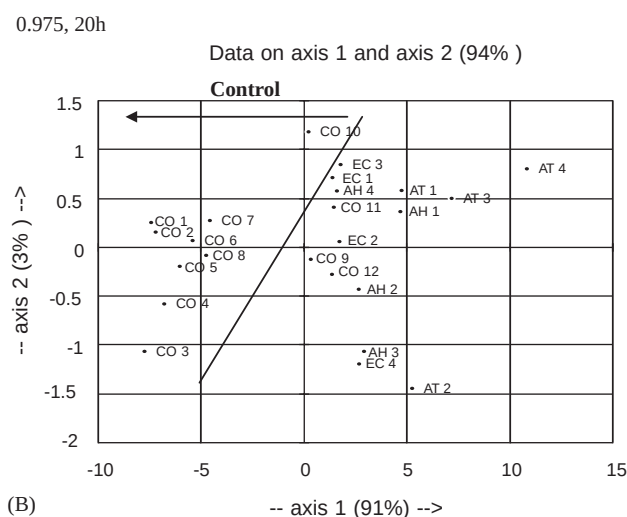
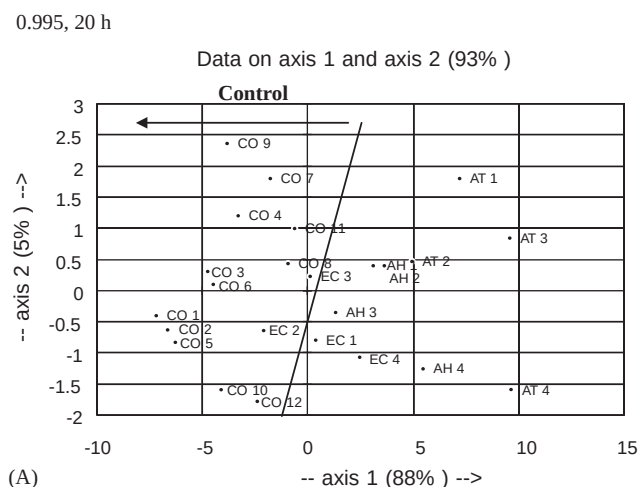


Fig. 2. PCA map of in vitro studies at (A) 0.995 a_w and (B) 0.975 a_w , with 20 h incubation at 25°C. Key for treatments: CO (1–12), control; AT (1–4), *A. terreus*; AH (1–4), *A. hollandicus*; EC (1–4), *E. chevalieri*.

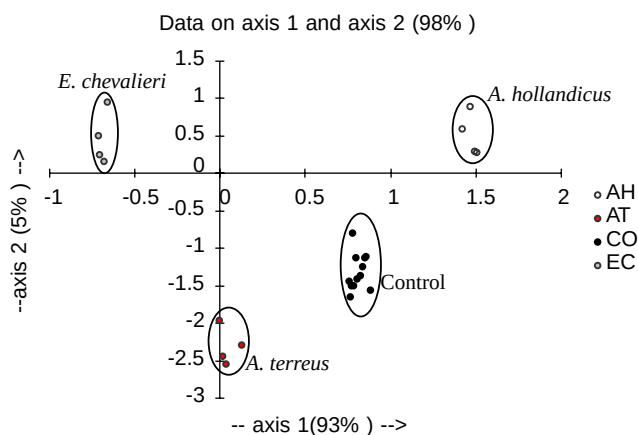


Fig. 3. DFA of in vitro studies at 0.975 a_w incubated for 20 h at 25°C. Key for treatments: CO, control; AT, *A. terreus*; AH, *A. hollandicus*; EC, *E. chevalieri*.

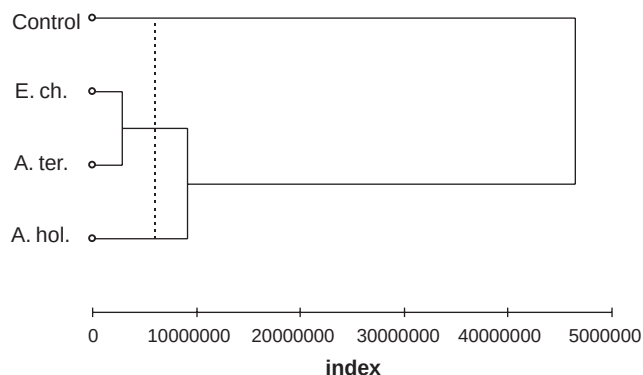


Fig. 4. Cluster analysis showing the clear separation of the control from in vitro samples at 0.995 a_w inoculated with the three types of fungi (*E. ch.*, *E. chevalieri*; *A. ter.*, *A. terreus*; *A. hol.*, *A. hollandicus*). Samples were incubated for 40 h at 25°C.

