PROJECT REPORT No. 295

DEVELOPMENT AND TESTING OF A SENSOR TO DETECT MICROBIOLOGICAL SPOILAGE IN GRAIN

NOVEMBER 2002

Price £7.50

PROJECT REPORT No. 295

DEVELOPMENT AND TESTING OF A SENSOR TO DETECT MICROBIOLOGICAL SPOILAGE IN GRAIN

by

H.M. Brown[1], N. Ratcliffe[2], P.A. Voysey[1], J.S. Williams[1]
P. Spencer-Phillips[2], B.P.J. de Lacey Costello[2], N. Beales[1], S. Salmon[1], H. Gunson[2],
P.S. Sivanand[2] and R.J. Ewen[2]

[1]Campden & Chorleywood Food Research Association,
Chipping Campden, Gloucestershire, GL55 6LD

[2]The University of the West of England
Faculty of Applied Sciences, Department of Chemical & Physical Sciences
Bristol Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY

This is the final report of a 42-month project that started in April 1998. This work was funded by a grant of £299,053 from HGCA (project 2026).

CONTENTS

Page No.

ABSTRACT

The handling and storage of wheat can result in the formation of off-odours in grain, in particular musty, sour, 'green' and fishy odours. The sources of these off-odours during storage are due to a combination of fungal or bacterial activity when grain is held at elevated levels of moisture (>14%) and temperature in the grain store. Off-odours can be difficult to detect in both farm and grain processing environments due to the presence of other odours. By the time that spoilage odours can be detected by human senses, spoilage is likely to have spread widely within the store. Grain spoilage when it occurs costs the industry significant amounts of money in lost raw materials, down time and cleaning.

The aim of this project was to develop a sensing device to give an early warning of the onset and presence of microbiological spoilage in grain and to evaluate the sensor device in an industrial environment. The availability of a sensor-based instrument to detect incipient spoilage, and thus potential mycotoxin production, on wheat intake would reduce the need for human odour assessment of grain, which is desirable on safety grounds, and provide an opportunity to standardise an approach to assessing grain spoilage. Laboratory based culturing systems using irradiated grain conditioned to 25% moisture, inoculated with specific spoilage organisms, were developed to generate characteristic grain spoilage odours. These systems provided a means of defining spoilage odours and the time course over which they develop using the human sense of smell. They provided a means for the chemical identification of suitable volatile compounds for use as early warning indicators, to which sensors had to be developed. Novel sensors, constructed from a range of materials, were tested against pure compounds identified as potential early warning indicators of spoilage and spoiled grain. The most promising sensors were incorporated into a prototype device suitable for use in a grain intake laboratory. Sampling conditions (times and temperatures) were optimised and a 'laboratory threshold' defined using samples of spoiled grain submitted from commercial practice, the laboratory-based spoilage systems, and mixtures of spoiled and sound grain.

The prototype sensor system was evaluated in a commercial wheat intake facility where over 100 samples were tested. All samples identified as having an odour related problem were rejected by the prototype system. A number of samples that were passed by the intake laboratory were rejected by the prototype system – this may indicate early detection of spoilage. Of 37 newly harvested wheat samples of different varieties from different geographical locations in the UK, the wheat intake assessors rejected one sample and six were rejected by the prototype sensor system. The 'laboratory defined threshold' may need adjustment to minimise the number of false positives.

The trial highlighted the promise of this prototype for the detection of spoilage in wheat grain. A larger trial is now needed to ascertain the reliability and long-term stability of the device and its usefulness to the industry.

EXECUTIVE SUMMARY

The aim of this project was to develop sensors to give an early warning of the onset and presence of microbiological spoilage odours in grain. The sensors were to be used to develop a low cost instrument and the prototype was to be tested in the industrial environment during the life of the project.

Sensors available in 'off the shelf' electronic nose devices had been found to not be sufficiently sensitive to detect odour active molecules or were oversensitive to changes in moisture content. The approach in this work was to design a solution specific to the problem of spoilage of wheat grain. To this end, the project had three main aspects: to identify causes and indicators of microbial spoilage in wheat grain storage and handling; to develop sensing techniques that can give an early warning of the onset and presence of micro-biological spoilage in grain; and to develop a prototype low cost instrument using these sensors.

To identify microorganisms associated with grains that generate spoilage odours, organisms were isolated from spoiled grain collected from the industry by CCFRA. Whilst the presence of a microorganism did not necessarily indicate that it was responsible for the spoilage odour, its presence did agree with a review of the literature on microorganisms associated with grain spoilage. For example, environmental scanning microscopy showed teliospores of *Tilletia caries* to be present on grain with a fishy odour, and sporophores of *Aspergillus* and *Penicillium* species present on musty grain. From this information, ten microorganisms were identified as candidates to generate spoilage odours in laboratory based culturing systems: *Alternaria alternata, Aspergillus flavus, Aspergillus niger, Fusarium culmorum*, *Mucor sp.*, *Penicillium aurantiogriseum, Penicillium vulpinum*, *Rhizopus sp., Streptomyces griseus* subsp. *griseus* and *Wallemia sebi*.

Inoculation of sound grain with *Streptomyces griseus, Fusarium culmorum* and *Penicillium aurantiogriseum* produced inconsistent results in terms of spoilage odours, possibly because of the diversity of microbial growth arising from the natural microflora in addition to the introduced organism. All surface sterilisation regimes (ethanol, Tween 80, sodium hypochlorite and Plant Preservative Mixture) failed to eliminate microorganisms associated with the grain except for irradiation at 12kGy. Therefore irradiated grain was used as the substrate onto which the selected organisms were inoculated. A series of trials in which the ten microorganisms were individually inoculated onto intact sterile (irradiated) grain that had been conditioned to a specified moisture content ranging from 16% to 25%, then incubated at 22ºC, were conducted. The results were used to define the parameters for laboratory based culturing systems in which recognisable spoilage odours could be generated. Important factors for the production of recognisable and consistent odours were the moisture content - grain had to be conditioned to 25% moisture; and the level of the starting inoculum – a starting inoculum of approximately 10^3 colony forming units per gram rather than 10^5 resulted in increases in numbers during incubation and the generation of spoilage odours, i.e. growth was important in odour production.

The time course for the development of spoilage odours in the laboratory based culturing system ideally required a period to enable monitoring of the early stages of spoilage that are not detected by human smell and subsequently the production of volatile compounds that are responsible for characteristic spoilage odours. An informal panel, trained by reaching a consensus on the odours in the spoiled samples submitted from industry, was used to define spoilage odours and the time course over which they developed. In some cases, spoilage odours were evident after 6 days and spoilage odours generally peaked after 15 to 22 days. Musty, fermented, green and sour spoilage odours were generated. The laboratory based culturing system developed was used in studies to characterise and identify indicator molecules during the early onset of spoilage in wheat and subsequently as part of the programme to test the performance of developed sensors.

The time course of production of volatile compounds generated by three individual microorganisms (*Fusarium culmorum, Aspergillus niger* and *Penicillium aurantiogriseum*) in laboratory based culturing systems over incubation times of up to 15 days was monitored by taking triplicate sub-samples of the culture on six occasions. Grains were purged with nitrogen gas and the volatiles trapped on to Tenax-TA adsorbent. Volatiles were then transferred to a gas chromatography-mass spectrometry system by thermal desorption using a concentrated headspace injection system and on-column cryofocusing. Chromatograms were analysed to find volatile compounds meeting criteria for early warning spoilage indicators and which were suitable for detection using metal oxide based sensors. Criteria for potential early warning indicators were: that concentration increased with spoilage; that detection by the sensors was achievable at a concentration below that which is detected by odour; and that the indicator volatiles do not increase in sound grain.

A total of 147 volatile compounds were identified, many of which have previously been detected in wheat. Of these volatiles, 27 were found to increase in abundance with spoilage time, and 16 of these compounds were alcohols. Other compounds that increased with time were esters and carbonyl compounds. Increases in the abundance of total volatile alcohols with time followed the same trend as the increase in colony forming units for each of the three microorganisms studied, suggesting a relationship between the total alcohol abundance and biomass of the inoculated organism. Sterile sound grain conditioned to 25% moisture did not show an increase in the abundance of total volatiles. The level of total alcohols in inoculated grain was estimated to increase to around $10 mg.kg^{-1}$ at the end of the incubation period, which is more than adequate for the metal oxide based sensors proposed for the sensing device.

The development of sensors to be incorporated into a prototype device was based upon a previous observation that a composite sensor of tin dioxide and zinc oxide under dry conditions (0% relative humidity) gave a higher sensitivity to organic vapours than if either of these materials were used alone. As the moisture level of grain to be tested by the sensor device is variable, it was necessary to nullify the effects of variable humidity on the sensors. This was achieved by operating the sensors under a flow of high humidity air.

An extended range of composite materials using various combinations of tin dioxide, zinc oxide, niobium oxide and indium oxide was prepared and applied to interdigitated electrodes to prepare sensors. These were tested in an in-house injection test rig with volatiles identified as being associated with the microbial spoilage of grain during storage. The sensitivity of the composite sensors to known concentrations (0.1-5ppm) of volatile organic compounds (alcohols, ketones, aldehydes, hydrocarbons, cyclic aromatics) was measured over a range of relative humidities (0-100%) at a constant temperature. All of the oxide materials showed a decrease in sensitivity at high humidity; the magnitude was dependent on the organic volatile and there was not a clear relationship between sensitivity and humidity. The tin dioxide/indium oxide composite sensor showed the least loss of sensitivity to ethanol and the tin dioxide/zinc oxide sensor showed the least loss of sensitivity to 3-octanoate.

Four composite sensors were selected for incorporation into a prototype device: tin dioxide/zinc oxide composite; tin dioxide/indium oxide composite; indium oxide; and tin dioxide/indium oxide/zinc oxide. Each sensor was mounted in an individual stainless steel chamber and a constant flow (1L/minute) of humidified air (achieved by passing BOC cylinder air through distilled water in a Dreschel bottle) was passed over them. The prototype device was portable – a sealed lead acid battery could power it. Software was written programming a complex series of valve operations to enable a controlled sampling and purge regime of the sensors. The sensor outputs were displayed on a LCD mounted on the outer casing of the prototype; alternatively, the system can be interfaced to a personal computer. Subsequently an autosampler, facilitating incubation and analysis of up to 10 samples, was added.

The prototype sensor device was tested using the laboratory based culturing systems developed at CCFRA. The aim was to determine whether the sensors could discern the spoilage process as it progressed with time. Irradiated wheat, conditioned to 25% moisture, and inoculated with individual spoilage microorganisms (*Aspergillus niger*, *Aspergillus flavus*, *Penicillium aurantiogriseum*, *Penicillium vulpinum, Penicillium verrucosum, Streptomyces griseus* and *Fusarium culmorum)* was held at 22°C for up to 42 days. Sensors were exposed to the headspace at intervals throughout this time. The sensor output increased during the first 14-21 days of storage, peaked and then decreased. The magnitude of the sensor output and the time taken to peak varied between organisms, but in general the sensor was successful in tracking the progress of infection and spoilage from the early stages. This increase in sensor output in the early stages of infection mirrored the increase in the selected volatile compounds in the GC-MS studies carried out at CCFRA.

To determine whether the prototype system could be used for on-site monitoring of grain storage facilities, a 70kg silo of grain was constructed with a 700g ‘hotspot’ of spoiled grain taken from a laboratory culturing system. At fifteen different points throughout the silo, temperature and humidity were measured and air was sampled by pumping and passed directly over the sensors. Variations in humidity at the different sampling points affected the sensor response, confirming the need for the humidity control that had been built into the

prototype device. This emphasised the difficulties in making a truly portable device when the control of humidity proves necessary.

To optimise the availability of volatiles and minimise the sampling time, the effect of grinding the sample, sample equilibration temperature and sampling time were investigated. Grinding of the sample was not considered to provide any overall benefit. Sample equilibration at 80°C for 15 minutes rather than 50°C for 1 hour, with a sampling time of 20 seconds and a sensor recovery time of 20 seconds improved differentiation of spoiled (1.5% w/w) from sound wheat and the precision of the measurements. This also reduced the overall sampling time required from receipt of sample to gaining a result. Using this optimised procedure, a preliminary threshold sensor response was defined to differentiate sound and infected grain.

The levels of microbial infection that the sensor was exposed to using the laboratory culturing system were high. Whilst this may reflect the conditions that could occur in hotspots in grain stores, it is more likely that the levels in commercial samples would be considerably less. The sensitivity of the sensor device to volatiles emitted by musty grain was tested by mixing grain (25% moisture) that had been inoculated with *Penicillium aurantiogriseum* and held for 42 days with sound grain (13% moisture) at concentrations ranging from 0.5% to 10% (w/w). The sensor system output increased with the percentage of spoiled grain present. Spoiled grain at 1.5% (w/w) was clearly differentiated from sound grain alone. It may be possible to differentiate lower levels but with less confidence.

The prototype device and threshold value were evaluated by operating in a commercial wheat intake laboratory. The prototype system was in place for two working weeks. During this time, over 100 grain samples taken for routine testing from trucks delivering to the processing plant were analysed. The samples were assessed by trained wheat grain intake laboratory technicians and also by the prototype sensor device. Different mathematical approaches to calculate the sensor output were compared with a view to determining that which gave the greatest discrimination between spoiled and sound samples. Using the preferred mathematical approach and a threshold value of 180, of the 106 samples assessed, the prototype gave 5 "false positives" (i.e. rejected when the intake assessors said accept). If the threshold value was raised to 185, then the problem of the "false positives" would be overcome and all of the samples with odour related problems would still have been rejected, though care should be taken with this approach as all "false positives" may not be false – the system was designed to detect incipient spoilage, i.e. earlier detection than human assessors. Only a limited number of samples were tested during this period and there was a very low frequency of spoiled samples. It is necessary to test the prototype further on an extended range of commercial intake samples to define a threshold value that equates with a commercially acceptable sample. It would be most appropriate to do this at the time of year when grain has been stored for long periods when there tends to be a higher occurrence of odour related spoiled grain samples.

Whilst located at the commercial grain intake facility, 37 newly harvested wheat samples of different varieties from different geographical locations in the UK were tested using the prototype. Using a threshold of 180, six samples were rejected by the prototype system; the intake assessors would have rejected only one of these. This was the sample that gave the highest sensor output.

In summary the trial highlighted the promise of the prototype device for the detection of spoilage in wheat grain. The sensor device warrants further testing in a larger trial where it is exposed to a larger number of spoiled samples and where the reliability and long-term stability can be tested to confirm its usefulness to the cereals industry.

When grain spoilage occurs, the cost to the industry is significant in terms of lost raw materials, down-time and cleaning, particularly as in some cases the spoilage odours only become evident after the first stage of further processing. Use of this simple, sensor-based device to detect incipient spoilage on wheat intake would reduce the need for human odour assessment and provide an opportunity to standardise grain spoilage assessment.

PAPER 1

DEVELOPMENT OF LABORATORY-BASED CULTURING SYSTEMS TO INDUCE AND MONITOR THE ONSET OF SPOILAGE ODOURS IN GRAIN

P.A. Voysey, H.M. Brown, N. Beales, J.S. Williams, S. Salmon and B.M. Williams
Campden & Chorleywood Food Research Association, Chipping Campden, Glos, GL55 6LD

1.1 ABSTRACT

The aim of this work was to identify microorganisms associated with grain that generate spoilage odours and to develop a laboratory based culturing system to reliably induce them to grow and consistently generate spoilage odours. The time course for spoilage ideally required a period that would enable monitoring of the early stages of spoilage that are not detected by human smell and subsequently the production of volatile compounds that are responsible for characteristic spoilage odours.

An informal odour assessment team was trained using spoiled samples collected from the industry. Four distinct odour types were defined – sour, musty, fishy and green, though variations within these categories were acknowledged. This team was used to assess the odour development in laboratory based culturing systems.

Ten microorganisms were selected that have been associated with the production of spoilage odours in grain. The organisms were sourced and cultured to produce an inoculum. Organisms were inoculated onto intact sterile (irradiated) grain that was conditioned to specified moisture contents ranging between 16% and 25%, then incubated at 22°C. At time intervals up to 34 days, samples of grain were taken to assess odour, the number of colony forming units per gram of grain and moisture content. Important factors for the production of recognisable and consistent odours were: the moisture content – significant spoilage odours were only achieved when the grain was conditioned to 25% moisture; and the level of starting inoculum - approximately 10^3 colony forming units per gram (cfu) resulted in an increase in numbers during incubation whereas higher starting inoculum levels did not. In some cases, spoilage odours were evident after 6 days incubation.

The culturing system developed was subsequently used to monitor and identify volatiles that are produced in the early and later stages of spoilage at CCFRA, and following transfer of the procedure to project collaborators at UWE, for testing the response of sensors to spoilage odours.

1.2 INTRODUCTION

The handling and storage of wheat can result in the formation of off-odours and toxins in grain. In particular, musty, sour and fishy odours have been described in grain samples. The problem of spoiled grain is found to occur at regular intervals with respect to harvest year. The sources of these off-odours during storage are generally due to a combination of fungal or bacterial contamination and elevated levels of moisture and temperature in the grain store.

Off-odours can be difficult to detect in both farm and grain processing environments due to the presence of other odours. By the time that spoilage odours can be detected by human senses, spoilage is likely to have spread widely within the store. The availability of a sensing device to give an early warning of the onset and presence of microbiological spoilage in grain would be of benefit to both farmers and processors. Early detection may provide an opportunity to intervene to avoid spoilage in grain stores and to assist processors assessing grain at wheat intake. Besides removing the subjectivity of assessing spoilage odours in grain, it would also reduce the need for human odour assessment, which is desirable on safety grounds. Smelling grain to assess odours carries potential hazards of dust inhalation and the dust particles carrying mycotoxins, many of which are confirmed carcinogens.

In order to develop a sensing device it was necessary to identify indicators of microbiological spoilage in grain storage and handling. This paper describes work to identify microorganisms associated with grain that generate spoilage odours and the development of a laboratory based culturing system to reliably induce spoilage and generate spoilage odours in wheat grain. This system was subsequently used to determine suitable volatile indicators of spoilage (Paper 3) and to test the sensors and sensing device that was developed (Papers 5 and 6).

The laboratory based culturing system was based upon inoculation of a suitable substrate with microorganisms known to be associated with the production of spoilage odours in wheat or grain. The moisture content of the substrate was adjusted to achieve a time period without spoilage odours detectable by human assessors, leading to a period of production of strong, consistent spoilage odours.

1.3 METHODS

1.3.1 Identification of Organisms Causing Spoilage Odours in Grain

1.3.1.1 Collection of Spoiled Samples

In order to determine the range of spoilage odours that occur and to provide spoiled samples for study, the industry was requested to provide samples of spoiled grain they encountered to CCFRA. Samples were assessed for odour and stored in sealed glass jars at 12-15°C at 60-70% relative humidity.

1.3.1.2 Identification of Organisms for Inoculation onto Grain

A selection of the spoiled wheat samples submitted by industry was used to culture and identify the microorganisms present on spoiled wheat. In addition, a review of the literature (Salmon *et al*., 2002) was used to identify microorganisms that have been associated with spoilage odours in grain.

1.3.2 Development of an Odour Assessment Method for Grain

Volatiles were purged from grain using nitrogen gas and the gas flow passed through a 0.45μm filter to a glass funnel 'sniffer port'. Different approaches to maximise the odours being stripped from the grain were tried: different flow rates; purging across the surface of temperature equilibrated wheat versus purging gas between wheat grains. A standard operating procedure was produced that could be used by project collaborators at the University of the West of England.

Spoiled samples submitted from the industry were used to familiarise an informal group of odour assessors with the range of odours likely to occur. Definition of the odour types was discussed to achieve consensus on the descriptors used to classify odour type. Four key descriptors were agreed – musty, fishy, sour and green. There was variation within each of these categories, but they were considered to encompass the range of odours present in the spoiled samples that had been obtained.

1.3.3 Development of a Laboratory Culturing System for Generating Spoiled Grain

Development of a laboratory based culturing system in which spoilage odours could be produced reliably was based upon the following:

1.3.3.1 Mould Cultures

The following moulds were obtained: *Fusarium culmorum* (FMBRA 17), *Rhizopus* sp. (FMBRA 24), *Mucor* sp. (FMBRA 25, 26), *Penicillium aurantiogriseum* (IHEM 698); *Penicillium vulpinum* (CCF 2198); *Wallemia sebi* (CCF 1689); *Alternaria alternata* (IMI 300776); *Aspergillus niger* (IMI 017454); *Aspergillus flavus* (obtained from Cranfield Biotechnology Centre); *Streptomyces griseus* subsp. *griseus* (NCIMB 8136).

1.3.3.2 Substrate for Inoculation

A number of substrates were considered for inoculation studies. These included 2% wheat agar extract, whole grain and whole sterilised grain. Whole sterilised grain was prepared by irradiation (12kGy) (Isotron, Swindon).

1.3.3.3 Preparation of Mould Cultures for Inoculation onto the Substrate

In preparation for inoculation onto a suitable substrate for generating odours, moulds were picked off the working plate and cultured by placing in the centre of 5 pre-poured plates of medium (RBCA (Rose Bengal Chloramphenicol Agar) or MEA (Malt Extract Agar) with or without 20% sucrose). This was incubated at 25°C for 5-10 days, depending on the culture. Following growth, 5ml Maximum Recovery Diluent (MRD) was poured onto the surface of one of the culture plates. The spores were harvested by gently moving a sterile spreader over the surface of the mould colony. Using a sterile liquipette, the spores suspended in MRD were removed and added to 15ml MRD. This was repeated for all the plates, taking the 5ml 'MRD' from the now 20ml spore suspension to obtain a total spore suspension. The final number of spores was estimated using a haemocytometer. Usually 10^7 to 10^8 were obtained. To determine the actual spore count, serial dilutions were prepared from duplicate 1ml volumes of the spore suspension. Using the pour plate technique, the dilutions were plated out onto the appropriate growing medium, depending on what they were previously grown on. Plates were incubated at 25°C for 5 days, then the number of colony forming units per ml (cfu/ml) were counted. The remaining 18ml spore suspension was used to inoculate 1kg of whole sterile grain.

1.3.3.4 Moisture Adjustment and Inoculation of the Substrate

The volume of water required to adjust the moisture content of sterile grain to that required was calculated as follows:

$$\text{volume of water required (cc) for 1kg grain} = \frac{(M2 - M1) \times 1000}{(100 - M2)}$$

where M1 = moisture content before conditioning
M2 = required moisture content

The volume of water to be added was adjusted to account for the volume of liquid present in the inoculum used for adding microorganisms to the grain. The inoculum volume was subtracted from the volume calculated in the equation above.

The moisture content of the irradiated grain prior to conditioning was established using a water activity meter (Aqualab CX2, Labcell Ltd, Hampshire) and converted to equivalent moisture content as described by Henderson (1987) and Ayerst (1965). The volume of water calculated above was added to the grain, followed by the inoculum (18ml spore suspension was used to inoculate 1kg of grain). The grain was shaken for 10-20 minutes and again after 1 hour. It was then left at 22°C for 24 hours to absorb the moisture. This resulted in the grain being conditioned to the required moisture content. This was checked using a water activity meter.

1.3.3.5 Odour Assessment of Grain

Wheat (25g) was weighed into a boiling tube and sealed with a Dreschel head connected at one end to a supply of ECD grade nitrogen and at the other end to a 0.45µm filter and subsequently to a glass sniffing funnel. All transfer lines were PTFE. The sample tube was submerged in a water bath and allowed to equilibrate to 50°C for 30 minutes. A nitrogen purge was applied for 20s and each odour assessor assessed the odour eluting from the funnel.

On each sampling occasion, a known control sample of grain was included to provide a reference point for assessors. For each unknown sample, assessors were asked to state whether the odour of the unknown was the same as the control ('control') or different from the control ('not control'). If the sample was assigned as 'not control', then the assessor was asked which of the following descriptors best described the odour - musty, sour, fishy or green. If none of these terms best described the odour then the assessor was asked to describe the odour. At the end of the session the panel discussed such samples in an attempt to reach a consensus opinion. If panel members could not reach agreement, then 'no consensus' was recorded.

1.3.3.6 Monitoring of Cultures Following Inoculation

Following inoculation of the grain, an initial 40g sample of grain was analysed. A 1 in 10 dilution using MRD was prepared, shaken and left to stand for 1 hour. Using the pour plate technique, a dilution series was prepared using MRD. Pour plates (1ml) were prepared using Rose Bengal Chloramphenicol Agar (RBCA, Oxoid CM549, SR078E). For *Wallemia sebi*, Dichloran 18% Glycerol Agar (DG18, Oxoid CM729) was used. The plates were allowed to set, inverted and incubated at 25°C ± 1°C for 5 days. Following incubation, the plates were examined for typical mould colonies and microscopic examination was performed to confirm species.

Subsequently, at suitable intervals during incubation, grain was removed under aseptic conditions to determine the water activity of the grain and the number of cfu/g (20g), and for odour assessment (50g).

1.3.4 Use of Laboratory Culturing System to Establish Conditions Required to Induce Spoilage

The approaches described above were used to determine the moisture content and incubation temperature required to induce spoilage odours that were strong enough to describe reliably and within a time scale suitable for the needs of the project. In a series of trials, irradiated wheat (Hereward) (1-3kg) was conditioned to moisture contents ranging from 16% to 25%, inoculated with each of 10 selected micro-organisms and held at 22°C to determine the conditions and incubation time required to allow spoilage to proceed and generate spoilage odours.

1.4 RESULTS AND DISCUSSION

1.4.1 Development of an Odour Assessment Method

Smelling grain to assess odours carries potential hazards of dust inhalation and the dust particles carrying mycotoxins, especially when handling spoiled grain. A method for preventing inhalation of dust whilst enabling odours to be assessed was devised and risk assessed. The most effective approach for stripping odours from the wheat was to purge with gas between the grains. This was achieved by placing whole wheat grains (50g) in a boiling tube stoppered with a Dreschel head with the inlet tube reaching between the grains to the bottom of the boiling tube (Figure 1). Purging between the grains in this way gave better results than passing the nitrogen stream over the surface of grain in a round bottomed flask. The outlet tube passed to a 0.45µm filter to retain dust particles and onto a glass funnel suitable for enclosing the nose of odour assessors. All connecting tubing was PTFE to prevent absorption of volatiles. The boiling tube was submerged in a water bath at 50°C, ensuring that all of the grain was below water level. This was allowed to

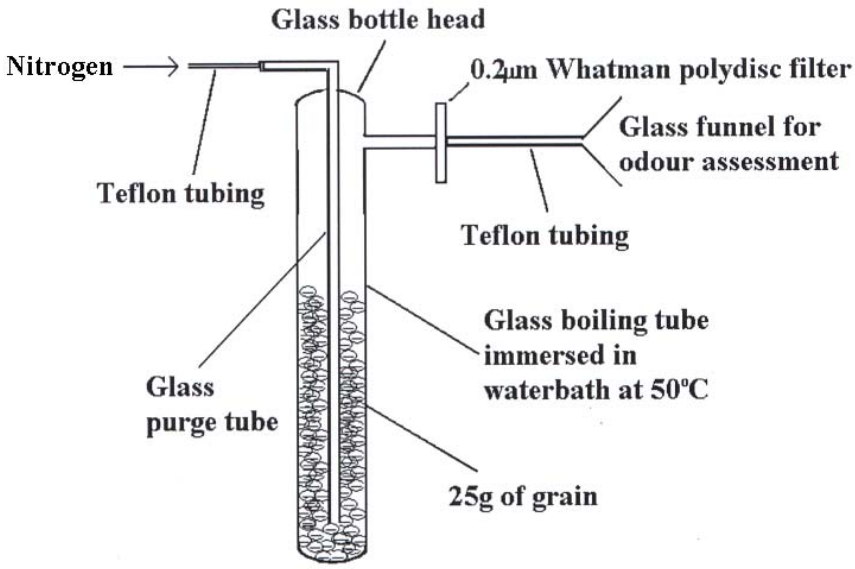


Figure 1: Apparatus for Odour Assessment of Grain

temperature equilibrate for 30 minutes. The odours from the grain were released by purging with nitrogen at 1L/min. After 1 minute of purging, individual assessors placed their nose close to the glass funnel to assess the odours released.

1.4.2 Selection of Organisms to Use to Induce Spoilage Odours in Grain

1.4.2.1 Spoiled Samples from Industry

The spoiled grain received from industry was used to agree terms to use to describe odours and train the informal odour assessment panel. The spoilage odours could generally be classified as sour, musty, fishy or 'green'. During the project, 58 samples of spoiled grain were received from the industry; of these, 50 were suitable for odour assignment with the following numbers falling into each category: 8 sour, 17 musty, 5 fishy and 9 green; there were 11 where no odour was detected. There were variations in the odours of samples within a category, for example musty included variations upon the descriptor such as 'damp mustiness', 'earthy' 'mushroom' and 'mouldy', and fishy included 'ammonia'.

The odour assignment of some samples changed over time – some samples initially assigned as green that were re-assessed after holding for some months were later assigned as sour and others initially assigned as green were later considered to be musty. As the spoilage process proceeds, different organisms may

predominate or different odour volatiles may be generated, suggesting the need for volatile indicators of spoilage to be generic in nature.

Nineteen of the spoiled samples received from industry were used to identify the microorganisms present. The microorganisms present and the spoilage odour of the grain from which they were isolated are shown in Table 1.

Of the micro-organisms isolated from spoiled grain, *B. licheniformis* was common to all spoilage types, including control grain. This may be because this organism grows at higher water activity than most. Most organisms were associated with more than one type of spoilage odour but none of the moulds isolated were common to all spoiled sample types. Nine different moulds were isolated. The literature indicates that six of these have been associated with mycotoxin formation.

This approach for identifying organisms responsible for spoilage was considered to be limited as the presence of an organism on grain does not necessarily mean that it is the cause of spoilage. To identify correlations between spoilage odours and the presence of spoilage organisms would have required large numbers of spoiled samples. Therefore in addition to this work, the literature was also used to identify suitable organisms for generating spoilage odours in laboratory based culturing systems.

1.4.2.2 Literature Review

Information from a literature review relating spoilage odours and specific microorganisms (Salmon *et al*., 2002) was used to provide further information for selection of the micro-organisms to be used to inoculate grain to induce spoilage (Table 2).

On the basis of organisms isolated from the industry samples, information from the literature and a desire to represent a wide range of mould genera, ten microorganisms were selected for inoculation studies:

- *Acremonium (Cephalosporium) strictum*
- *Alternaria alternata*
- *Aspergillus flavus*
- *Fusarium culmorum*
- *Mucor* sp.
- *Penicillium aurantiogriseum*
- *Penicillium vulpinum*
- *Rhizopus* sp.
- *Streptomyces griseus*
- *Wallemia sebi*

Table 1: Microorganisms Present on Control and Spoiled Grain and the Associated Spoilage Odour

Micro-organism	Control	Musty	Musty/Sour	Musty/Fishy	Mouldy	Green/Silage	Green/Sour	Sour	Fishy
Bacteria									
Pseudomonas fluorescens						1	1	1	1
Bacillus licheniformis	1	1	1	1	1	1	1	1	1
Brevibacillus brevis	1	1	1			1			1
Strep. xylosus				1					
Pantoea spp.	1	1	1		1	1	1	1	1
Bacillus cereus								1	
Bacillus pumilus							1		
Enterobacter cloacae									1
Yeasts									
Pichia ohmeri									1
Pichia spp.	1								
Rhodotorula spp.	1								1
Moulds									
Fusarium *spp*.		1							
Aspergillus spp.	1	1	1	1	1			1	1
Penicillium spp.	1	1	1			1	1		1
Cladosporium		1	1			1			1
Mucor spp.				1	1	1	1		
Rhizopus spp.				1	1	1	1		
"White" (fluffy)								1	
"White" (not fluffy)	1	1	1	1	1				
Wallemia sebi									

Table 2: Odours Associated with Specific Organisms from Literature

Organism	Odour	Reference
Asp. flavus	Musty, putrid	Kaminski *et al.* (1985)
Asp. niger	Sulphurous, musty, mouldy	Börjesson *et al.* (1992)
P. aethiopicum	Earthy, red beet, musty	Börjesson *et al.* (1992)
P. aurantiogriseum	Musty, mushroom, mouldy	Börjesson *et al.* (1990)
P. roquefortii	Mushroom, musty paper	Harris *et al.* (1986)
P. vulpinum	Wet concrete, plastic, mouldy	Börjesson *et al.* (1992)
Rhizopus sp.	Putrid	Kaminski *et al.* (1985)
Strep. griseus	Musty, earthy	Abo Gnah & Harris (1985)
Strep. odorifer	Musty, earthy	Abo Gnah & Harris (1985)
Tilletia caries	Fishy (common bunt)	Hanna (1932)

P. – *Penicillium;* Asp. – *Aspergillus;* Strep. – *Streptomyces*

1.4.3 Development of a Laboratory Culturing System for Generating Spoiled Grain

Initially the use of 2% wheat agar extracts was investigated. The agar was assessed for odour *per se* and organisms were inoculated onto the agar for growth and assessment of odours. From this perspective, 2% wheat agar provided a suitable substrate. However, whole wheat grains were considered to be more representative of the substrate that moulds would encounter and interact with, therefore a means of generating sterile grain, inoculating it with the selected organisms and generating growth and development of spoilage odours was required. Methods for sterilising grain were investigated (see Paper 2). These included hypochlorite and peroxide washes and irradiation. Hypochlorite was ruled out as it has the potential to produce taints, and washes do not penetrate the crease of the grain where the highest numbers of organisms are likely to be located. Irradiation of grain at 12kGy was most effective and subsequently used throughout the project.

The aim of the laboratory culturing system was to determine conditions under which spoilage would be induced consistently over a predictable time course. Ideally the time course was required to consist of a period in which no spoilage odours were detected by human senses (early warning period), subsequently leading to detectable, characteristic and sustainable spoilage odours.

Grain for storage is considered to be at risk of spoilage until dried to a moisture content of ≤14.5% (HGCA, 1999). Therefore the moisture content of grain for the first trial of the laboratory culturing systems was conditioned to 16% which is the lower end of the moisture content required for growth of many of the microorganisms selected for inoculation (Salmon *et al*., 2002). Following inoculation of *Streptomyces griseus, Mucor spp.* and *Rhizopus spp.*, only *Streptomyces griseus* appeared to show an increase in numbers (Table 3). Spoilage odours that differed from the control were detected after 5 days but changed in nature on subsequent sampling occasions (Table 3). This was considered to be because the odours were weak in nature and difficult to assign with confidence. No increase in numbers was detected in the irradiated control; however, the odour did differ from that of the reference grain, suggesting that adjustment of moisture content may also affect other characteristics of the grain, e.g. enzymic or chemically induced odour changes.

Table 3: Development of Laboratory Based Culturing Systems – Effect of Inoculation of Irradiated Wheat Conditioned at 16% Moisture with *Streptomyces griseus, Mucor sp.* and *Rhizopus sp.*

	Day	Mould			Irradiated Control
		Streptomyces griseus	*Mucor sp.*	*Rhizopus sp.*	
cfu/g	0	5.7E+05	8.9E+05	1.0E+06	<5
Odour	1	Not control	Not control	Not control	Sour
cfu/g	3	1.7E+07	8.4E+04	1.9E+06	<5
Moisture	3	16%	16%	16%	
Odour	5	Green	Sour/control	Sour	Not control
Odour	7	Fishy	Not control	Not control	Not control
cfu/g	7				<5
Odour	9	Musty/green	Sour/control	Sour/control	Sour/control

Batch of irradiated grain: CAM010 0053283; volume of grain inoculated: 3kg; container: 10L Nalgene pot; weight to volume ratio: 1:3.3; volume of inoculum: 19ml (control 19ml MRD); storage temperature: 25°C

The moisture content of the grain was increased slightly in an attempt to enhance spoilage. In trials using grain conditioned to 17% moisture inoculated with a wider range of organisms, increases in numbers of *Wallemia sebi* and *Acremonium strictum* took place over the first 14 days but odours did not differ from the control until 27 days (Table 4). In most cases, odours assigned as 'not control' were not strong or consistent enough to be clearly defined. Very little if any increase in numbers was detected for the organisms other

than *Wallemi sebi* and *Acremonium strictum*. Both of these organisms were inoculated at low starting levels compared to the organisms that did not show any change.

As the cfu/g did not increase and/or produce distinct, consistent spoilage odours, the moisture content of the grain for inoculation was increased further. With grain conditioned to 18% moisture content, inoculations were carried out for a range of organisms (Table 5) and some organisms were selected for inoculation in duplicate (Table 6).

At 18% moisture there was some increase in numbers and some spoilage odours were produced after 15 days. However, the odours were still not consistent between assessment days or strong enough to be assigned with confidence. This was demonstrated when some of the inoculations were repeated and replicated. The same odours were not described on the second occasion of setting up nor between replicate inoculations set up at the same time (Table 6) for selected microorganisms.

It was considered that the apparent lack of increase in numbers may be due to competition at the higher inoculum levels used (1E+06-1E+07/g), therefore the number of organisms inoculated onto grain was reduced to 1E+03-1E+04 or 1E+04-1E+05/g. This resulted in an increase in numbers of the moulds and the development of characteristic spoilage odours. However spoilage odours were still weak and varied between replicate inoculations (A and B in Table 7).

The moisture content was therefore increased further to 25%. Under these conditions the spoilage odours were more clearly detected as spoilage odours and assessors had more confidence in assigning spoilage descriptors, though the descriptors were still not necessarily consistent over time (Table 8). It is assumed that in biological systems it is feasible that the secondary metabolites responsible for the spoilage odours can vary, both in terms of level and type produced.

The procedure for the laboratory based culturing systems and cultures of the moulds were transferred to project collaborators at The University of the West of England (UWE) for them to set up and test further. This provided a test of the reproducibility of the procedures, and familiarised UWE with the spoilage odours being generated (see Paper 3 in this report). The data generated (Paper 2) confirmed that consistent spoilage odours were produced at 25% moisture. This procedure was considered to be suitable for use to characterise and identify the indicator molecules during the early onset of spoilage in wheat (Paper 3) and subsequently as part of the programme for testing the performance of developed sensors (see Paper 5).