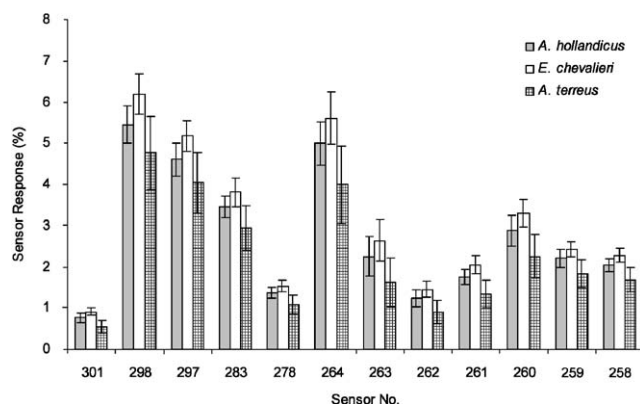


Fig. 5. Sensor responses to the fungal volatiles produced by the three species on paper C at 100% RH, showing the different responses for each species.

the control samples and paper strips (paper type A) inoculated with the fungal species, at two RH levels, (A) 75% and (B) 100%. Fig. 7 reveals that successful discrimination between the different paper types was possible. However, it was not in this case possible to separate the fungal treatments from the controls within each paper treatment. Changing the environmental conditions, the e-nose was able to differentiate between the control samples and those inoculated with fungi in the two humidity conditions at 75 RH and 100 RH (Fig. 8).

4. Discussion

The response of the sensors to volatiles produced by the spoilage fungi used showed that different types and amounts were probably slightly different. Since the e-nose is qualitative and not quantitative, it gives a good fingerprint of the differences between control and other treatments. Previous studies have investigated the volatile production of microorganisms in different substrates. For example,

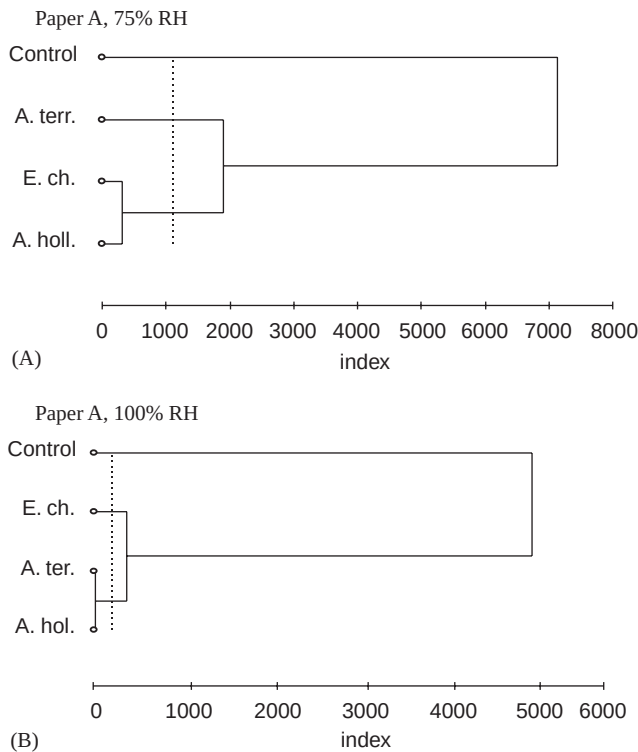


Fig. 6. Cluster analysis showing the discrimination between the different fungal species inoculated in paper type A (Whatman 1CHR), at (A) 75% RH, and (B) at 100% RH. Key for treatments: E.ch., *E. chevalieri*; A.ter., *A. terreus*; A.hol., *A. hollandicus*.