Table 4: Development of Laboratory Based Culturing Systems – Effect of Inoculation of Irradiated Wheat Conditioned at 17% Moisture with Various Microorganisms

	Day	Mould							Irradiated Control
		Pen. aurantogriseum	*Aspergillus flavus*	*Fusarium culmorum*	*Pen vulpinum*	*Alternaria alternata*	*Wallemia sebi*	*Acremonium strictum*	
cfu/g	0	4.3E+06	6.3E+06	3.6E+06	4.9E+06	9.5E+05	2.0E+02	4.1E+02	<5
Moisture		17%	17%	17%	17%	17%	17%	17%	17%
Odour	1	Control	Not control	Control	Control	Control	Control	Control	Not control
Odour	6	Not control	Control	Control	Control	Control	Control	Control	Control
Odour	8	Not control	Sour/ control	Control	Control	Green/ control	Not control	Control	Control
Odour	12	Musty/control	Control	Control	Control	Control	Control	Control	Control
cfu/g	14	3.0E+06	3.6E+06	1.8E+06	3.2E+06	3.3E+05	1.3E+05	2.0E+06	<5
Moisture		16-17%	16-17%	16-17%	16-17%	16-17%	16-17%	16-17%	
Odour	16	Sour/ control	Control	Control	Sour/ control	Control	Control	Control	Control
Odour	27	Sour/ control	Not control	Control	Not control	Control	Not control	Not control	Control
cfu/g	30	9.0E+06	7.5E+06	4.5E+06	8.0E+06	3.4E+06	2.1E+07	1.4E+07	<5
Moisture		17%	17%	17%	17%	17%	17%	17%	
Odour	34						Sour	Control	Control

Batch of irradiated grain: CAM010 0053283; volume of grain inoculated: 1kg; container: 4l Nalgene pot; weight to volume ratio: 1:4w/v; volume of inoculum: 18ml; control: 18ml MRD

Table 5: Development of Laboratory Based Culturing Systems – Effect of Inoculation of Irradiated Wheat Conditioned at 18% Moisture with Various Microorganisms

	Day	Mould									Irradiated Control
		Asp. niger	*Asp. flavus*	*Asp. glaucus* Group	*Alternaria alternata*	*Cladosporium herborum*	*Fusarium culmorum*	*Pen. vulpinum*	*Pen. aurantio-griseum*	*Strep. griseus*	
cfu/g	0	1.4E+06	4.0E+05	1.3E+06	1.5E+06	1.6E+06	2.3E+05	3.6E+05	1.5E+06	1.1E+05	<5
Moisture	1	18%	18%	18%	18%	18%	18%	18%	18%	17%	18%
Odour	1	Control	Control	Control	Control	Control	Control	Control	Control	Control	Not control
Odour	6	Control	Control	Not control	Control	Sour/control	Control	Sour/ control	Control	Control	Control
cfu/g	8	4.7E+08	3.5E+08	3.3E+08	2.1E+08	3.0+08	1.3E+07	6.1E+07	1.9E+08	2.9E+07	<5
Moisture	8	18%	18%	18%	18%	18%	18%	18%	18%	18%	18%
Odour	8	Sour/ control	Control	Control	Musty/ control	Control	Control	Fishy	Not control	Control	Control
Odour	13	Control	Sour/ control	Control	Control	Control	Control	Not control	Control	Control	
Odour	15	Not control	Sour/ control	Control	Control	Sour/ control	Musty	Control	Musty	Not control	Control
Odour	20	Not control	Sour	-	Control	Sour/control	Musty/sour	Not control	Musty	Not control	Control
cfu/g	30										<5

Batch of irradiated grain: FIII316 11/4/99; volume of grain inoculated: 0.5kg; container: 10L Nalgene pot; weight to volume ratio:1:20w/v; volume of inoculum (S*trep. griseus* 2kg, 36ml): 9ml, control: 9ml MRD

Table 6: Development of Laboratory Based Culturing Systems – Duplicate Inoculation of Irradiated Wheat Conditioned at 18% Moisture with *Penicillium vulpinum*, *Aspergillus niger* and *Aspergillus flavus*

	Day	Control	Mould					
			Pen. vulpinum		*Asp. niger*		*Asp. flavus*	
			A	B	A	B	A	B
cfu/g	0	<5	7.3E+05	4.8E+05	1.2E+06	1.2E+06	1.0E+06	1.2E+06
Moisture		18%	18%	18%	18%	18%	18%	18%
cfu/g	1	<5	1.0E+06	1.2E+06	1.4E+06	1.3E+06	1.2E+06	1.3E+06
Moisture		18%	18%	18%	18%	18%	18%	18%
Odour		N/A	Slightly musty/sour	N/A	N/A	N/A	Slightly not control	N/A
cfu/g	4	<5	1.0E+06	6.3E+05	1.4E+06	1.4E+06	1.3E+06	1.4E+06
Moisture		18%	18%	18%	18%	18%	18%	18%
Odour		Control	Not control	Control	No consensus	Control	‘Ovaltine’	Control
cfu/g	6	<5	7.2E+06	9.0E+06	1.1E+07	9.8E+06	9.0E+06	9.5E+06
Moisture		18%	18%	18%	18%	18%	18%	18%
cfu/g	8	<5	1.3E+07	1.3E+07	1.5E+07	1.4E+07	1.4E+07	1.4E+07
Moisture		18%	18%	18%	18%	18%	18%	18%
Odour		N/A	Control	Sour	N/A	Control	Control	Control
Odour		Control	No consensus	Slightly sour	Control	No consensus	Control	Control

Table 6: Development of Laboratory Based Culturing Systems – Duplicate Inoculation of Irradiated Wheat Conditioned at 18% Moisture with *Penicillium vulpinum*, *Aspergillus niger* and *Aspergillus flavus* (Continued)

	Day	Control	Mould					
			Pen. vulpinum		*Asp. niger*		*Asp. flavus*	
			A	B	A	B	A	B
cfu/g	12	5	2.0E+05	1.8E+05	2.2E+05	2.4E+05	1.5E+05	1.8E+05
Moisture		18%	18%	18%	18%	18%	18%	18%
Odour		Control	No consensus	Control	Control	Control	Control	Control
cfu/g	15	15	4.2E+04	2.9E+04	9.6E+04	6.5E+04	8.2E+04	6.2E+04
Moisture		18%	18%	18%	18%	18%	18%	18%
Odour		Control	Control	Not control	No consensus	Control	Control	No consensus
cfu/g	19	10	1.4E+05	1.6E+05	1.6E+05	1.7E+05	1.4E+05	1.7E+05

( ) no. of assessors

Volume of grain inoculated: 1kg; container: 2l Duran bottle; weight to volume ratio: 1:2w/v; volume of inoculum: 18ml

Table 7: Development of Laboratory Based Culturing Systems – Reduced Levels of Inoculum and Duplication of Inoculation of Irradiated Wheat Conditioned at 18% Moisture with *Fusarium culmorum, Aspergillus aurantiogriseum* and *Streptomyces griseus*

	Day	Mould								
		Fusarium culmorum		*Penicillium aurantiogriseum*		*Streptomyces griseus*		*Streptomyces griseus*		Irradiated Control
		A	B	A	B	A	B	A	B	
cfu/g	0	5E+03	4.35E+03	4.5E+05	5.8E+05	1.55E+04	1.6E+04	2.3E+04	2.8E+04	<5
Moisture		18%	18%	18%	18%	18%	18%	18%	18%	18%
Odour		Control	Control	Control	Control	Control	Control	Control	Control	Control
cfu/g	5	6.8E+04	2.5E+04	6.9E+06	7.3E+06	9.9E+04	1.3E+05	9.8E+04	1.1E+05	<5
Odour		Control	Control	Control	Control	Control	Sour	Musty	Musty	Green
cfu/g	7	8.1E+04	7.3E+04	8.7E+07	9.2E+06	1.2E+05	1.5E+05	1.1E+05	1.3E+05	<5
Odour		Musty	Musty/ green/sour	Sour	Musty	Musty/ green/sour	Musty/ green/sour	Control	Musty	Sour
cfu/g	21	2.1E+07	1.8E+07	4.8E+07	5.1E+07	2.3E+07	2.4E+07	2.2E+07	2.8E+07	<5
Odour		Musty	Sour	Musty/green	Sour/green	Green	Sour	Sour	Control	Control
cfu/g	27	5E+07	4.5E+07	8.2E+07	8.5E+07	5.9E+07	6.4E+07	6.8E+07	7.2E+07	<5

Batch of irradiated grain: FIII316 11/4/99; volume of grain inoculated: 1kg; container: 4L Nalgene pot; weight to volume ratio: 1:4w/v; volume of inoculum (*Strep. griseus* 0.5kg, 9ml): 18ml

Table 8: Development of Laboratory Based Culturing Systems – Reduced Levels of Inoculum and Inoculation of Irradiated Wheat Conditioned at 25% Moisture with *Penicillium vulpinum, Aspergillus niger*, *Penicillium aurantiogriseum*, *Alternaria alternata*, *Fusarium culmorum Aspergillus flavus* and *Aspergillus niger*

	Day	Control	Mould					
			Pen. vulpinum	*Fusarium culmorum*	*Alternaria alternata*	*Pen. aurantiogriseum*	*Asp. flavus*	*Asp. niger*
cfu/g	0	<5	1.0E+03	1.13E+03	8.2E+02	7.8E+02	9.5E+02	1.2E+03
cfu/g	1	<5	1.1E+03	1.4E+03	9.9E+02	8.9E+02	1.0E+03	1.33E+03
Moisture		25%	25%	25%	25%	25%	25%	25%
cfu/g	4	<5	1.35E+04	5.8E+05	3.8E+03	2.3E+04	4.8E+05	1.45E+04
Moisture		25%	25%	25%	25%	25%	25%	25%
Odour	6	Control	Control	Not control	Control	Musty	Sweet biscuit	Control
cfu/g	7	<5	3.7E+06	3.3E+07	4.3E+05	1.65E+06	3.5E+07	2.0E+06
Moisture		25%	25%	25%	25%	25%	25%	25%
Odour	12	Musty	No consensus	No consensus	Musty	Musty	No consensus	No consensus
cfu/g	13	<5	1E+08	3.4E+08	1.8E+07	9E+06	2.9E+08	2.7E+07
Moisture		25%	25%	25%	25%	25%	25%	25%
cfu/g	18	<5	6.1E+08	9.2E+08	2.9E+08	3.3E+07	9.2E+08	2.3E+08
Moisture		25%	25%	25%	25%	25%	25%	25%
Odour	19	Control	Musty	Not control	No consensus	Musty	Not control	Fishy

Volume of grain inoculated: 1kg; container: 5L Duran bottle; weight to volume ratio: 1:5; volume of inoculum: 18ml

1.5 REFERENCES

Abo Gnah, Y.S. and Harris, N.D. (1985) Journal of Food Science **50** 132-135.

Ayerst, G. (1965) Journal of the Science of Food and Agriculture **16** (2), 71-78.

Börjesson, T, Stollman, U., Schnurer, J (1990) Applied and Environmental Microbiology **56** 3705-3710.

Börjesson, T., Stollman, U. and Schnurer, J. (1992) Applied and Environmental Microbiology **58** (8), 2599-2605.

Harris, N.D., Karahadian, C. and Lindsay, R.C. (1986) Journal of Food Protection **49** (12), 964-970.

HGCA (1999) Home-Grown Cereals Authority, Caledonia House, 223 Pentonville Road, London N1 9HY.

Hanna, W.F. (1932) Phytopathology **22**, 978-979.

Henderson, S. (1987) Journal of Stored Products Research **3** (3), 143-147.

Kaminski, E., Przybylski, R. and Wasowicz, E. (1985) Journal of Cereal Science **3**, 165-172.

Salmon, S.E., Voysey, P.A., Williams, J.S. and Brown, H.M. (2002) CCFRA Review 35, Campden & Chorleywood Food Research Association, Chipping Campden, Glos.

PAPER 2

EFFECT OF SURFACE STERILISATION, IRRADIATION AND MALODOURS ON THE VIABILITY AND MICROFLORA OF WHEAT GRAINS, AND METHODS FOR REPRODUCING MALODOURS BY INOCULATION *IN VITRO*

H. E. Gunson, P.T.N. Spencer-Phillips and N.M. Ratcliffe

Faculty of Applied Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY

2.1 ABSTRACT

Malodours in 25 commercial wheat grain samples collected in the UK were related to grain viability and causal fungi. Attempts to replicate malodours in the laboratory by inoculating sound grain with specific microorganisms are also reported. Five control samples with no odour varied significantly in their ability to germinate: three showed <25% and two >96% germination. It is likely that samples with poor germination were killed by excessive heat used to dry the grain after harvest, and will be more liable to colonisation by storage fungi. Of the other samples, those with musty/mouldy odours tended to have the lowest viability (n = 11, mean 16.6% germination), followed by the sour (n = 3, mean 76.7%), green (n = 3, mean 85.7%) and fishy (n = 3, mean 91.7%) samples. Environmental scanning electron microscopy showed that the fishy samples were contaminated by teliospores of the pathogen *Tilletia caries*, whilst the musty samples showed sporophores of *Aspergillus* and *Penicillium* species. These also developed on sound grain of cv. Hereward with 18% moisture content and incubated at 22°C, as did the field fungus *Fusarium culmorum* and other microorganisms. Attempts to reproduce musty odours by inoculating control grain with *F. culmorum, Streptomyces griseus* and *Penicillium aurantiogriseum* produced inconsistent results. This was explained by the diversity of microbial growth arising from the natural microflora in addition to the introduced organism. All surface sterilisation regimes failed to eliminate microorganisms associated with the grain. However, inoculation of irradiated grain at 25% moisture content with *P. aurantiogriseum* and incubation at 22°C consistently produced musty odours in all replicates. The time-course of colonisation and odour development is described, with infection of the embryo giving the characteristic "blue eye" symptoms and preceding further colonisation. The intensity of musty odours peaked between 15 and 22 days, before declining at 29 days. Consistent infections and odours were also produced when irradiated grain at 25% moisture content was inoculated with *Asp. flavus*, *Asp. niger, F. culmorum, P. vulpinum, P. verrucosum* and *S. griseus*.

2.2 INTRODUCTION

Heads of grain have a diverse microflora, with more than 150 species of fungi. Most are field fungi, such as species of *Alternaria, Cladosporium* and *Fusarium*. Storage fungi, including *Aspergillus* and *Penicillium* spp., are also widespread although not dominant on plant surfaces in the field, but may develop to dominate the ecosystem of stored grains if environmental conditions are favourable. Key factors are temperature and moisture content, with post-harvest spoilage likely to occur if the latter is above 14%. Consequences of spoilage include reduced germination, caking, heating (leading to spontaneous combustion, in exceptional cases) and discoloration, with the most serious outcome being grain rendered unfit for consumption due to mycotoxins and malodours. Losses are compounded because odours may not be detectable by the human nose before the final processed product. Present methods for detecting fungal spoilage are simply visual inspection and odour assessment with the human nose. The latter has serious health implications, and the method is subject to the limits of human ability. Therefore, a more reliable and sensitive method is needed for early detection of the growth of spoilage organisms and the odours produced.

The development of sensors specific for volatile organic compounds (VOCs) that comprise the characteristic odours produced by spoilage organisms, and their incorporation into a prototype electronic nose, is described elsewhere. Development of the sensors first required a method by which grain could be infected by specific spoilage organisms. This would enable the characteristic VOCs to be identified by GC-MS, and then rigorous testing of the selected sensors against grain samples with malodours that could be replicated reliably in the laboratory.

The present paper reports experiments to select a sterilisation method to ensure that grain became infected by the introduced organism and the effect of the selected method on grain viability and development of malodours, in comparison with 25 commercial wheat grain samples collected in the UK.

2.3 MATERIALS AND METHODS

2.3.1 Surface Sterilisation of Wheat Grains

A range of treatments was assessed for surface sterilising of grains of *Triticum aestivum* cv. Hereward. The methods used the combinations of Tween 80, sodium hypochlorite, ethanol and Plant Preservative Mixture (Plant Cell Technology Inc., USA) listed in Table 1. Approximately 100 wheat grains (4.4 g) were exposed to each of the 14 different treatments, and then three replicate batches of five grains from each sample were plated onto Potato Dextrose Agar (PDA). Microbial contamination was assessed visually after incubation of the plates in the dark for 7 days at 19°C.

Table 1: Methods of Surface Sterilisation of *Triticum aestivum* cv. Hereward Grains

	Pre-treatment	Main Treatment	Post-treatment
1.	None	None	None
2.	None	3 x SDW wash	None
3.	None	6% NaOCl (10 min)	3 x SDW wash
4.	None	6% NaOCl + Tween 80 (10 min)	3 x SDW wash
5.	None	70% EtOH dip/5% NaOCl (10 min)	3 x SDW wash
6.	None	70% EtOH dip/5% NaOCl + Tween 80 (10 min)	3 x SDW wash
7.	None	0.5 % Plant Preservative Mixture (1 hour)	3 x SDW wash
8.	SDW + Tween 80 (1 hour)	6% NaOCl (10 min)	3 x SDW wash
9.	SDW + Tween 80 (2 hour)	6% NaOCl (10 min)	3 x SDW wash
10.	70% EtOH dip SDW + Tween 80 (1 hour)	6% NaOCl (10 min)	3 x SDW wash
11.	70% EtOH dip SDW + Tween 80 (2 hour)	6% NaOCl (10 min)	3 x SDW wash
12.	SDW + Tween 80 (1 hour)	70% EtOH dip/6% NaOCl (10 min)	3 x SDW wash
13.	SDW + Tween 80 (2 hour)	70% EtOH dip/6% NaOCl (10 min)	3 x SDW wash
14.	SDW + Tween 80 (2 hour)	None	None

SDW = sterile distilled water; NaOCl = sodium hypochlorite; EtOH = ethanol; plant preservative mixture supplied by Plant Cell Technology Inc., USA; Tween 80 at 0.1% v/v.

2.3.2 Grain Germination Test for Effect of Irradiation and Odour

Twenty-five commercial samples of wheat grains collected in the UK from a variety of unknown cultivars, and grains of cv. Hereward that had either been treated with a 12kGy dose of irradiation or were untreated, were supplied by CCFRA. Three replicates of 100 grains were prepared to assess the germination rate of each sample. One hundred grains were laid out in a grid pattern on a damp paper towel, before being covered by another towel and then rolled up. The rolls of paper towel were placed in a penicillin assay dish, covered with the lid and incubated in the dark at 19°C for 10 days. The experiment with irradiated grain was repeated twice.

2.3.3 Inoculation of Non-sterile Grain (cv. Consort) with Spoilage Fungi

Seed quality grain of wheat cv. Consort was obtained from the Plant Breeding Institute, Cambridge. It had not received any chemical seed treatments and had a 92% germination rate. The moisture content was 15%, as determined with a Protimeter Digital Grainmaster, and was conditioned following the CCFRA protocol to give 18% moisture before inoculation.

The grain was inoculated following a protocol provided by CCFRA, with the following modifications. The amount of grain inoculated was either 250g or 500g, and was incubated in 500ml or 1000ml glass Duran media bottles respectively at 22°C in the dark. *S. griseus* and *F. culmorum* were cultured on V8 agar (Pitt and Hocking, 1997) at 22°C for 5-10 days and the spores harvested in maximum recovery diluent (MRD), then adjusted to the required density. Inoculated grain samples were incubated at 22°C in the dark, and odours were assessed at various intervals.

2.3.4 Inoculation of Untreated and Irradiated Grain (cv. Hereward) with Spoilage Fungi

Wheat cv. Hereward was used for experiments with irradiated grain. The moisture content before conditioning was 12% and 13% for the non-irradiated and irradiated grain, respectively. The grain was conditioned as before to give 18% moisture for the non-irradiated grain and 25% for the irradiated grain.

The grain was inoculated as before using batches of 500g incubated in the 1000ml glass media bottles. *Asp. flavus*, *Asp. niger, F. culmorum, P. aurantiogriseum, P. vulpinum, P. verrucosum* and *S. griseus* were cultured as previously, with spores harvested in either MRD or SDW, depending on which was used for suspending spores to inoculate the grain.

2.3.5 Assessment of Odour of Inoculated Grain

A protocol developed by CCFRA (Paper 1) was followed, except that compressed air was used instead of nitrogen. A boiling tube (20cm length, Quickfit No: MF24/3/8) containing the wheat sample was fitted with a bottle head for a Dreschel assembly (Quickfit No: MF27/3/13), connected to a filter (Whatman Polydisc, 0.2μm pore size) and then a glass funnel by Teflon tubing. Due to problems getting the glass purge tube into the test tube when it contained the 50g of grain specified in the CCFRA protocol, a 25g sample of wheat grains was used instead. A sensory panel of four assessors was employed to describe the odours emanating from the glass funnel as the compressed air was passed through the system.

2.3.6 Environmental Scanning Electron Microscopy

Wheat grains were examined using a Philips XL30 environmental scanning electron microscope (ESEM). They were mounted directly with no pre-treatment on 9mm diameter self-adhesive carbon discs (Agar Scientific Ltd, Cambridge) attached to stainless steel stubs before being placed on the Peltier stage of the ESEM at 5°C. The grain surface was examined at an accelerating voltage of 150kV and a vapour pressure of between 6.8 and 7.4 Torr.

2.4 RESULTS

2.4.1 Effectiveness of Surface Sterilisation Treatments

All plates showed microbial growth from all grains following the treatments listed in Table 1, showing that they remained colonised by viable microorganisms. Thus none of the surface sterilisation methods were effective. It was noted, however, that contamination was slower to appear in treated samples than in the control samples (1, 2 and 14 in Table 1). Contamination was more apparent as mycelial fungal colonies in treated samples than as either yeasts or bacteria; in contrast, colonies arising from control grains were a mixture of mycelial and unicellular fungi, as well as bacteria.

2.4.2 Effect of Irradiation and Odour: Grain Germination and Microbial Contamination

Normal germination was considered to have occurred when the grain produced a seedling with an intact shoot and at least two main roots.

In the experiment to assess the effect of irradiation on grain viability, non-irradiated control samples showed a mean germination of 86 and 94% in the two experiments (Table 2). No grains germinated normally with the irradiated samples, and where germination did occur either the root and shoots stopped growing after they were about 1cm in length, or the grain only produced a root or a shoot but not both. This was termed irregular germination.

Irradiated grains plated onto PDA showed no microbial growth after 7 days incubation.

Grain samples with distinctive odours (Table 3) varied in their ability to germinate according to the type of odour. Control grain, with a normal “wheat” odour, produced a shoot greater than 2.5cm long and roots

Table 2: Germination of Wheat (cv. Hereward) Grains

(Grains either had been irradiated with a 12kGy dose or were untreated (non-irradiated), and had been incubated in the dark at 19°C for 7-10 days. Three replicates of 100 grains were prepared for each sample, which were assessed in two separate experiments (1 and 2). Germination was considered to have occurred when the grain produced a seedling with an intact shoot and at least 2 main roots.)

Grain Sample	Germination (%)			
	1	2	3	Mean
Experiment 1				
Non-irradiated	94	98	89	94
Irradiated	0	0	0	0
Experiment 2				
Non-irradiated	92	85	81	86
Irradiated	0	0	0	0

greater than a total length of 4.5cm in 89 to 94% of the grains. Two control samples (FC/38242/001 and 025) showed a similar pattern of germination to previous results for non-irradiated control grain (Table 2). In contrast, control samples FC/38242/007, 002 and 022 had 75%, 94% and 96%, respectively, of their grains not germinating at all, with only 007 and 002 showing very low levels of normal germination.

Seed with a musty or mouldy component to the odour was always severely damaged in its ability to germinate, with the greatest normal germination at 21% in the musty/fishy sample 006. The grains did not germinate at all in six of the musty or mouldy samples, and close inspection showed that most were supporting the growth of discrete fungal colonies after the 10 day incubation period. Non-germinated grains of other musty samples also often had fungal colonies associated especially with the embryo end of the grain. Where germination had occurred, it often was classed as irregular. This was defined as for the irradiated grains, but where the shoots and roots were less than 2.5cm and 4.5cm long, respectively.

Grain samples with either a green or sour component to the odour generally had higher rates of germination than those with musty or mouldy odours. Some of these, and two of the three samples with a fishy odour, showed rates of germination close to the highest rate found for control grain.

Table 3: Germination of Wheat Grains from 25 Samples Collected in the UK and Ascribed an Odour by CCFRA

(Germination assessed as in table 2, but with irregular germination defined as where shoots and roots were less than 2.5cm and 4.5cm long, respectively; grain with a normal wheat odour is described as control)

Grain Sample Code	Odour	Germination (%, Mean of 3 Replicates)		
		None	Irregular	Normal
FC/38242/025	Control	3	3	94
FC/38242/001	Control	4	12	84
FC/38242/007	Control	75	11	14
FC/38242/002	Control	94	4	2
FC/38242/022	Control	96	4	0
FC/38242/003	Musty	100	0	0
FC/38242/024	Musty	35	65	0
FC/38242/035	Musty	54	38	8
FC/38242/008	Musty/mouldy	100	0	0
FC/38242/009	Mouldy	100	0	0
FC/38242/004	Musty/sour	100	0	0
FC/38242/010	Musty/sour	100	0	0
FC/38242/016	Musty/green	100	0	0
FC/38242/018	Musty/green	82	18	0
FC/38242/019	Musty/green	73	25	2
FC/38242/006	Musty/fishy	73	6	21
FC/38242/034	Sour/control	11	12	77
FC/38242/021	Sour	4	7	89
FC/38242/017	Sour	55	17	28
FC/38242/012	Green/sour	5	3	92
FC/38242/020	Green	4	10	86
FC/38242/023	Green	34	17	49
FC/38242/013	Fishy	5	3	92
FC/38242/014	Fishy	2	5	93
FC/38242/015	Fishy	18	27	55

Examination with the ESEM showed that sporophores of *Aspergillus* (Figure 1) and *Penicillium* (Figure 2) spp. were typically associated with musty/mouldy grain. All samples of grain with a fishy odour had teliospores of *Tilletia tritici* (also known as *T. caries*, the bunt or stinking smut fungus) attached to the grain surface (Figure 3).

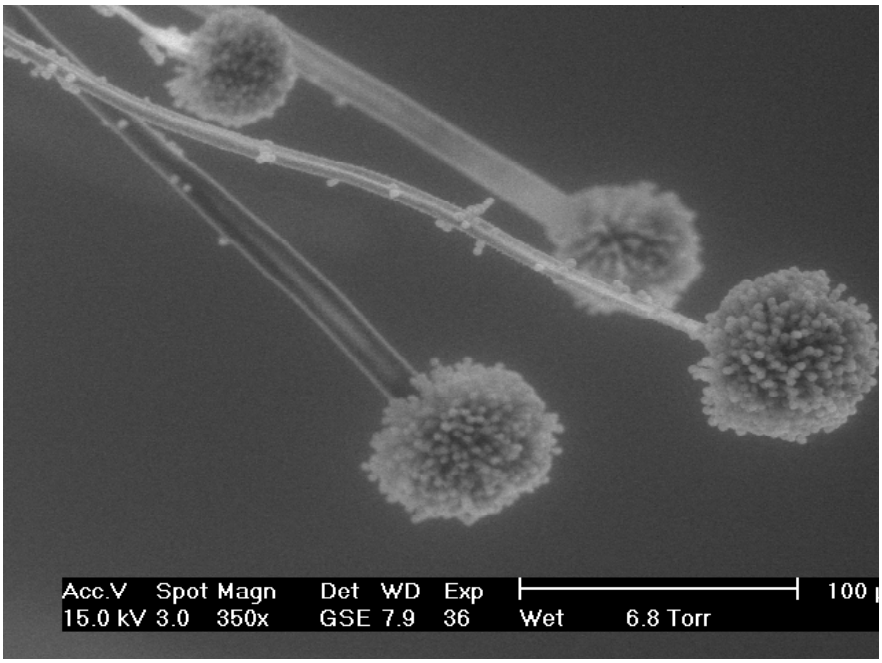


Figure 1: Sporophores of *Aspergillus* spp. Observed by Environmental Scanning Electron Microscopy on a Commercial Grain Sample with a Musty Odour

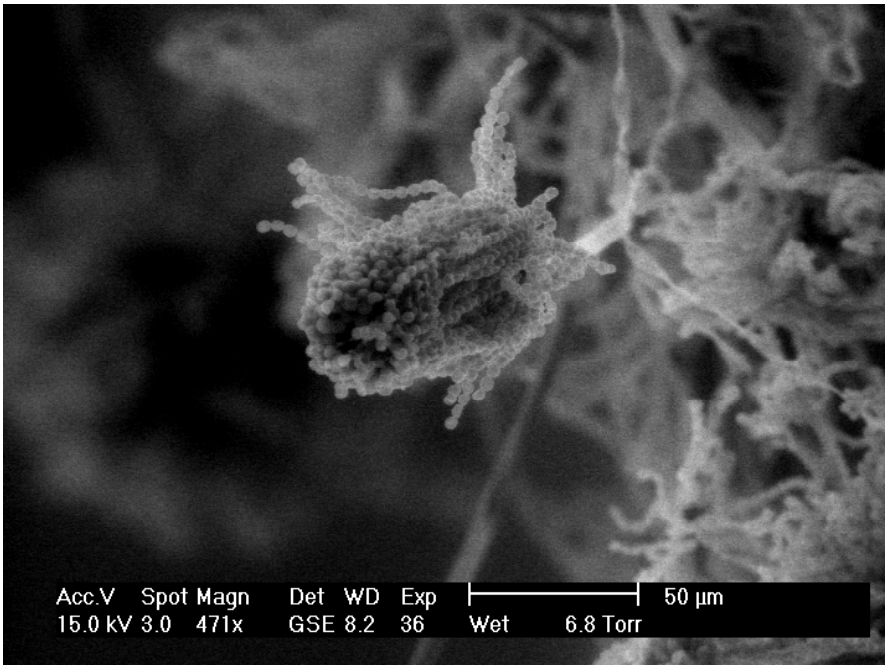


Figure 2: Sporophore of *Penicillium* spp. Observed by Environmental Scanning Electron Microscopy on a Commercial Grain Sample with a Musty Odour

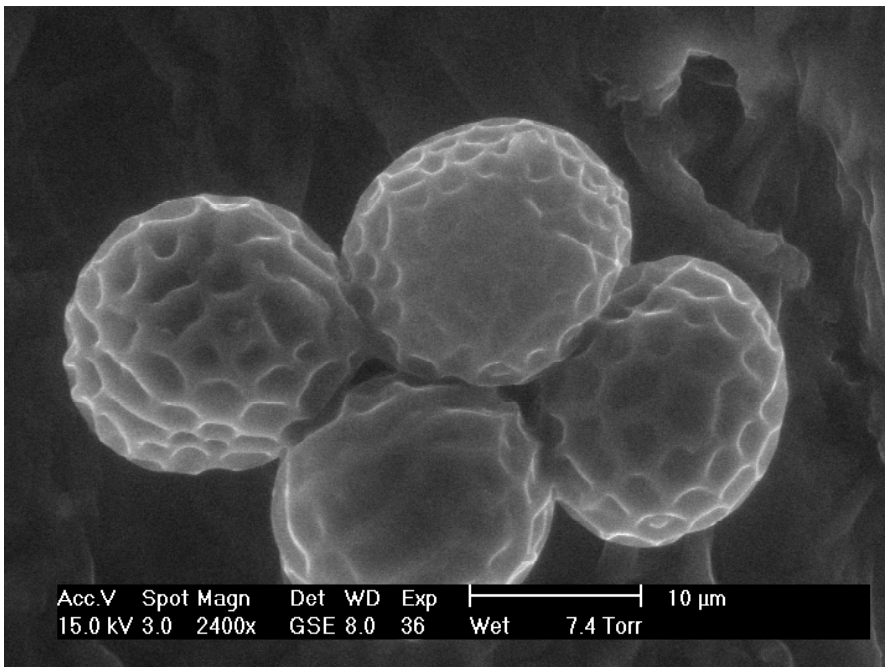


Figure 3: Teliospores of *Tilletia tritici* Observed by Environmental Scanning Electron Microscopy on a Commercial Grain Sample with a Fishy Odour

2.4.3 Inoculation of Grain with Spoilage Fungi and Development of Malodours

After 36 days following inoculation of untreated grains of cv. Consort with MRD, *F. culmorum* or *S. griseus,* musty odours were detected. However, these odours were not limited to any one particular grain treatment (Table 4). Other odours were present but not detected consistently. In a second experiment with this grain, no significant malodours were detected until 27 days after inoculation. The control wheat samples inoculated with MRD developed an obvious musty odour whilst the *F. culmorum* and *S. griseus* infected grain developed an odour described as "damp" (Table 5). Other odours were apparent, but the sensory panel did not agree consistent descriptors.

With grains of cv. Hereward, malodours were detected after 12 days in several of the non-irradiated samples (Table 6), but were not linked to a specific grain treatment. Where no consensus was reached, odours were detected at very low levels and descriptors were varied. After 36 days, all assessors agreed that malodours were present in all the grain samples. There was a consensus of opinion that there was a musty component to the odours, but no consensus on the descriptors of the other odours present. It was noted that the odours seemed to peak at 22-29 days, and decline in intensity at 36 days.

Table 4: Odour Assessment of Grains of Wheat (cv. Consort)

(Inoculated with Maximum Recovery Diluent (MRD) alone, or with MRD containing spores of *Fusarium culmorum* or *Streptomyces griseus* at a density of 6 x 10^4 spores ml^{-1} (2.7 x 10^5 spores per 250g grain), at 18% moisture content and after 36 days incubation at 22°C in the dark (3 replicates of each treatment))

Treatment		Odour
1.	MRD	Musty
2.	MRD	Musty
3.	MRD	Other [1]
4.	*F. culmorum*	Other [2]
5.	*F. culmorum*	Musty [3]
6.	*F. culmorum*	Musty [4]
7.	*S. griseus*	Other [4]
8.	*S. griseus*	Musty/other [4]
9.	*S. griseus*	Musty/damp

[1] Woody, damp grass

[2] Damp, chlorine, stale sock

[3] Stale sock, damp

[4] Damp, slightly malty

Table 5: Odour Assessment of Wheat Grains (cv. Consort)

(Inoculated as in Table 4, but at a density of 1 x 10^4 spores ml^{-1}

(9 x 10^4 spores per 500g grain) and over a 33 day period)

Treatment		Odour (Days Post-inoculation)				
		5 Days	12 Days	19 Days	27 Days	33 Days
1.	MRD	Sour	Yeasty	Wheaty	Musty [7]	Musty [1]
2.	MRD	Sour	Wheaty	Wheaty [2]	Musty [7]	Wheaty/green [9]
3.	MRD	Sour	Wheaty	Wheaty [2]	Musty [7]	Wheaty/musty/green [9]
4.	*F. culmorum*	Sour	Wheaty	Other [3]	Musty	Musty [10]
5.	*F. culmorum*	Sour	Other [1]	Wheaty [6]	Damp [8]	Other [9,10]
6.	*F. culmorum*	Sour	Other [1]	Wheaty/other [4]	Damp [8]	Other [9,10]
7.	*S. griseus*	Sour	None	Wheaty [5]	Damp [8]	Wheaty/musty/green [9,10]
8.	*S. griseus*	Sour	Wheaty	Wheaty [4]	Damp [7,8]	Musty [9,10]
9.	*S. griseus*	Sour	Wheaty	Wheaty [6]	Damp [7]	Other [8,9]

[1] Damp chlorine

[2] Dry chlorine, woody, mushroom

[3] Grassy, mushroom, dry chlorine, wet soil, wheaty, musty

[4] Chlorine, mushroom soup, amine

[5] Chlorine, mushroom soup

[6] Woody

[7] Stale sock

[8] Slight musty

[9] Damp, chlorine, metallic, dusty

[10] Fragrant, mealy

Table 6: Odour Assessment of Non-irradiated Wheat Grains (cv. Hereward)

(Inoculated as in Table 5, but including *Penicillium aurantiogriseum*)

Treatment		Odour (Days Post-inoculation)			
		12 Days	22 Days	29 Days	36 Days
1.	MRD	No consensus	Green	Musty/green	Musty
2.	MRD	Musty	Wheaty/sour	No consensus	Musty
3.	MRD	Wheaty	Wheaty	Not tested	Not tested
4.	*F. culmorum*	No consensus	No consensus	Musty	Musty
5.	*F. culmorum*	No consensus	Wheaty	No consensus	Musty
6.	*F. culmorum*	Green	Not tested	Not tested	Not tested
7.	*S. griseus*	Wheaty	Musty	Musty	Musty
8.	*S. griseus*	Wheaty/musty	Wheaty	Musty	Musty
9.	*S. griseus*	Wheaty	Not tested	Not tested	Not tested
10.	*P. aurantiogriseum*	Wheaty	No consensus	Musty	Musty
11.	*P. aurantiogriseum*	Musty	Wheaty	Musty	Musty
12.	*P. aurantiogriseum*	Musty	Not tested	Not tested	Not tested

Microscopic examination of non-irradiated grain confirmed the presence of a variety of microorganisms, including *F. culmorum*, as well as growth of the organism originally introduced as inoculum.

Irradiated grain of cv. Hereward developed musty odours in three out of four samples tested after 8 days, in all four samples after 15 and 22 days and in all eight samples after 29 days following inoculation with *P. aurantiogriseum* (Table 7). There was a very strong odour and complete consensus that it was the same for all samples. After 29 days the musty odour was still dominant, but other odours were noted although no consensus on descriptors was reached. The grain in all samples had a blue-green coloured growth visible macroscopically after 8 days, with the embryo becoming colonised preferentially giving the typical "blue eye" symptoms of infection by *P. aurantiogriseum*. No significant differences were noted between the grains inoculated with the different spore densities, nor with MRD or SDW as diluent. The MRD treated samples had clearly become contaminated at the inoculation stages.

Table 7: Odour Assessment of Irradiated Wheat Grains (cv. Hereward)

(Conditioned to 25% moisture content and inoculated as in Table 6, but with MRD alone, and spores (1 x 10^4 or 1 x 10^6 ml^{-1}) of *P. aurantiogriseum* (PA) suspended in either MRD or SDW)

Treatment		Odour (Days Post-inoculation)			
		8 Days	15 Days	22 Days	29 Days
1.	MRD	Musty	Musty	Musty	Musty
2.	MRD	Not tested	Not tested	Not tested	Musty
3.	PA 10^4/MRD	Musty	Musty	Musty	Musty
4.	PA 10^4/MRD	Not tested	Not tested	Not tested	Musty
5.	PA 10^6/MRD	Wheaty	Musty	Musty	Musty
6.	PA 10^6/MRD	Not tested	Not tested	Not tested	Musty
7.	PA 10^4/SDW	Musty	Musty	Musty	Musty
8.	PA 10^4/SDW	Not tested	Not tested	Not tested	Musty

The same inoculation technique was then applied to a range of other spoilage organisms, at two different spore densities (Tables 8 and 9). At a spore density of 5 x 10^5 spores ml^{-1} (Table 8) musty odours were apparent 2 days after inoculation for all organisms and in one of the SDW controls. The difference between the two control samples may be because sample 2 was tested blind, whilst sample 1 was used as the known control to prime the sensory panel. Sample 1 also had air passed through it for the duration of the odour assessment for the first three sampling periods, hence potentially flushing a significant amount of the detectable odour out. At the subsequent sampling times the known control was sampled at the beginning and halfway through the sampling. Both control samples developed odours although visually they appeared uncontaminated. In contrast, the inoculated grain samples showed discoloration characteristic of each organism by day 14.

There was a general trend for the odours to increase in intensity up to day 14, after which some declined in intensity. *P. vulpinum* demonstrated the most notable decrease in odour between days 14 and 28, with little difference in odour from the control by day 28. It was also noted that the odour changed during the assessment event; for example with *S. griseus* the odours detected at day 21 were initially described as earthy/musty, but after a few minutes had changed to fishy.

Table 8: Odour Assessment of Irradiated Wheat Grain at 25% Moisture (cv Hereward)

(Inoculated with *Penicillium vulpinum, Fusarium culmorum, Penicillium aurantiogriseum, Penicillium verrucosum*, *Aspergillus niger*, *Aspergillus flavus* and *Streptomyces griseus* at 5 x 10^5 spores ml^{-1}, suspended in sterile distilled water (SDW)

Treatment		Odours (Days Post-inoculation)								
		1 Day	2 Days	3 Days	5 Days	7 Days	14 Days	21 Days	28 Days	35 Days
1.	SDW	Wheaty	Wheaty	Wheaty	Wheaty/musty	Wheaty	Wheaty/other	Faint musty	Wheaty	Wheaty/fruity
2.	SDW	Wheaty	Musty	Musty	Musty	Musty	Musty	Faint musty	Wheaty/musty	Musty
3.	*P. vulpinum*	Wheaty	Wheaty	Musty	Musty	Musty	Musty	Musty	Wheaty/musty*	Musty
4.	*P. vulpinum*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Wheaty/musty*	Musty
5.	*F. culmorum*	Wheaty	Wheaty/musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
6.	*F. culmorum*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
7.	*P. aurantiogriseum*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
8.	*P. aurantiogriseum*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
9.	*P. verrucosum*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
10.	*P. verrucosum*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty/fishy	Musty
11.	*Asp. niger*	Wheaty	Wheaty/musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
12.	*Asp. niger*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
13.	*Asp. flavus*	Wheaty	Musty	Musty	Musty	Musty	Musty/fishy	Musty	Musty	Musty
14.	*Asp. flavus*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty
15.	*S. griseus*	Wheaty	Wheaty	Musty	Musty	Musty	Musty	Musty/fishy	Musty/fishy	Musty
16.	*S. griseus*	Wheaty	Musty	Musty	Musty	Musty	Musty	Musty	Musty	Musty

* Faint odour

Table 9: Odour Assessment of Irradiated Wheat Grain at 25% Moisture

(Inoculated as in Table 8, but with 1 x 10^6 spores ml^{-1})

Treatment		Odour (Days Post-inoculation)			
		7 Days	14 Days	21 Days	28 Days
1.	SDW	Wheaty	Wheaty	Wheaty	Wheaty
2.	SDW	Wheaty	Wheaty	Wheaty	Wheaty
3.	*P. vulpinum*	Musty	Musty	Musty	Not tested
4.	*P. vulpinum*	Musty	Musty	Musty	Not tested
5.	*F. culmorum*	Musty	Musty	Musty	Musty/sour
6.	*F. culmorum*	Musty	Musty	Musty	Musty
7.	*P. aurantiogriseum*	Musty	Musty	Musty	Not tested
8.	*P. aurantiogriseum*	Musty	Musty	Musty	Not tested
9.	*Asp. niger*	Musty	Musty	Musty	Musty
10.	*Asp. niger*	Musty	Musty	Musty	Musty
11.	*Asp. flavus*	Musty	Musty	Musty	Musty
12.	*Asp. flavus*	Musty	Musty	Faint musty	Faint musty
13.	*S. griseus*	Wheaty	Musty	Musty	Musty
14.	*S. griseus*	Musty	Musty	Musty	Musty
15.	*P. verrucosum*	Musty	Faint musty	Faint musty	Not tested
16.	*P. verrucosum*	Musty	Faint musty	Faint musty	Not tested

The description "musty" gives no indication of the variety of odours produced by these organisms. Whilst recognising that odour descriptors are subjective, it was possible to ascribe different characteristic odours to the different organisms following their inoculation onto irradiated wheat. All of the *Penicillium* isolates produced a very characteristic brazil nut-type odour, with *P. vulpinum* having earthy undertones and *P. verrucosum* having socky/damp undertones. *Asp. niger* developed a fragrant, earthy and nutty odour, whilst *Asp. flavus* had a more damp, socky and savoury odour. *S. griseus* produced the musty/earthy odour characteristic of *Streptomyces* spp. *F. culmorum* developed an odour which was hard to describe but had earthy, sweet and bitter type components to its mustiness.