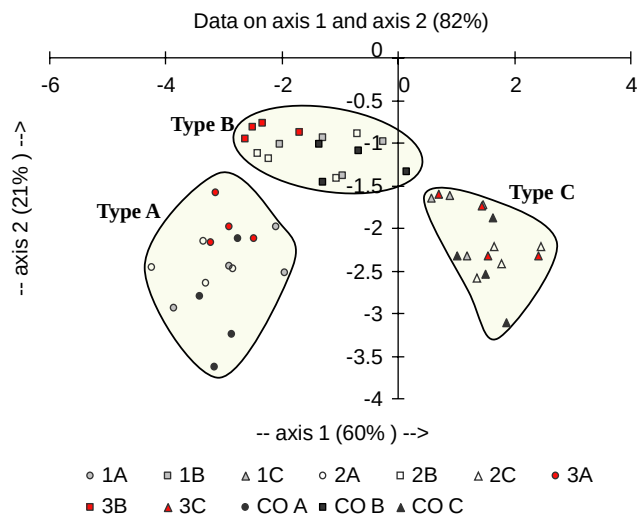


Fig. 7. DFA of all types of paper (A—Whatman 1CHR; B—“Freelife vellum”; and C—“Old mill” ivory), different fungal species (1, *A. hollandicus*; 2, *E. chevalieri*; and 3, *A. terreus*), and control samples (CO A, CO B and CO C), at 100% RH.

Fiedler et al. (2001) used GC-MS combined with HS-SPME to explore the volatile metabolites released by different fungal species grown on different substrata. They concluded that more than 150 compounds could be identified but not

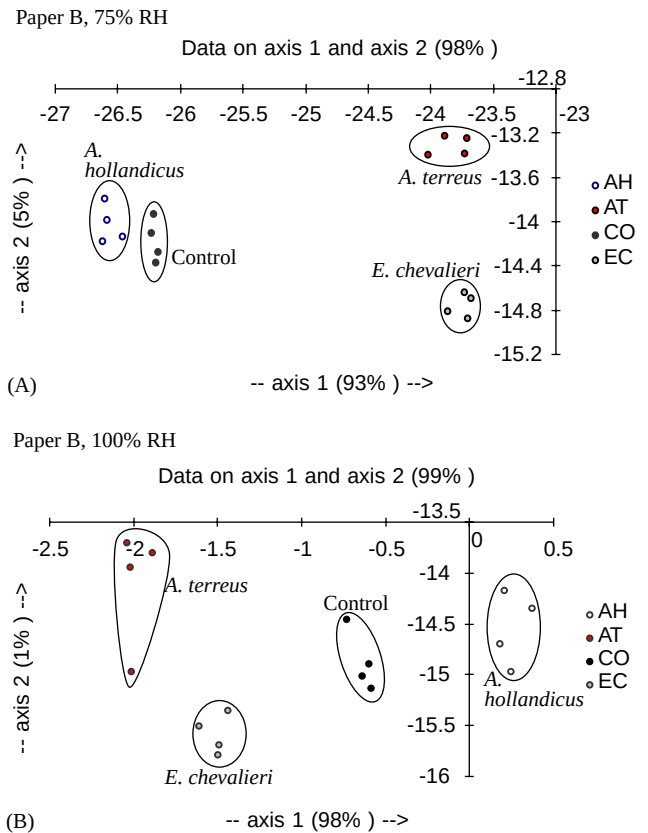


Fig. 8. DFA of paper type B inoculated with different fungal species (1, *A. hollandicus*; 2, *E. chevalieri*; and 3, *A. terreus*), at (A) 75% RH, and (B) 100% RH. Letters (a–d) stand for replicates.

a single compound was common to all species. Further studies (Claeson et al., 2002) investigated the emission of volatile compounds by fungi from building materials and cultures grown in solid media. They found that the production of volatile compounds by fungi varied greatly between different growth media. Environmental factors such as RH also have an effect on volatile production patterns. Korpi et al. (1997) investigated the influence of RH on the growth of microorganisms, including fungi. Their results showed that RH affects the volatile emission and also the growth rate of the different fungal species.