One sample each of *Asp. flavus*, *S. griseus* and *P. verrucosum* developed fishy odours at various times (see Table 8).

All organisms produced musty odours at the greater inoculum density (10^6 spores ml^{-1}, Table 9) that were not significantly different (as detected by human nose) from those in previous experiments.

2.5 DISCUSSION

None of the surface sterilisation treatments proved effective, which suggests that contaminating microorganisms were protected from the treatments used. The observation that contamination was slower to appear in treated than in control samples supports this notion, with mycelial fungi being well adapted to colonising internal tissues. It is likely that the crease and embryo regions of the grain are areas affording special protection. Some of the microorganisms may also be endophytic (i.e. growing within the internal tissues), which is why irradiation alone was able to inactivate them.

The predominance of mycelial fungi following surface sterilisation was not due to dehydrated fungal spores being resistant to the sterilising chemicals. The pre-treatments which involved wetting the grains with Tween 80 prior to the main treatments (8, 9, 12 and 13 in Table 1) would have allowed fungal spores to hydrate and germinate and therefore be more vulnerable to the sterilising agents.

The germination experiments clearly demonstrated that the irradiated grain was severely damaged in its ability to germinate. Although many grains germinated with both shoot and root initiation occurring, seedlings did not develop any further than this stage, with none developing to the same extent as non-irradiated control grains. Irradiation is likely to modify nutrients available to the natural microflora and spoilage microorganisms. This may influence the predominance of certain microbial species and the progress of infection, and hence odour production.

There appears to be a correlation between type of odour and grain viability, although this conclusion is based on a relatively small number of commercial samples. Typically, musty odours were associated with non-viable grain. Therefore sterilisation by irradiation would appear to be appropriate for preparing grain to be inoculated with specific spoilage microorganisms in order to reproduce these odours in the laboratory. However, it is not clear whether the grains either became musty because they were non-viable, and hence were more readily infected by spoilage fungi, or were non-viable as a result of infection. The first scenario is significant because it may be a consequence of over-heating grain during the drying process after harvest, and merits investigation. In this regard, it was interesting to observe that some control samples had greatly reduced viability (FC/38242/007, 002 and 022 in Table 3), which was most probably caused by either heat damage or some other post-harvest effect. A reduction in seed viability may be a significant factor in the

establishment of infections leading to musty odours in stored wheat grains. It would have been useful to know the origins of these control samples, but the information was not available.

Fishy, sour and green odours were associated with grain with a greater viability than musty odours. Irradiated grain with reduced viability therefore may not be an appropriate substrate for reproducing these odours in the laboratory. The presence of *T. tritici* spores on the fishy samples provides conclusive evidence that this odour was not a consequence of fungal infection whilst in storage. *T. tritici* causes Bunt or Stinking Smut of wheat in the field, with the characteristic odour due to production of trimethylamine.

With non-irradiated grain inoculated in the laboratory, there seemed to be no treatment specific effects: raising grain moisture content to 18 % and adding MRD either alone or with spores of the organisms generally gave off-odours. This may indicate that at an inoculum density of 10^4 spores ml^{-1}, the introduced organism does not consistently become established and predominate. Off-odours are therefore a cocktail resulting from the activities of the natural microflora, with the odours developing not always consistent amongst different batches of the same sample of grain.

Inoculation of irradiated grain at 25% moisture content, however, reliably produced the same type of musty odour characteristic for each of the organisms examined. These results replicated independently those generated by CCFRA. The systems were subsequently used for GC-MS analyses of the VOCs emitted and for routine laboratory testing of novel sensors for the early detection of spoilage organisms.

2.6 REFERENCE

Pitt, J.I. and Hocking, A.D. (1997) Fungi and Food Spoilage. Second Edition, Pitt, J.I. and Hocking, A.D. (Eds.), Blackie Academic & Professional, London.

PAPER 3

CHARACTERISATION OF VOLATILE INDICATOR MOLECULES FOR THE EARLY ONSET OF SPOILAGE IN WHEAT GRAIN AND ESTABLISHMENT OF A LINK WITH FORMATION OF SPOILAGE ODOURS

J.S. Williams, H.M. Brown, N. Beales and B.M. Williams
Campden & Chorleywood Food Research Association, Chipping Campden, Glos, GL55 6LD

3.1 ABSTRACT

The aim of this work was to identify volatile compounds that are early warning indicators of spoilage of wheat grain and are suitable for detection by a metal oxide sensor-based device. A total of 147 volatile compounds were identified in three separate trials where sterile grain (irradiated), conditioned to 25% moisture and inoculated with one of *Penicillium aurantiogriseum, Fusarium culmorum* and *Aspergillus niger* was held at 22ºC for 15 days during which time spoilage odours were produced. Of the volatile compounds identified, 27 were shown to increase in abundance with storage time, and 16 of these volatiles were alcohols. Increases in the abundance of total volatile alcohols with time followed the same trend as the increase in colony forming units. The level of total alcohols was estimated to increase to around 10mg.kg^{-1} at the end of the incubation period. This would be sufficient for detection by a metal oxide-based sensor device. Sterile grain conditioned to 25% moisture that was not inoculated with micro-organisms did not show the equivalent increase in total volatile alcohols over a 15 day incubation period.

3.2 INTRODUCTION

One of the most common causes of the post-harvest spoilage of wheat is growth of spoilage organisms. Microbial spoilage is prevalent when the wheat has a high moisture content and renders it unsuitable for food use due to the possible formation of toxins and the development of off-odours.

The overall aim of this project is to develop a sensing device to provide an early warning of the onset of spoilage in grain. The device is to use sensors designed to detect volatiles that have been identified as suitable to indicate the onset or early stages of microbial spoilage. Extensive research has been conducted into the identification of volatiles responsible for off-odours in microbially spoiled wheat (for example Kaminski *et al* 1974; Borjessen *et al* 1994). The aim of the work reported here was to identify volatile compounds formed at an early stage of the spoilage process that may be suitable for use as early warning indicators of spoilage.

In an earlier part of the project, a laboratory culturing system (see Paper 2) was developed to enable sterile grain to be inoculated with spoilage microorganisms and incubated so that the spoilage process proceeded to the production of spoilage odours. In this paper, work to assess the most suitable approach for monitoring volatiles produced, and its application to the characterisation and identification of volatile indicator compounds for detecting the early onset of spoilage is described.

3.3 METHODS

3.3.1 Approaches to Extraction and Concentration of Volatiles from Wheat

Initial method development was carried out using spoiled samples submitted to CCFRA from the industry (see Paper 1).

Direct headspace analysis was investigated by holding approximately 50g wheat in a sealed glass bottle to temperature equilibrate to 50°C and 70°C. Headspace gas (100µl) was withdrawn through a septum using a gas tight syringe and injected directly on to a GC-MS system (Hewlett Packard 6890 gas chromatograph and Hewlett Packard 5973 mass spectrometer).

Closed loop stripping apparatus (CLSA) was used to compare volatile profiles of good (control) and musty samples of wheat. A sample (100g) of wheat was placed in a sample flask in a waterbath at 50°C. This then formed part of a closed loop stripping apparatus. The headspace above the sample was circulated around the system for 2 hours with the volatiles being collected onto a 1.5mg charcoal trap. The volatiles were eluted from the charcoal trap with 3 x 10µl aliquots of 'Spectrosol' grade carbon disulphide. The extracts were sealed in glass vials and stored in a freezer prior to analysis. Analyses were carried out on a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 mass spectrometer. GC-MS conditions were: column – 25m x 0.25mm fused silica with CPWax 52CB polar stationary phase; helium carrier gas, flow rate 1ml.min^{-1}; column temperature: 2 min at 50°C then 5°C.min^{-1} to 250°C; ambient injector temperature; injection volume 1µl on column; ionising voltage: 70eV; scan range 29-350 daltons and selected ion monitoring (SIM) for ions characteristic of compounds that cause musty taints in foods and beverages. Peaks were identified by spectral matching with the Wiley library of mass spectra.

Purge and trap using Tenax-TA and a concentrated headspace injection system (CHIS) from SGE was used to monitor volatiles generated during spoilage of grain. An aliquot (50µl) of a 500µg.kg^{-1} 1-bromohexane in pentane solution was measured into the bottom of a 50ml boiling tube. Wheat (20g) was added, and the boiling tube sealed with a Dreschel head assembly, connected at one end to a Tenax-TA packed trap (3mm ID, SGE) and at the other end to a supply of ECD grade nitrogen. The sample tube was submerged into a

water bath (80ºC) and a nitrogen flow (40ml.min^{-1}) was passed through the sample and the trap for 55 minutes. A one minute dry purge was used to remove moisture from the adsorbent. Volatiles were thermally desorbed from the Tenax-TA trap onto a GC column using a CHIS heated to 250ºC. Cryofocusing was achieved by cooling a small section (approximately 10cm) of deactivated pre-column in solid carbon dioxide throughout the 5 minute desorption. GC-MS conditions were: column: 25m × 0.25mm fused silica with CPWax 52CB polar stationary phase; carrier gas: helium; column temperature: 7 min at 50ºC (including a 5 min desorption period with cryofocusing), then 5ºC.min^{-1} to 250ºC; ionising voltage: 70eV; acquisition: scan range 29-350 daltons.

Chromatographic peaks were tentatively identified by mass spectral matching with the Wiley library. Compounds were quantified by normalising the integrated total ion chromatographic peak area with the integrated total or extracted ion chromatographic peak area of the internal standard, 1-bromohexane.

3.3.2 Identification of Volatiles as Early Warning Indicators of Wheat Spoilage

3.3.2.1 Literature

A literature review (Salmon *et al*., 2002) was used to identify volatile compounds that have previously been reported to cause spoilage in grain including, where possible, the levels of the compounds that have been reported. It was also used to identify volatile compounds that may have potential as early warning indicators of spoilage.

3.3.2.2 Laboratory Culturing Systems

These were set up and monitored as described in Paper 1 except that samples were taken to determine the volatile profile at various times throughout storage. Three organisms were used in monoculture: *Penicillium aurantiogriseum* (IHEM 698), *Aspergillus niger* (IMI 017454), and *Fusarium culmorum* (Chorleywood collection (17)). Wheat (Hereward from 1999 harvest) was sterilised by irradiation (12kGy, Isotron, Swindon, UK). The moisture content of the sterile grain was adjusted to 25%. Microorganisms were inoculated at approximately 10^3 colony forming units per gram of grain. Alongside the inoculated samples a control sample was also prepared, in which only sterile distilled water was added to adjust the moisture content. All were set up in duplicate. Inoculated grain was incubated at 22°C ± 1°C for up to 15 days. At intervals throughout this time, samples were taken for the following: to check the moisture content; to carry out a colony count (cfu/g grain); for odour assessment by an informal odour assessment panel (each of these is described in Paper 1); and for analysis of volatile compounds. Analysis of volatile compounds was carried out on six sampling occasions during the storage period. Triplicate samples were taken at each sampling time and analysed by purge and trap (Tenax-TA) and CHIS-gas chromatography/mass spectrometry.

3.4 RESULTS AND DISCUSSION

3.4.1 Approaches to Extraction and Concentration of Volatiles from Wheat

Use of direct headspace analysis to measure volatiles was the preferred approach as this equates most readily to a sensor system in which there is no concentration of volatiles from the headspace. However, insufficient numbers of volatile compounds were detected using this approach, so extraction and concentration techniques were investigated.

The closed loop stripping apparatus in which the headspace above a sample is circulated continuously through an absorbent charcoal trap and back to the sample has been used effectively at CCFRA to extract and concentrate volatile compounds responsible for taints. Therefore it was appropriate to use to identify compounds responsible for the spoilage odours in grain. Comparison of the volatile profile of good grain (Soisson) with that of a sample described as musty (variety unknown) (Figure 1) showed differences between them. Only 7-octen-4-ol (peak 1) was present exclusively in the spoiled sample compared with the control. No specific odour has been described for this compound but other isomers of octenol are described as 'mushroomy'. The peak area of *m*-methoxyanisole (peak 2) was greater in the spoiled sample than the control. The odour due to this compound is described as 'hazelnut' and 'earthy' and has been identified as a cause of musty taints in wheat (Kaminski and Wasowicz, 1991). Other compounds that are known to cause musty taints in foods were looked for specifically by using the GC-MS in SIM mode (Table 1). 2,3,6-Trichloroanisole (2,3,6-TCA) was detected only in the musty wheat whereas 2,6-dichloroanisole, 2,4,6-trichloroanisole (2,4,6-TCA), pentachloroanisole and 2,4,6-tribromoanisole (2,4,6-TBA) were all detected in both the spoiled and the control wheat samples. 2,4,6-TCA and 2,3,6-TCA were at higher levels in the musty wheat. An estimate of the concentration of each analyte calculated by normalising against 20$\mu g.l^{-1}$ standards and assuming 100% recovery, showed that 2,4,6-TCA and 2,4,6-TBA in the musty and control wheat, and 2,3,6-TCA in the musty wheat, exceeded the odour thresholds of these compounds in water. It is highly likely therefore that the latter was contributing to the musty odour of the spoiled sample.

Having demonstrated the use of the CLSA for identifying and semi-quantifying compounds responsible for the spoilage odours in wheat, it confirmed that the levels of many of the compounds responsible for the spoilage odours are too low for detection by the sensors being considered, where ppm levels are required for detection (Paper 4). Compounds considered more suitable for detection by the sensors were small volatile molecules such as ammonia and short chain alcohols and aldehydes. Such small molecules are not detected using the CLSA approach as the solvent desorption step to remove volatiles from the charcoal trap results in a solvent peak when injected onto the GC-MS. This solvent peak masks highly volatile compounds with short retention times such as those of interest for the application of sensors. Therefore a purge and trap approach using thermal desorption to transfer volatiles to the GC-MS and a cryofocusing step to concentrate them on the column was considered to be essential for the identification of small molecules that may be early

File : C:\HPCHEM\1\DATA\RLWHT24B.D
Operator :
Acquired : 6 Aug 1999 15:50 using AcqMethod BWTEA3F
Instrument : GC/MS Ins
Sample Name: FC/38242/024
Misc Info : 1µl ONTO CARBOWAX 52-CB COLUMN
Vial Number: 2

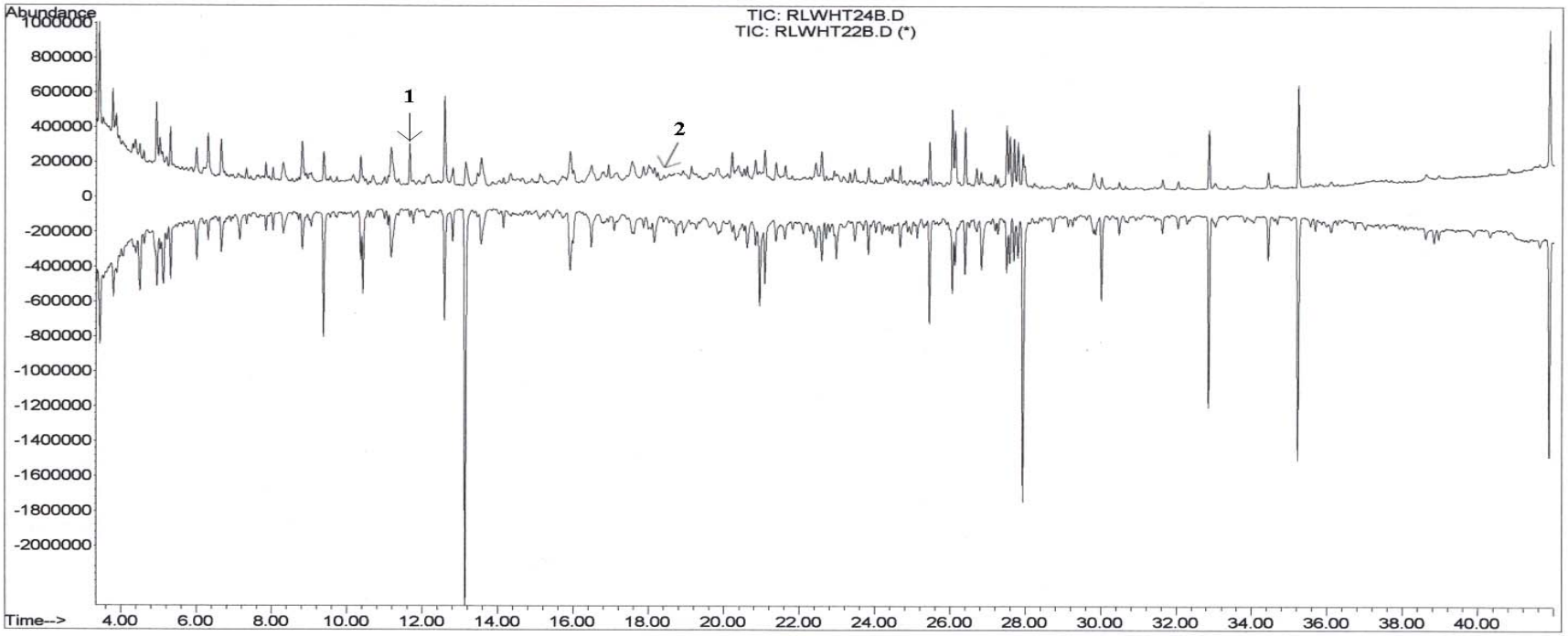


Figure 1: Comparison of Volatiles Extracted from Sound and Spoiled Wheat

Table 1: Anisole Compounds Known to Cause Musty Taints in Foods that were Present in the Wheat Samples Analysed in SIM Mode

Compound	Concentration in Sample Assuming 100% Recovery (ng/l)		Odour Threshold in Water (ng/l)
	Control Wheat (Soissons)	'Musty' Wheat	
2,6-dichloroanisole	0.17	0.14	400
2,4,6-trichloroanisole	0.59	4.51	0.03
2,3,6-trichloroanisole	-	0.19	0.0003
Pentachloroanisole	16.5	7.5	3,000
2,4,6-tribromoanisole	0.17	0.33	0.008

warning indicators of the onset of spoilage suitable for detection by the sensors. This resulted in the use of Tenax-TA to trap volatiles and a CHIS to transfer them to the GC-MS for identification.

3.4.2 Volatiles Selected on the Basis of Information in the Literature

A number of volatile compounds have been reported in the literature to be responsible for spoilage odours in grain (Table 2). Many of these compounds have very low odour thresholds (ppb), therefore it is unlikely that they will be detected by the sensors before their spoilage odours are evident to the human sense of smell.

The literature was also reviewed to identify non-odiferous volatile compounds present at higher levels in spoiled grain than in sound grain that may provide an alternative for early warning of the onset of spoilage (Table 3). There are also a number of compounds that other workers have suggested may be suitable indicators of spoilage (see below).

On the basis of information collated from the literature the following volatile compounds were suggested to project collaborators at The University of the West of England (UWE), as starting points for sensor testing and development whilst work to characterise and identify further potential indicator compounds was ongoing. Volatile compounds as potential indicators of wheat spoilage were:

- 2-methyl-1-propanol
- 3-methyl-1-butanal
- 3-methylfuran
- 1-octen-3-ol
- 3-octanol
- 3-octanone
- carbon dioxide
- Geosmin
- 2-methylisoborneol

Table 2 : Compounds Likely to be Responsible for Off Odours

(Reported by Kaminski and Wasowicz, 1991)

Compound	Estimated Concentration in Grain with	
	Normal Odour (ppb)	Musty Odour (ppb)
1-octen-3-ol	24.1	63.4
3-octanone	15.6	19.3
2-octanone	14.1	17.2
3-octanol	13	20
Methoxybenzaldehyde	-	55
2-methylisoborneol	-	1.4
Geosmin	-	17.1
Methylpyrazine	-	4
2,6-dimethylpyrazine	-	20
Benzaldehyde	38.9	67.5
1,2,3 trimethylbenzene	17	30
4-vinylguaiacol	80.3	106.6

Table 3: Compounds which Differed in Level Between Normal and **Musty Wheat whose Odours are not Characteristic of Spoilage Odours**

(Kaminski and Wasowicz, 1991)

Compound	Estimated Concentration in Grain with	
	Normal Odour (ppb)	Musty Odour (ppb)
Ethylbenzene	168	257
3 methyldecane	11	21
2-nonenal	11.1	102.2
Dodecane	49	113

3.4.3 Identification of Early Warning Volatiles Using Laboratory Culturing Systems

Work was carried out in a series of trials using three organisms. Irradiated grain was adjusted to 25% moisture and inoculated in monoculture with *P. aurantiogriseum*, *F. culmorum* or *A. niger* and held at 22°C ± 1°C for up to 15 days. The number of colony forming units was shown to increase in inoculated samples and remained negligible in control samples (Tables 4-6). The moisture content was relatively unchanged in all samples throughout the trials. Agreement between duplicates was generally good, suggesting that the laboratory culturing systems were reasonably reproducible.

Table 4: Colony Forming Units and Water Activity of Wheat Inoculated with *P. aurantiogriseum*

Day	Sample A		Sample B		Control
	cfu/g	Moisture (%)	cfu/g	Moisture (%)	cfu/g
0	2.8×10^3		3.8×10^3		<5
1	3.5×10^3	24	4.3×10^3	24	<5
2	8.9×10^3		8.4×10^3		<5
2	Control		Control		Control
4	3.1×10^6	24	2.8×10^6	24	<5
4	Slightly musty		Slightly musty		Control
6	2.7×10^7	24	2.3×10^7	24	<5
6	Musty		Musty		Control
12	6.8×10^7	24	6.1×10^7	24	<5
15	1.2×10^8	25	8.5×10^7	24	<5
15	Musty		Musty		Control

Table 5: Colony Forming Units and Moisture Content of Wheat Inoculated with *F. culmorum*

Day	Sample A		Sample B		Control	
	cfu/g	Moisture (%)	cfu/g	Moisture (%)	cfu/g	Moisture (%)
0	4.1×10^3		3.3×10^3		<5	
1	1.75×10^3	25	5.4×10^2	24	<5	25
2	4.5×10^3		5.3×10^3		<5	
4	6.9×10^4	25	7.6×10^4	24	<5	25
6	5.1×10^5	25	4.7×10^5	24	<5	25
6	Control		Control			Control
11	3.8×10^6	25	3.4×10^6	24	<5	25
11	Slightly sour		Slightly sour			Control
14	3.9×10^7	25	4.1×10^7	24	<5	25
14	Sour		Sour			Control

Table 6: Colony Forming Units and Moisture Content of Wheat Inoculated with *A. niger*

Day	Sample A		Sample B		Control	
	cfu/g	Moisture (%)	cfu/g	Moisture (%)	cfu/g	A_W (%)
0	2.3×10^3		3.3×10^3		<5	
1	4.2×10^3	25	4.4×10^3	25	<5	25
2	4.0×10^4	25	3.3×10^4	25	<5	25
5	5.9×10^5	25	6.1×10^5	25	<5	25
5	Control		Control			Control
8	8.1×10^5	25	9.4×10^5	25	<5	25
8	Fermented		Fermented			Control
12	1.9×10^6	25	1.9×10^6	25	<5	25
12	Fermented		Fermented			Control
15	8.0×10^6	25	7.2×10^6	25	<5	25
15	Sour/fruity		Sour/fruity			Control

Each of the inoculated samples was found to generate off-odours during the course of the trial. Wheat inoculated with *P. aurantiogriseum* developed a distinctive musty odour after four days. The odour of wheat inoculated with *F. culmorum* could not be differentiated from the odour of control wheat until the eleventh day, when a sour off-odour became evident. Wheat inoculated with *A. niger* was found to have a 'fermented' odour after eight days, which developed into an odour described as 'sour/fruity' after fifteen days.

Analysis of volatile profiles generated large amounts of data. Manual cryofocusing of volatiles following desorption resulted in shifts in retention time of the same compound between chromatograms such that chromatograms could not be compared directly by overlaying. To overcome this, initially one chromatogram was selected from three sampling occasions, usually days 1, 6 and 15, and comprehensive matching of extracted ion chromatograms across all three analyses was carried out. A total of 147 volatile compounds were identified in the three separate trials (Table 7). Most of the compounds identified have been previously reported from wheat. Semi-quantification was achieved by normalisation against the peak area of the internal standard. This initial analysis was used to identify those compounds that increased in abundance over storage time (Table 8) and those that decreased.

This served as a starting point for a more detailed investigation of the changes in concentration of selected groups of compounds using the data from all 18 chromatograms generated for each organism. For example, if the initial analysis showed that aliphatic alcohols increased in abundance, the subsequent analysis involved identification of all aliphatic alcohols detected in the samples and estimation of their abundance and the total abundance of aliphatic alcohols. Although methoxybenzene and 1,3-dimethoxybenzene did not increase with incubation time, they have been proposed as spoilage indicators (Ram *et al,* 1998), so were included in the second stage of analysis. For each organism inoculated, the compounds showing a consistent increase in abundance with incubation time for the triplicate analyses (see plots in Appendices I, II, and III) were considered as potential early indicators of spoilage.

The majority of these compounds were alcohols, particularly in wheat inoculated with *P. aurantiogriseum* and *F. culmorum*. In wheat inoculated with *A. niger*, a number of esters and carbonyl compounds were also found to increase in abundance with incubation time.

For a volatile compound to be considered as an early warning indicator of fungal spoilage for the sensor device being developed, it must fulfil a number of criteria. Ideally it should increase in abundance as spoilage occurs. All of the compounds listed in Table 8 meet this requirement. It must be detected by the sensor device at a concentration below that which it is detectable as an odour, so for example, 1-octen-3-ol increased in *Aspergillus niger* cultures (Appendix III), has a distinctive 'mushroom-like/musty' odour and a sensory threshold of $1\mu g.kg^{-1}$ (Buttery *et al*, 1988). Therefore, this compound alone is unlikely to be suitable for use as an early warning indicator unless the sensors can be made extremely sensitive to this compound. However, of the 27 volatile compounds found to increase in abundance as spoilage progressed (Table 8),

Table 7: Volatile Compounds Detected in Inoculated Wheat Samples

	P. aurantiogriseum	*F. culmorum*	*A. niger*		*P. aurantiogriseum*	*F. culmorum*	*A. niger*
(E)-1-Nonenal		✓		*trans*-2-Hexenal	✓		
(E)-2-Decenal	✓			2-Methyl-1-butanol		✓	✓
(E)-2-Hexen-1-ol	✓			2-Methyl-1-propanol	✓	✓	✓
(E)-2-Octenal	✓			2-Methyl-2-butenal	✓		
(E)-2-Tridecen-1-ol	✓			2-Methyl-3-heptene	✓		
(E)-4-Methyl-2-pentene	✓			2-Methyl-3-pentanol	✓		
(E,E)-2,4-Decadienal	✓		✓	2-Methylbutanoic acid, ethyl ester		✓	
(E,E)-Nona-2,4-dienal	✓		✓	2-Methylfuran	✓	✓	✓
(Z)-2-Heptenal	✓			2-Methylisoborneol	✓		
1,2,3-Trimethylcyclohexane			✓	2-Methylpropanal			✓
1,4-Dichlorobenzene	✓			2-Methylpyridine		✓	
1.3-Dimethoxybenzene	✓			2-Nonanol			✓
1-Butanol	✓	✓	✓	2-Nonanone		✓	✓
1-Ethyl-2,4-dimethylbenzene	✓			2-Nonen-1-ol	✓		
1-Ethyldibenzothiophene	✓			2-Octanol	✓	✓	✓
1-Heptanol	✓	✓	✓	2-Octanone		✓	✓
1-Heptene	✓		✓	2-Octen-1-ol	✓		
1-Hexanol	✓	✓	✓	2-Octenal		✓	
1H-Pyrrole	✓	✓		2-Pentanol	✓	✓	✓
1-Nonanol	✓			2-Pentylfuran	✓	✓	
1-Octanol			✓	2-Undecanone	✓	✓	
1-Octen-3-ol	✓	✓	✓	3-Heptanol	✓		
1-Pentanol	✓	✓	✓	3-Hexen-1-ol	✓		
1-Penten-3-ol	✓	✓	✓	*cis*-3-Hexenol	✓		
1-Propanol	✓	✓	✓	3-Hydroxy-2-butanone	✓	✓	✓
2,3-Dihydro-4-methylfuran		✓		3-Methyl-1-butanol	✓	✓	✓
2,3-Pentanedione			✓	3-Methyl-2-butanol			✓
2,4-Heptadienal	✓			3-Methyl-2-buten-1-ol	✓		✓
2-Butanol	✓	✓	✓	3-Methyl-2-butenal	✓		✓
2-Buten-1-ol	✓		✓	3-Methyl-2-butenoic acid, ethyl ester		✓	
2-Butenal		✓		3-Methyl-2-heptanone	✓		
2-Butenoic acid, ethyl ester			✓	3-Methyl-3-buten-1-ol		✓	✓
2-Decanone	✓			3-Methylbutanal	✓		✓
2-Ethyl-1-hexanol	✓	✓	✓	3-Methylbutanoic acid, ethyl ester	✓		✓
2-Ethyldibenzothiophene	✓			3-Methylfuran			✓
2-Heptanol	✓	✓	✓	3-Octanol	✓	✓	
2-Heptanone		✓	✓	3-Octanone	✓	✓	✓
2-Hexanol		✓	✓	3-Pentanol	✓	✓	✓
2-Hexanone	✓			3-Penten-2-one		✓	✓

Table 7: Volatile Compounds Detected in Inoculated Wheat Samples (Continued)

	P. aurantiogriseum	*F. culmorum*	*A. niger*		*P. aurantiogriseum*	*F. culmorum*	*A. niger*
4,5,5-Trimethyl-2(5H)-furanone		✓		Ethyl caproate			✓
4-Ethyl-2-methoxyphenol		✓	✓	Ethyl octanoate	✓	✓	
4-Ethylphenol	✓			Ethylphenylacetate		✓	✓
4-Heptanol	✓			Ethyl sorbate	✓		
4-Methylcyclohexane		✓		Ethyl tiglate		✓	✓
4-Methylheptane	✓			Furfural	✓	✓	
5-Nonanone		✓		Germcrene-d		✓	
6-Methoxy-2-hexanone		✓		Heptanal	✓	✓	✓
6-Tert-butyl-m-cresol	✓			Heptane	✓		✓
Acetic acid, ethyl ester	✓			Hexadecane	✓		
Acoradiene		✓		Hexanal	✓	✓	✓
Aristolene	✓			Isobutanal		✓	
Benzaldehyde	✓	✓	✓	α-Longipinene	✓	✓	
Benzeneacetaldehyde	✓		✓	*p*-Menth-2-en-1-ol		✓	
Benzeneethanol	✓			Methoxybenzene	✓		
Benzoic acid, ethyl ester	✓	✓	✓	Nonanal	✓	✓	✓
epi-Bicyclosesquiphellandrene		✓		Octa-1,5-dien-3-ol	✓		
Cadina-1,4-diene	✓	✓		Octanal		✓	
γ-Cadinene		✓		Octanol	✓		
Calarene	✓	✓		Pentadecane	✓	✓	
trans-Caryphyllene	✓			Pentanal	✓		
α-Chamigrene	✓	✓		Pentane	✓	✓	✓
Chloroform	✓			*cis*-Piperitol		✓	✓
α-Copaene		✓		*trans*-Piperitol		✓	
α-Cubebene		✓		Piperitone		✓	
ß-Cubebene			✓	Pyridine	✓	✓	
Cyclopentanol	✓	✓	✓	Sabinene		✓	
Cyclopentanone	✓	✓	✓	Styrene		✓	
p-Cymene	✓			α-Terpineol		✓	
Diacetyl	✓			γ-Terpinene		✓	
Dihydro α-curcumene		✓		Tetradecane	✓	✓	
α-Elemene	✓	✓		Thiazole	✓	✓	
Ethanol	✓	✓	✓	Thiobismethane		✓	
Ethyl acetate			✓	Toluene	✓	✓	✓
				Trimethylamine	✓		

Table 8: Volatile Compounds Found to Increase in Concentration with Incubation

Time in Laboratory Culturing Systems Inoculated with Microorganisms

Compound	*P. aurantiogriseum*	*F. culmorum*	*A. niger*
Ethanol	✓		✓
2-methylbutanoic acid, ethyl ester			✓
3-methyl-2-butanol			✓
2-methyl-1-propanol			✓
1-butanol	✓	✓	
2-butanol		✓	
2-buten-1-ol	✓		
2-pentanol	✓		✓
3-pentanol			✓
2-octanol			✓
1-octen-3-ol			✓
2-ethyl-1-hexanol		✓	✓
ß-cubebene			✓
2-butenoic acid, ethyl ester			✓
3-methyl-2-butenoic acid, ethyl ester			✓
3-methyl-2-butenal			✓
3-octanone			✓
3-methyl-1-butanol	✓	✓	✓
Cyclopentanol	✓		
3-methyl-3-buten-1-ol	✓	✓	
1-hexanol			✓
2-heptanol		✓	
Benzoic acid, ethyl ester			✓
cis-piperitol			✓
(E,E)-nona-2,4-dienal			✓
Ethylphenylacetate		✓	✓
4-ethyl-2-methoxyphenol		✓	

16 were alcohols. Alcohols were the only class of chemical to have increased in abundance in all three inoculated samples. For all three organisms, abundance of total alcohols and numbers of colony forming units showed the same increasing trend with incubation time (Figures 2-4).

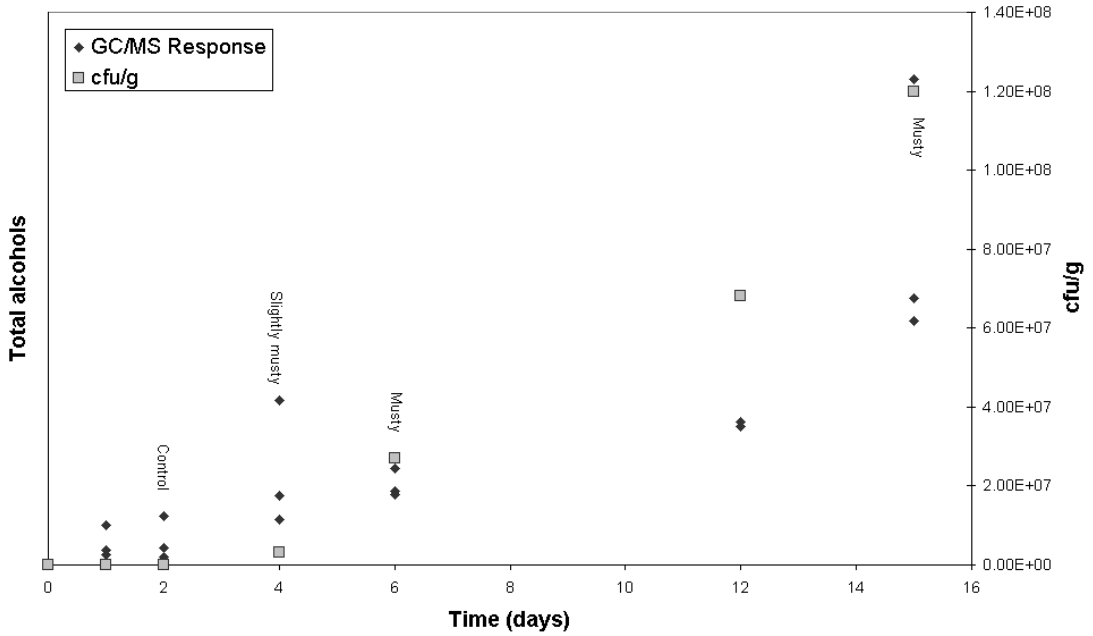


Figure 2: Plot of Total Alcohol Abundance (♦) and Number of Colony Forming Units (□) Against Time, for Wheat Inoculated with *P. aurantiogriseum*

(Odour is that described by the informal panel)

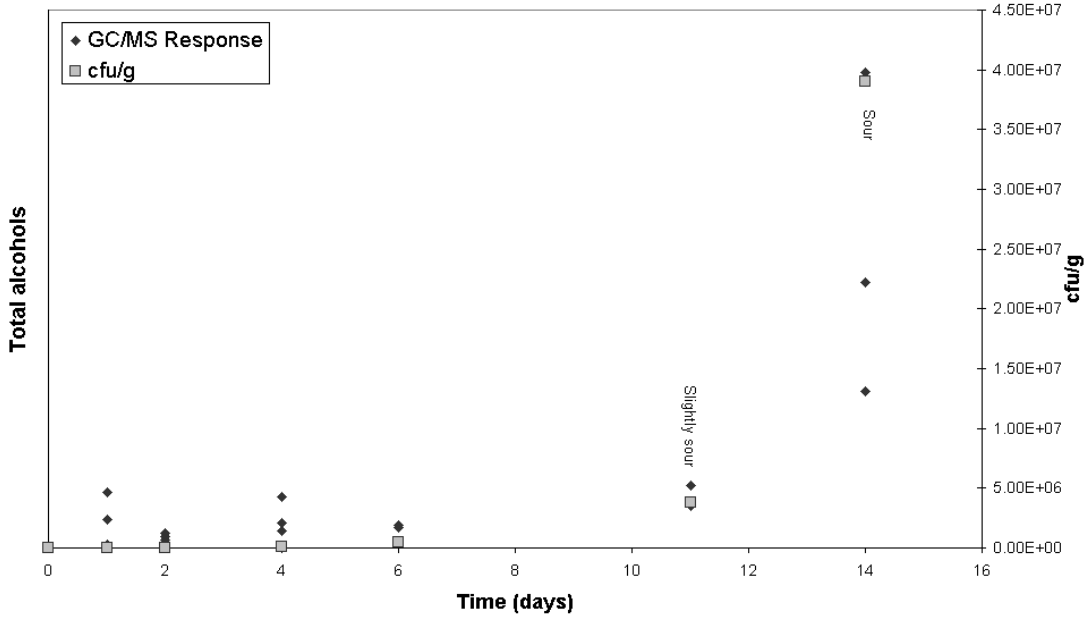


Figure 3: Plot of Total Alcohol Abundance (♦) and Number of Colony Forming Units (□) Against Time, for Wheat Inoculated with *F. culmorum*

(Odour is that described by the informal panel)

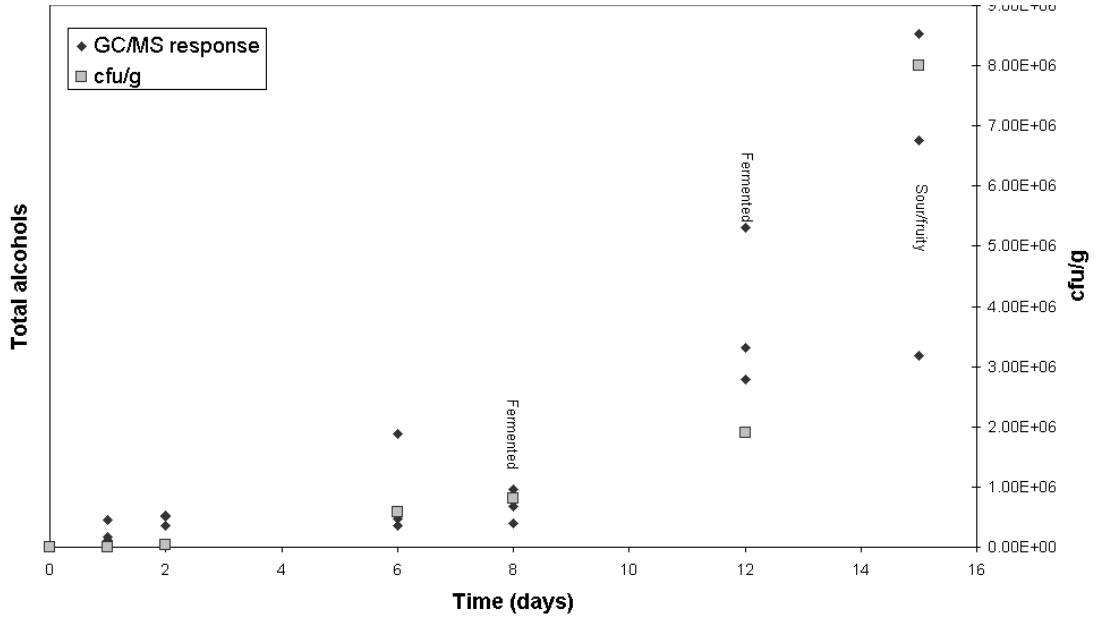


Figure 4: Plot of Total Alcohol Abundance (♦) and Number of Colony Forming Units (□) Against Time, for Wheat Inoculated with *A. niger*

(Odour is that described by the informal panel)

Therefore, rather than the use of single specific compounds as early warning indicators, alcohols as a chemical class may be more suitable for the sensor device to use as an early warning of mould spoilage in wheat. Alcohols are ubiquitous primary metabolites of fungal spoilage and are therefore likely to have a broad specificity. Alcohols are also generated in other spoilage processes (e.g. enzymic and lipid degradation) so may be of broader application than fungal spoilage. The data do suggest that subsets of alcohols, e.g. based on chain length or branched alcohols, could be a more specific approach, but ultimately the sensitivity and specificity of the sensor device will dictate whether this is feasible.