The in vitro studies revealed that successful differentiation could be obtained between spoilage fungi and the controls after 20 h incubation on a cellulose medium at $0.975a_w$. This is similar to previous studies with food spoilage fungi, where good discrimination was obtained between spoilage fungi and the controls, but where two closely related *Eurotium* species could not be differentiated (Keshri et al., 1998). Schiffman et al. (2000) found that the e-nose could detect and classify three fungi, *A. niger*, *A. flavus* and *Penicillium chrysogenum*, incubated in solid agar media after 24 h. Keshri et al. (2002) studied the detection of *Eurotium* spp. and *P. chrysogenum* inoculated in food matrices (bread), using a conducting polymer based e-nose and found that

successful detection could be made within 36 h, much faster than that which could be obtained using enzyme assays or traditional fungal counts. More recently, bacterial, yeast and filamentous fungal growth was successfully achieved using a similar e-nose system in food matrices. In this case at $0.95a_w$ differentiation was possible after 24 h (Needham et al., 2003). The potential for use of this approach has been demonstrated for a range of food, medical and environmental applications (Pearce et al., 2002; Tothill and Magan, 2003; Canhoto and Magan, 2003a).

The results obtained with different types of paper and differentiation of spoilage fungi, is the first report of this approach for library-based material. The results show that the e-nose was able to discriminate between control samples from paper inoculated with fungal spores, and between some species. The results indicate that the substrate plays an important role when paper samples are being used. This suggests that a different volatile production pattern is probably produced by each fungal species.

Dust accumulation may be an important factor in providing foci for fungal activity, provided enough moisture is available for spore germination and subsequent establishment. Early detection of activity of fungi has been demonstrated on food matrices where within 24 h the activity of fungal spores was discriminated, whereas using populations (CFUs) or enzyme assays required 48–72 h (Keshri et al., 2002). However, other approaches such as the use of ATP by effective extraction of samples may provide another method for determination of microbial/insect activity in wood-based materials (Nieto et al., 1997). However, the sensitivity and level of quantitative or qualitative information needed as part of quality assurance management system needs to be determined.

The advantage of the e-nose technology is that it can be used for effective monitoring in a storage situation, especially if linked to a real time neural network which has information on the volatile patterns from non-spoiled paper material. It could then be used to effectively provide a warning of when conditions may become compromised and effective management could be then employed for preventing loss of valuable cultural material. This has already been successfully demonstrated for detection of mouldy grain (Evans et al., 2000). The field of sensor technology is also advancing rapidly and other sensor arrays are now available with better sensitivity and stability in different abiotic environments. Potential thus exists for the development of a system for the detection of the growth of moulds in libraries, archives and museums long before it becomes perceptible to the human olfactory sense and thus preventing significant economic damage from occurring.

References

Bourgeois, W., Burgess, J.E., Stuetz, R.M., 2001. On-line monitoring of wastewater quality: a review. *Journal of Chemical Technology and Biotechnology* 76, 337–348.