Having identified alcohols as a class of compounds to be early warning indicators of spoilage, it was important to show that they do not increase in sound grain. It was estimated that the concentration of total alcohols (i.e. a combination of all of the alcohols in Table 8) increased to around 10mg.kg^{-1} (ppm) at 15 days after inoculation with spoilage organisms. Determination of the alcohol content in control grain (i.e. moisture adjusted to 25% but no inoculum) showed no similar increase over 15 days incubation (Figure 5).

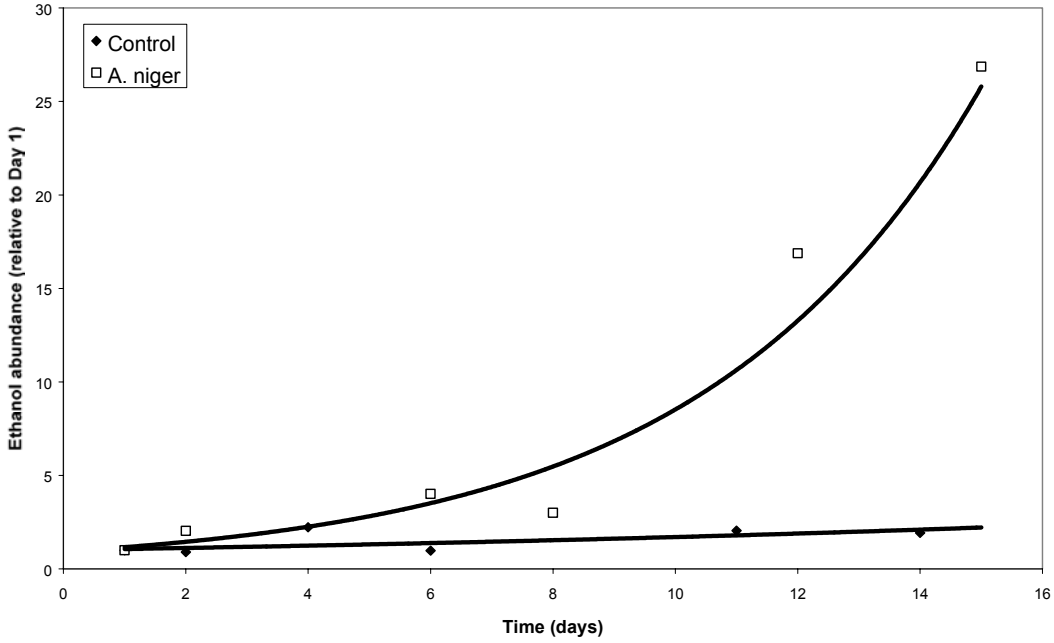


Figure 5: A Comparison of the Abundance of Ethanol in Wheat **with (□) and Without (♦) Inoculation with *A. niger***

3.5 CONCLUSIONS

The abundance of alcohols in sterile wheat samples at 25% moisture content inoculated with each of *Penicillium aurantiogriseum*, *Fusarium culmorum* and *Aspergillus niger* showed a similar increase with incubation time with the number of colony forming units. Either total volatile alcohols or the abundance of specific alcohols or groups of alcohols were suggested as compounds likely to be suitable for use as early warning indicators of spoilage by the sensors under investigation in Paper 4.

3.6 REFERENCES

Buttery, R.G., Turnbaugh, J.G. and Ling, L.C. (1988) Journal of Agriculture and Food Chemistry **43**, 1006-1009.

Borjesson, T., Stollman, U. and Schnurer, J. (1994) Cereal Chemistry **71** (1) , 16-20.

Kaminski, E., Stawicki, S. and Wasowicz, E. (1974) Applied and Environmental Microbiology **27**, 1001-1004.

Kaminski, E. and Wasowicz, E. (1991). In: *Cereal Grain: Mycotoxin Fungi and Quality in Drying and Storage*, Chelkowski, J. (Ed.), pp19-147, Elsevier, Amsterdam.

Ram, M.S., Seitz, L.M. and Rengarajan, R. (1998) Poster at 1998 Annual Meeting of the American Association of Cereal Chemists, 13th-17th September.

Salmon, S.E., Voysey, P.A., Williams, J.S. and Brown, H.M. (2002) CCFRA Review 35, Campden & Chorleywood Food Research Association, Chipping Campden, Glos.

Paper 4

THE FABRICATION OF COMPOSITE THICK FILM SENSORS BASED ON BINARY AND TERTIARY MIXTURES OF METAL OXIDES, THE ASSESSMENT OF THEIR SENSITIVITY TO ORGANIC VAPOURS AND THE EFFECTS ON THE SENSITIVITY OF OPERATING THE SENSORS AT A RANGE OF RELATIVE HUMIDITIES

B.P.J. de Lacy Costello, R.J. Ewen, N.M. Ratcliffe[1] and P.S. Sivanand

Department of Chemical & Physical Sciences, Faculty of Applied Sciences, The University of the West of England, Bristol Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY

[1] Centre for Research in Analytical, Material and Sensory Sciences, Faculty of Applied Sciences, The University of the West of England, Bristol Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY

4.1 ABSTRACT

Previously a composite sensor incorporating tin dioxide and zinc oxide was reported which exhibited an extremely high sensitivity to a range of organic vapours under dry conditions (0% relative humidity (RH)). Most importantly the composite sensor exhibited a significantly higher sensitivity than sensors constructed solely from either tin dioxide or zinc oxide when tested under identical experimental conditions.

Work is reported which was carried out on an extended range of composite materials, and involved testing these composites to a range of vapours that have been identified as being associated with the microbial spoilage of cereal grains in storage. Alcohol vapours were found to be the most significant volatile marker of early fungal infection in stored cereal grains.

Both previously and in this investigation, tests at 0% RH showed that the tin dioxide/zinc oxide sensor elucidated the highest sensitivity to alcohol vapours. However, for the purpose of a practical device it would be necessary to operate the sensors under a flow of high humidity in order to nullify the effects of variations in grain moisture levels. The sensitivity of the composite sensors to known concentrations of volatile organic compounds was measured over a range of relative humidities (0-100%) at a constant temperature. A composite sensor comprising 50:50 w/w tin dioxide/indium oxide was found to give the highest sensitivity when tested with alcohol vapours at 100% relative humidity.

The work also highlighted a difference in the response of sensors to classes of organic compounds when operated at different humidities. For example, the response to alcohols was reduced significantly when operated at high humidity, whereas the response to ketones and other carbonyl-containing compounds was relatively unaffected.

4.2 INTRODUCTION

Semiconducting metal oxides have been utilised extensively at elevated temperatures for the detection of simple gases (Gopel and Schierbaum, 1995). Sensors such as those based on tin dioxide or zinc oxide exhibit a high sensitivity but poor selectivity to organic vapours (Ippommatsu *et al*., 1991). These sensors are also highly sensitive to humidity. Previously base materials have been doped with catalytic metals or metal oxides to improve sensitivity, selectivity and also stability (Xu *et al*., 1992). Work has also been reported on the use of complex oxides (Reddy *et al*., 1999) that may also improve the sensor's stability and sensitivity, or allow operation of the device under a wider range of environmental conditions.

Recently composite materials such as those based on tin dioxide and zinc oxide have been utilised (de Lacy Costello *et al*., 1999; Yu and Choi, 1998). These composites were shown to exhibit a higher sensitivity than either tin dioxide or zinc oxide alone when exposed to sub-ppm concentrations of vapour (de Lacy Costello *et al*., 1999). Reasons for the apparent synergistic effects observed with these composites have been postulated. One suggestion invokes complementary catalytic activity (de Lacy Costello *et al*., 1999), whilst another is based on the formation of hetero-junctions and changes in the micro-structure on sintering (Yu and Choi (1998). However whatever the mechanism may be, it remains clear that composite materials of this type hold some key benefits for the construction of gas sensors.

When wheat is colonised by fungi such as *Penicillium* spp. and *Fusarium* spp. then volatile compounds are released (Magan and Evans, 1999). A range of compounds, consisting predominantly of alcohols, was identified which increased when wheat was in the early stages of an infection. The concentration and type of these compounds is indicative of the type and severity of any infection on a given substrate. If any sensor system is to be of use in the determination of wheat quality then it must give an early warning of this microbial spoilage and therefore be sensitive to sub-ppm levels of organic vapours. However, it must also be able to differentiate between sound wheat and wheat that was previously colonised by fungi, or even grain that was in contact with contaminated grain.

The work reported here investigates a range of composite materials based on binary and tertiary mixtures of single oxides, and includes a comparison with the single oxides alone. The work tests these composites to a range of vapours that have been identified to increase in the headspace above wheat infected with fungi. Due to the need to nullify the effects of moisture, a major focus of the work reported involved testing the composite sensors under flow conditions and at a range of relative humidities (0-100% RH). The aim was to find sensors with high sensitivity and long-term stability when operated under high humidity conditions.

4.3 MATERIALS AND METHODS

4.3.1 Sensor Preparation

4.3.1.1 Interdigitated Electrode Substrates

Uncoated substrates for the production of sensors were obtained from Capteur Sensors and Analysers, Abingdon, Oxon, U. K. These consisted of a 3-mm alumina square with interdigitated gold electrodes on one side and a platinum heater on the reverse. The substrates were mounted on eight-pin dual-in-line headers before use.

4.3.1.2 Thick Film Sensors

Zinc oxide powder (AnalaR grade, (99.5% BDH/Merck, Lutterworth, Leicestershire, UK), tin dioxide powder (99.999%, Janssen Chimica, Hyde, Cheshire, UK), niobium (V) oxide powder (99.99%, Aldrich Chemical Co., Gillingham, Dorset, UK) and indium oxide pieces (99.999%, Aldrich Chemical Company, Gillingham, Dorset, UK) were used in the production of the sensors.

Previous work had shown composite sensors incorporating 50:50 w/w tin dioxide: zinc oxide to exhibit the highest sensitivity when exposed to vapours. Therefore, composites incorporating 50:50 w/w tin dioxide: zinc oxide, 50:50 w/w tin dioxide: niobium oxide, 50:50 w/w tin dioxide: indium oxide, 50:50 w/w zinc oxide: indium oxide, and 33.3:33.3:33.3 w/w/w tin dioxide: indium oxide: zinc oxide were produced along with sensors incorporating indium oxide, tin dioxide and zinc oxide alone.

In each case, 1g of the oxide or oxide mixture was ground with a few drops of distilled water using an agate pestle and mortar for 1 hour to produce a thick paste. The paste was then applied to the interdigitated electrodes. The paste was dried for 24 h at 25^{o}C and the sensors were then placed into the test rig and heated at 350^{o}C for 24 h in dry blended air (MG Gas Products, Reigate, Surrey, UK) prior to testing.

4.3.2 Sensor Testing

4.3.2.1 Sensor Evaluation Equipment

The sensors were tested in an in-house designed injection test rig. The test chamber consisted of an aluminium base-plate which was covered by a detachable dome-shaped glass vessel (300 cm^3) which was sealed to the base plate. Zero-insertion-force sockets mounted on the base-plate permitted the easy fitting and

removal of the sensors, which were mounted in the standard dual-in-line packages, as described in section 2.1.1. Up to nine sensors could be tested at one time. The base-plate also contained two tubular feed-throughs (one inlet, one outlet), connected through electromagnetically operated on/off valves and a flow-measuring device to provide a continuous regulated flow of humidified air. The temperature inside the dome was measured using a semiconductor temperature sensor mounted on the base-plate.

The sensors were connected through a Keithley 7001 scanner to a Keithley 617 electrometer (Keithley Instruments, Reading, Berkshire, UK). Both Keithley instruments were connected via an IEEE488 interface to an IBM-compatible computer. Software written in-house permitted the automated gathering of sensor temperature, sensor conductance, chamber-temperature and purge flow rate data.

4.3.2.2 Humidity Control and Introduction of Test Vapour

The sensors were tested to the following vapours: ethanol, 1-propanol, 1-butanol, 1-hexanol, 3-methyl-1-butanol, 3-octanone, diacetyl, butanal, 2-butanone, ethyl benzene and decane. The vapours were tested at 6 different concentrations: 5ppm, 2.5 ppm, 1ppm, 0.5 ppm, 0.25 ppm and 0.1 ppm. The vapour was injected at the top of the dome using a gas tight syringe prior to the air inlet to ensure full mixing prior to contact with the sensors.

Initially the testing was carried out under dry conditions (0% RH) at a flow of 1 litre/min where the source was a dry blended air cylinder. To produce different humidities in the test rig air, from the same cylinder was directed through a number of Dreschel bottles maintained in a thermostatted water bath fitted with a cooling coil (Grant Instruments Ltd, Cambridge, UK).

4.3.2.2 Operation of the Testing Rig

The sensors to be tested were mounted in the chamber and heated to the operating temperature of 350^{o}C. The chamber was purged with dry blended air at a flow rate of 1 litre/min. The sensors were allowed to equilibrate for 24 hours prior to testing.

After the data-acquisition program had been initiated, the following procedure was undertaken:

a. The humidified air was initiated at a flow rate of 1 litre/min (not applicable if testing at 0% RH);
b. The sensors were left until a steady baseline was attained;
c. The test vapour was injected into the pre chamber and subsequently into the sensor chamber;
d. The response was monitored until the current measured had returned to a steady baseline value.

This procedure was repeated for different concentrations of the same vapour. Before testing to a different vapour, the test chamber was removed and cleaned and the same procedure was repeated.

4.4 RESULTS AND DISCUSSION

All the sensitivity results quoted were calculated using the following relationship: 100 $(I_g - I_o)/I_o)$, where I_g was the peak current flowing through the sensor after the injection of the test vapour, and I_o was the stable baseline current prior to injection.

Composite sensors incorporating niobium oxide were found to exhibit low sensitivity to organic vapours even when operated under dry conditions, and therefore composites of this type were not studied extensively. Figure 1 shows the typical responses of a range of thick film and composite sensors when exposed to 5 ppm ethanol vapour (all concentrations are quoted in ppm v/v) when operated under a flow of 1 litre/min dry air. Figure 2 shows the response of the same sensors when exposed to 5ppm ethanol vapour when operated under a flow of 1litre/min air at 100% RH. The results show that composites of tin dioxide/zinc oxide and tin dioxide/indium oxide gave enhanced sensitivity at both low and high humidities when compared with the single oxide sensors. For the case of composite sensors comprising zinc oxide and indium oxide, a reduction in sensitivity was observed when compared with sensors constructed of indium oxide or zinc oxide alone. The reasons for this are unclear but it highlights the significant differences in sensitivity and selectivity that can be obtained by mixing materials in this way to produce composite sensors. As mentioned above, a number of tentative mechanisms have been postulated to explain the synergistic effect observed with some composite sensors, but as yet none are definitive. In other work, evidence has been gained to suggest that the effect is at least in part a catalytic one. In studying the breakdown products of butan-1-ol and 2-butanone when passed over the composite material and the individual materials at 350°C there are important differences in the catalytic pathways operating in each material. For example, tin dioxide appears to catalyse an initial dehydrogenation whereas zinc oxide more efficiently catalyses the further breakdown of the dehydrogenated product. This catalytic explanation in part explains the synergistic effects observed with the composite sensors.

All the oxide materials exhibited a decrease in sensitivity when operated under high humidity conditions, suggesting a competitive effect with water vapour. In most cases the sensitivity decrease observed was at least 50%, as can be observed for the tin dioxide/zinc oxide composite sensor. However, for the case of the tin dioxide/indium oxide composite sensors the decrease was small, at *circa* 15%. Thus, when tested under high humidity conditions, the tin dioxide/indium oxide sensor exhibits the highest sensitivity to ethanol vapour of all the sensors tested. Therefore, composites based on tin dioxide/indium oxide offer not only higher sensitivity than either tin dioxide or indium oxide sensors, but also less variation in sensitivity (higher stability) when tested under different humidity conditions.