- Canhoto, O., Magan, N., 2003a. Potential for the detection of microorganisms and heavy metals in potable water using electronic nose technology. *Biosensors and Bioelectronics* 18, 751–754.
- Canhoto, O., Magan, N., 2003b. Electronic nose technology for the detection of microbial and chemical contamination. *Proceedings of the 10th International Symposium on Olfaction and Electronic Nose (ISOEN03)*, pp. 227–230.
- Claeson, A., Levin, J., Blomquist, G., Sunesson, A., 2002. Volatile metabolites from microorganisms grown on humid building materials and synthetic media. *Journal of Environmental Monitoring* 4, 667–672.
- Dickert, F.L., Hayden, O., Zenkel, M.E., 1999. Detection of volatile compounds with mass-sensitive sensor array in the presence of variable ambient humidity. *Analytical Chemistry* 71, 1338–1341.
- Evans, P., Persaud, K.C., McNeish, A.S., Sneath, R.W., Hobson, N., Magan, N., 2000. Evaluation of a radial basis function neural network for determination of wheat quality from electronic nose data. *Sensors and Actuators B* 69, 348–358.
- Fiedler, K., Schutz, E., Geh, S., 2001. Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. *International Journal of Hygiene and Environmental Health* 204, 111–121.
- Gardner, J.W., Schummer, H.V., Tan, T.T., 1992. Application of an electronic nose to the discrimination of coffees. *Sensors and Actuators B* 6, 71–75.
- Gardner, J.W., Shin, H.W., Hines, E.L., Dow, C.S., 2000. An electronic nose system for monitoring the quality of potable water. *Sensors and Actuators B* 69, 336–341.
- Gostelow, P., Parsons, S.A., Stuetz, R.M., 2001. Odour measurements for sewage treatment works: a review. *Water Research* 35, 579–597.
- Keshri, G., Magan, N., 2000. Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of *Fusarium* spp. using volatile production profiles and hydrolytic enzymes. *Journal of Applied Microbiology* 89, 825–833.
- Keshri, G., Magan, N., Voysey, P., 1998. Use of an electronic nose for early detection and differentiation between spoilage fungi. *Letters in Applied Microbiology* 27, 261–264.
- Keshri, G., Voysey, P., Magan, N., 2002. Early detection of spoilage moulds in bread using volatile production patterns and quantitative enzyme assays. *Journal of Applied Microbiology* 92, 165–172.
- Koestler, R.J., Sardjono, S., Koestler, D.L., 2000. Detection of insect infestation in museum objects by carbon dioxide measurement using FTIR. *International Biodeterioration & Biodegradation* 46, 285–292.
- Korpi, A., Pasanen, A.-L., Pasanen, P., Kalliokoski, P., 1997. Microbial growth and metabolism in house dust. *International Biodeterioration & Biodegradation* 40, 19–27.
- Magan, N., 1997. Fungal adaptation to environmental stress. In: Wicklow, D.T., Soderstrom, B., (Eds.), *MYCOTA, Environmental and Microbial Relationships*, Vol. IV, Springer, Berlin.
- Magan, N., Evans, P., 2001. Volatiles as an indicator of fungal activity and differentiation between species, and the potential use of electronic nose technology for the early detection of grain spoilage. *Journal of Stored Products Research* 36, 319–340.
- Needham, R., Williams, J., Magan, N., 2003. Early detection and differentiation of soilage micro-organisms of bakery products. *Proceedings of the 10th International Symposium on Olfaction and Electronic Nose (ISOEN03)* pp. 220–224.
- Nieto, F.E., Lee, J.J., Koestler, R.J., 1997. Assessing biodeterioration in wood using ATP photometry: Part I. Nucleotide extraction and wood interference. *International Biodeterioration & Biodegradation* 39, 9–13.
- Pavlou, A.K., Magan, N., Sharp, D., Brown, J., Barr, H., Turner, A.P.F., 2000. An intelligent rapid odour recognition model in discrimination of *Helicobacter pylori* and other gastroesophageal isolates in vitro. *Biosensors and Bioelectronics* 15, 333–342.
- Pearce, T.C., Schiffman, S.S., Nagle, H.T., Gardner, J.W. (Eds.), 2002. *Handbook of Machine Olfaction: Electronic nose technology*, Wiley-VCH, New York.

- Pinzari, F., Fanelli, C., Canhoto, O., Magan, N., 2003. Electronic nose technology applied to the early detection of fungal infections in libraries and archives. *Proceedings of Moulds, Health and Heritage Conference*, Braunschweig, Germany, 4–5 September 2003.
- Pitt, J.I., Hocking, A.D., 1985. *Fungi and spoilage*. CSIRO—Division of Food research, Sydney. Academic Press, Australia, 413pp.
- Schiffman, S.S., Wyrick, D.W., Gutierrez-Ozuna, R., Nagle, H.T., 2000. Effectiveness of an electronic nose for monitoring bacterial and fungal growth. In: Gardner, J.W., Persaud, K.C. (Eds.), *Electronic Noses and Olfaction*. IOP Publishing Ltd, UK.
- Tothill, I.E., Magan, N., 2003. Rapid detection methods for microbial contaminants. In: Tothill, I.E. (Ed.), *Rapid and on-line instrumentation for food quality assurance*. CRC Press, Woodhead Publishing Limited, UK, pp. 136–160.
- Turner, A.P.F., Magan, N., 2004. Electronic noses and disease diagnostics. *Nature Reviews Microbiology* 2, 161–166.
- Van Deventer, D., Mallikarjunan, P., 2002. Optimizing an electronic nose for analysis of volatiles from printing inks on assorted plastic films. *Innovative Food Science and Emerging Technology* 3, 93–99.
- Wang, P., Tan, Y., Xie, H., Shen, F., 1997. A novel method for diabetes diagnosis based on electronic nose. *Biosensors and Bioelectronics* 12, 1031–1036.
- Zyska, B., 1997. Fungi isolated from library materials: a review of the literature. *International Biodeterioration & Biodegradation* 40, 43–51.