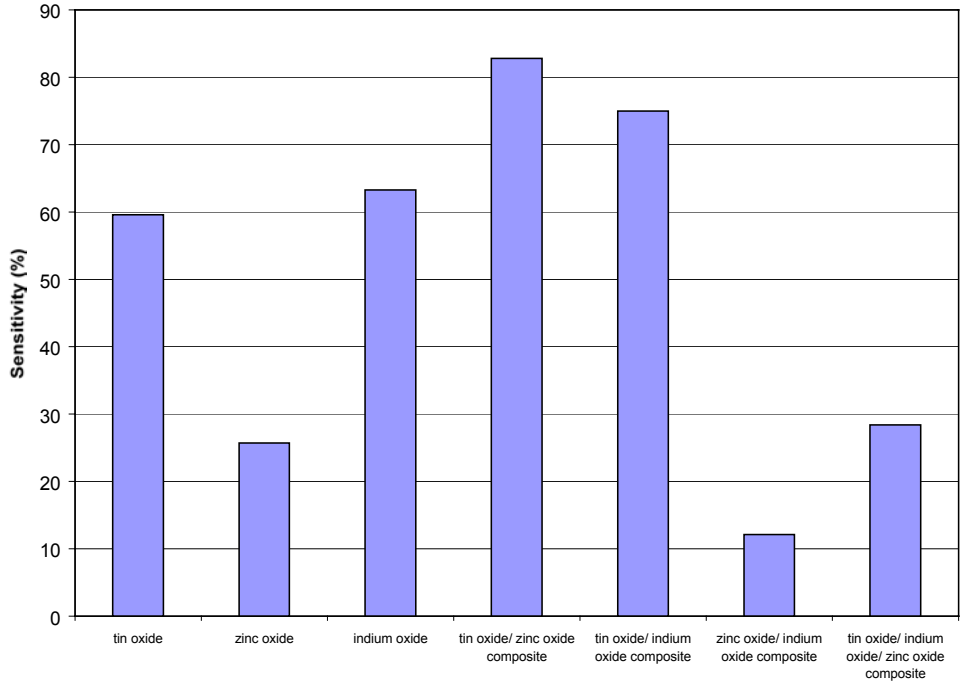
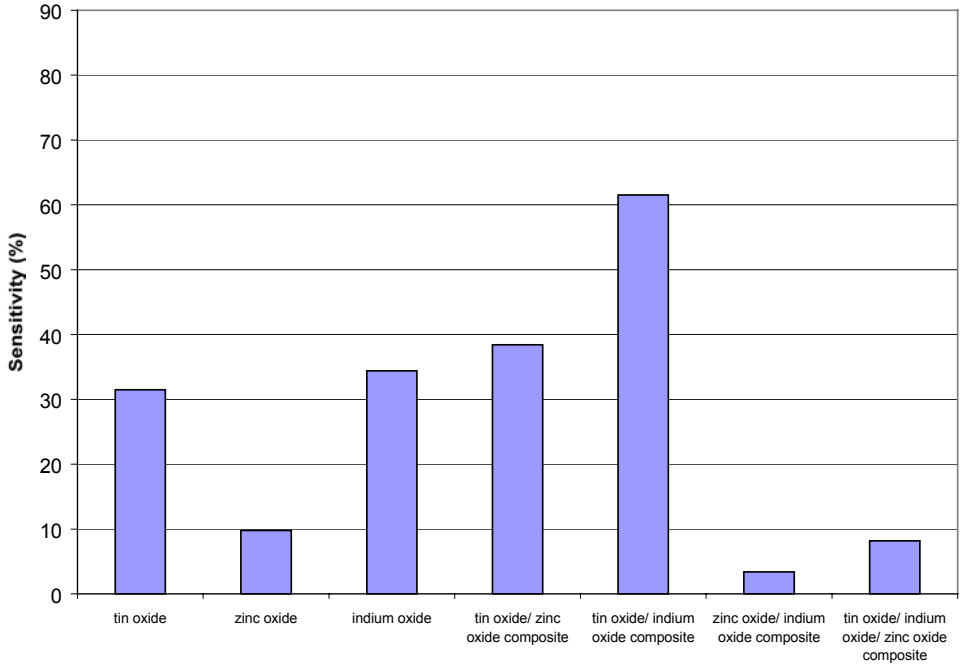


Figure 1: The Response of Single Oxide and Composite



Sensors to 5ppm of Ethanol Vapour at 0% RH

Figure 2: The Response of Single Oxide and Composite

Sensors to 5ppm Ethanol Vapour at 100% RH

The synergistic effects observed with tin dioxide/indium oxide composite sensors when tested at 0% RH were relatively small, with a 20% enhancement in sensitivity when compared with either tin dioxide or indium oxide sensors alone. When tested under 100% RH conditions the synergistic effect observed with this composite are greatly increased, with a 100% enhancement in sensitivity observed when compared with either tin dioxide or indium oxide sensors alone.

Figure 3 shows the responses of a range of sensors when exposed to ethanol vapour over the full range of relative humidities (0-100%) at 20°C. The sensitivity was seen to drop sharply for all sensors tested at 20% relative humidity. However, a subsequent increase in sensitivity was observed when the sensors were tested at 40% RH. This was then followed by another drop in sensitivity at 60 and 80% RH followed by a subsequent rise at 100% RH. Figure 4 shows the responses of a range of sensors when exposed to 5 ppm 3-octanone over a range of different humidities. The pattern is very different from that observed when ethanol was the target analyte. The decrease in sensitivity, particularly for the tin dioxide/zinc oxide composite sensor, was not as marked. The trends observed for individual sensors were also very different, with the tin dioxide/zinc oxide sensor decreasing gradually in sensitivity when tested between 0 and 40% RH, and then subsequently increasing when tested between 60 and 100% RH. This is in contrast to indium based, sensors particularly the indium oxide/tin dioxide composite, which exhibited a small increase in sensitivity when tested over the full range of humidities 0-100%. The results suggest that the competitive effects occurring between ethanol and water vapour on the surface of the sensors are more significant than those between water and 3-octanone. The effects of water on the surface reactions of oxide materials are complex and these results serve to emphasise this further. Some groups have postulated an enhanced sensitivity when water is present: for instance, the response of tin dioxide sensors when exposed to carbon monoxide (Barsan and Ionescu, 1993). It has been suggested that hydroxyl groups on the surface of tin dioxide increase the oxygen vacancies and therefore the number of surface oxygen ions. However, despite an unexpected pattern in the sensitivity results, the sensors tested in this study all suggest an overall competitive effect when water vapour is present, particularly when alcohols are the target vapours.

Figures 5-6 show the two best composite sensors, namely tin dioxide/zinc oxide and tin dioxide/indium oxide, when exposed to a range of 11 vapours at six concentrations between 0.1 and 5ppm under dry conditions. The results show that sensors of this type are easily capable of detecting sub-ppm levels of a range of organic vapours. The highest sensitivity was observed for polar compounds such as alcohols, ketones and aldehydes. Non-oxygen-containing and non-polar species such as decane and ethyl benzene gave much lower responses from the sensors. This suggests that the oxygen group of the organic vapour may serve to facilitate adsorption to the tin dioxide surface prior to any reaction at the surface.

When a range of primary alcohols were tested, an apparently linear increase in sensitivity was observed with increasing chain length for the tin dioxide/zinc oxide composite sensor. However, this relationship broke

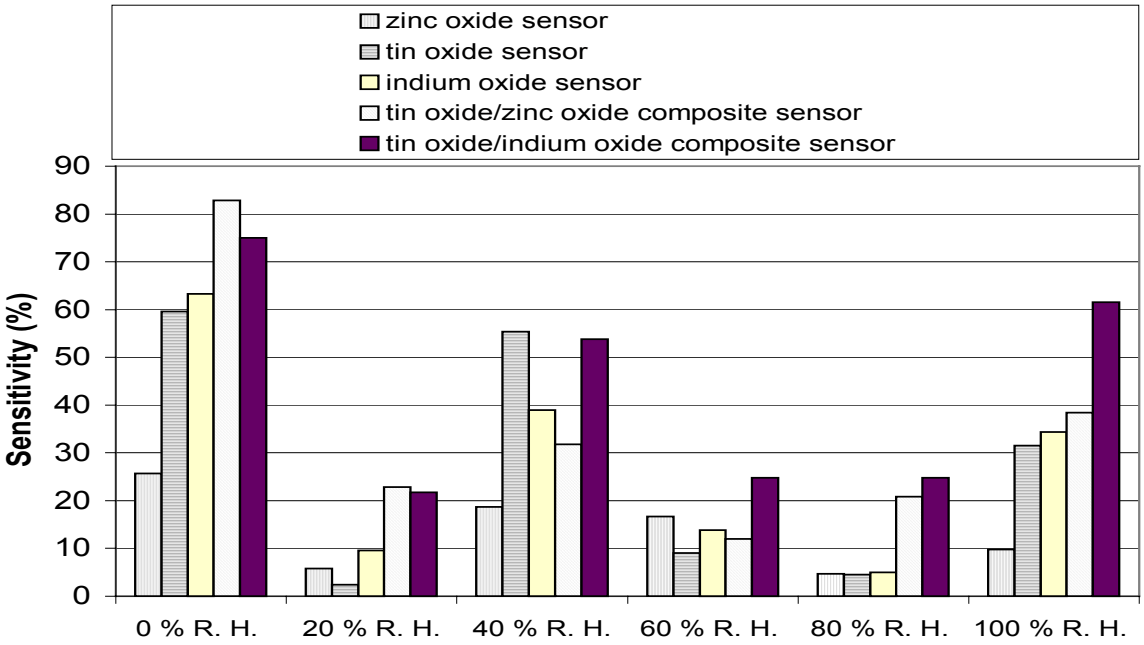
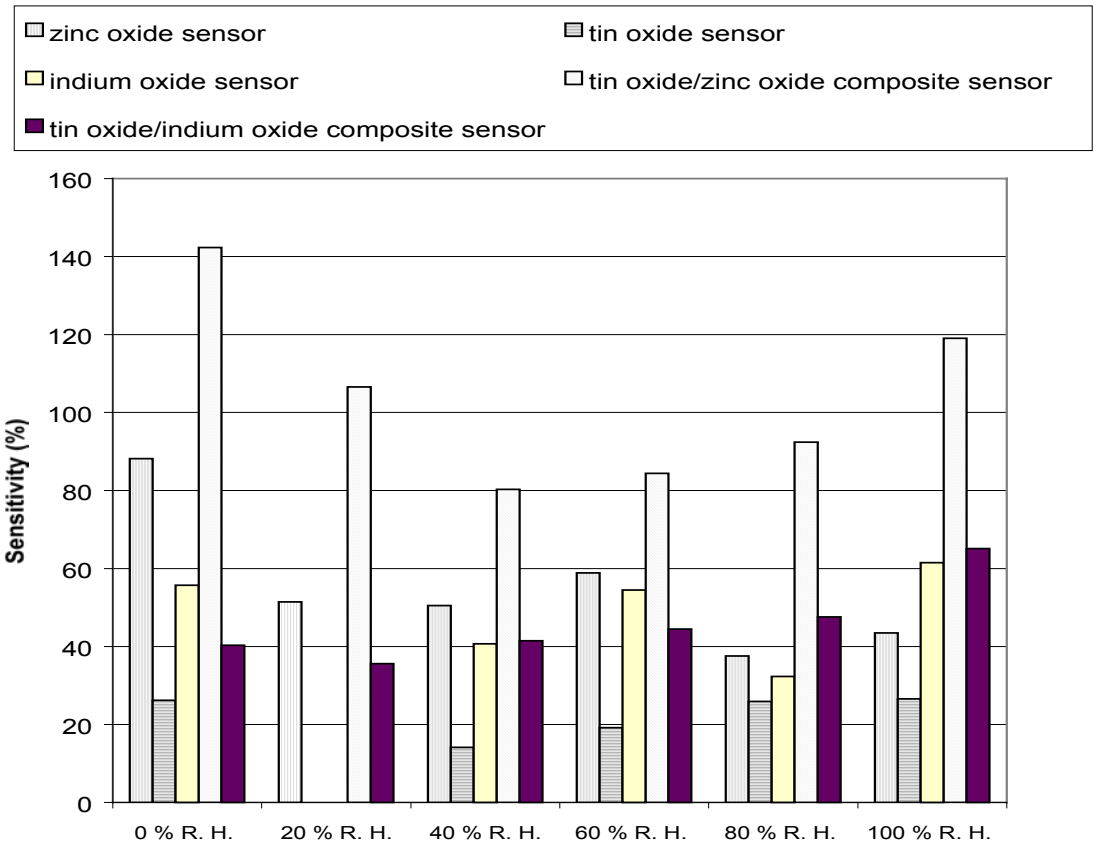


Figure 3: The Response of Single Oxide and Composite Sensors when Exposed to 5ppm of Ethanol Vapour at Six Different Humidities in the Range 0-100% RH

Figure 4: The Response of Single Oxide and Composite Sensors when Exposed to 5 ppm of 3-Octanone Vapour at Six Different Humidities in the Range 0-100% RH



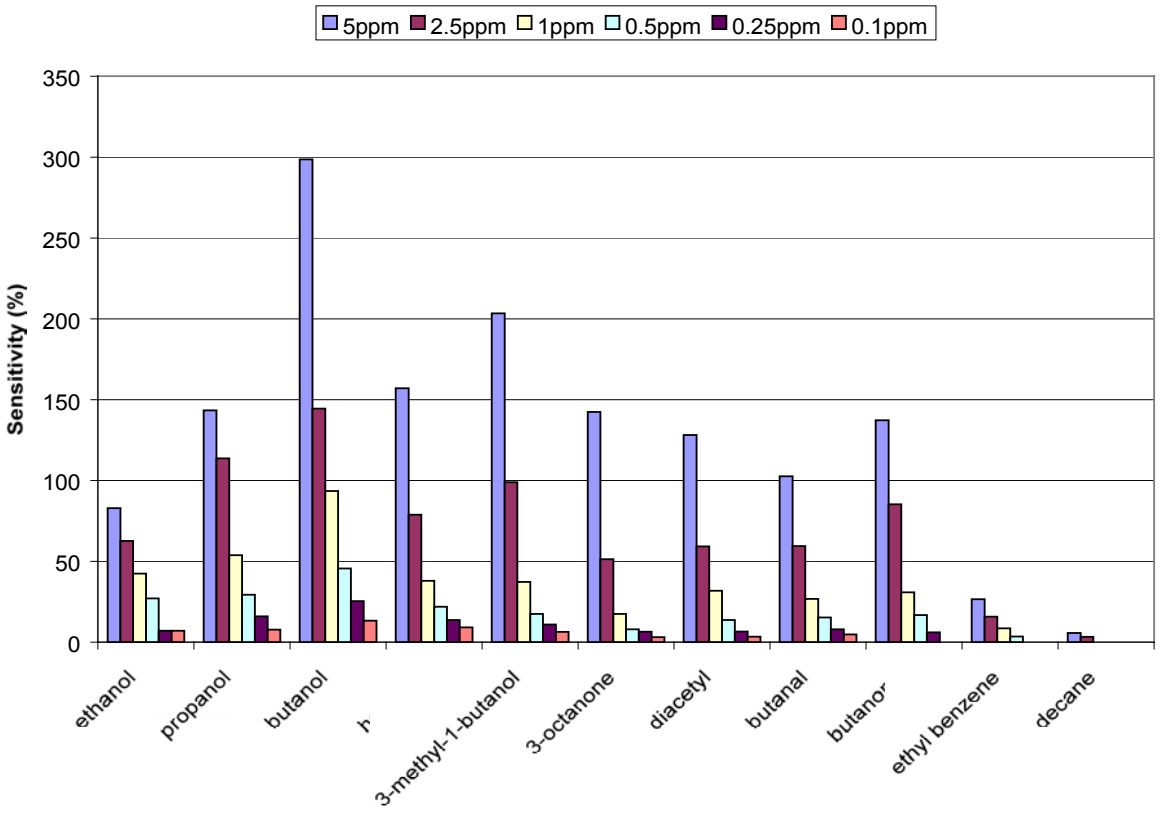


Figure 5: The Response Profile of the Tin Dioxide/Zinc Oxide Composite Sensor when Exposed to 11 Vapours at Six Different Concentrations under a Constant Flow of Dry Air at 1 litre/min

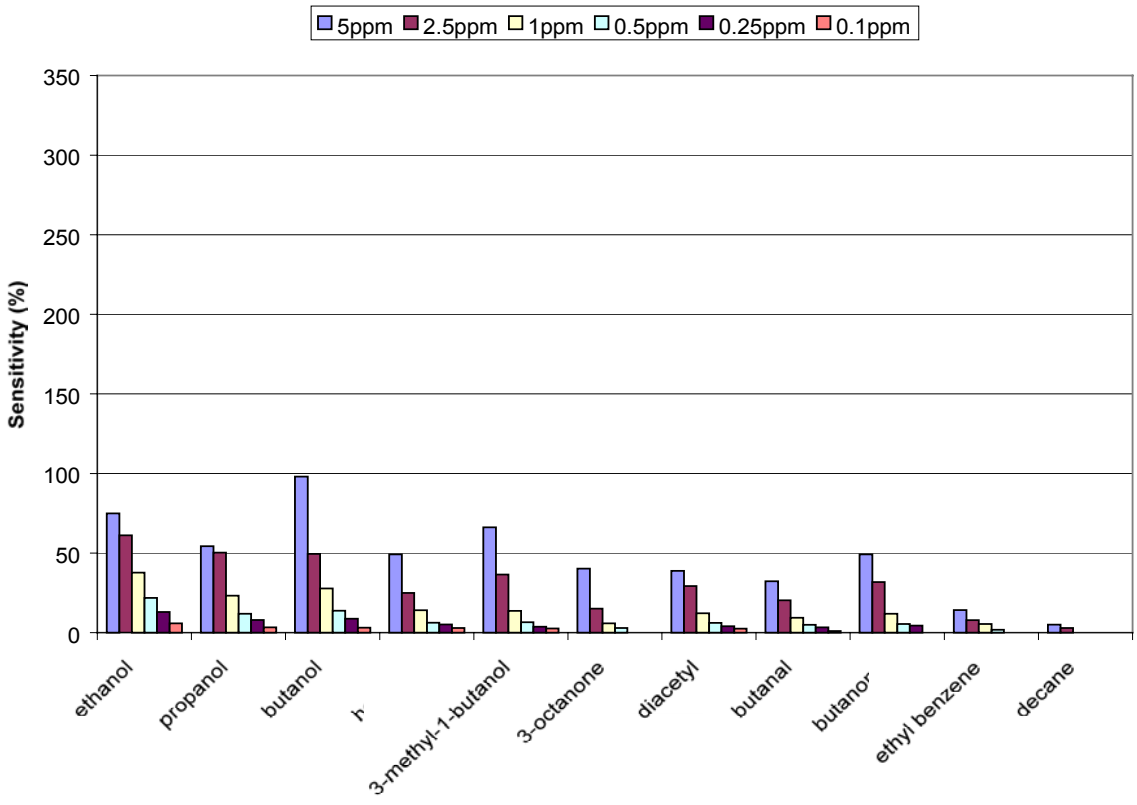


Figure 6: The Response Profile of the Tin Dioxide/Indium Oxide Composite Sensor when Exposed to 11 Vapours at Six Different Concentrations under a Constant Flow of Dry Air at 1 litre/min

down for the case of 1-hexanol and did not apply for secondary alcohols such as 3-methyl-1-butanol, but the reasons for this are unclear.

Under dry conditions the tin dioxide/zinc oxide sensor is more sensitive than the indium oxide/tin oxide composite sensor over the full spectrum of organic vapours tested, incorporating a range of chemical classes. Figures 7 and 8 show the same tests repeated at 100% RH. It can be seen that the indium oxide/tin oxide composite sensor exhibits higher sensitivity to a range of compounds at 100% RH, whereas the tin dioxide/ zinc oxide composite sensor displays a marked decrease in sensitivity, particularly to alcohol vapours. Furthermore, it can be seen that the indium oxide/tin dioxide sensor is now more sensitive to a number of the organic compounds when compared with the tin dioxide/zinc oxide composite sensor. The decrease in sensitivity of the tin dioxide/zinc oxide sensors was most marked when tested with alcohol vapours at 100% humidity whereas the responses to carbonyl-containing compounds, such as butanal and 3-octanone, were relatively unchanged. The responses to non-polar vapours such as decane and ethyl benzene were also not reduced drastically. For the case of indium oxide/tin dioxide sensors the responses to alcohol vapours were relatively unchanged when tested at 0% RH and 100% RH, with only a small reduction observed. However, the sensitivity to a range of carbonyl-containing compounds was increased when tested at 100% humidity compared to a parallel study in dry conditions. The response to non-polar compounds was also enhanced when operated at 100% relative humidity. This suggests there is some additive effect with water when using the tin dioxide/indium oxide sensor to detect various classes of organic compounds.

The mechanism of operation of single oxide sensors is little understood and studies which incorporate an extended range of relative humidities with mechanistic details of the effects of water vapour are rare. The use of composite sensors further complicates the complex nature of these interactions. However, composite materials of this type produce sensors that exhibit very high sensitivity and relatively high stability, and that can be made fairly reproducibly. This study has shown that with a limited range of materials and by employing facile production techniques a range of sensors that operate effectively under different environmental conditions can be produced. This is of use where a range of sensors with differing sensitivities and selectivities are required for array type devices. Previous work by our group and other groups has shown that changing the proportions of each material in the composite yields a wide range of sensor materials with very different sensing characteristics. This study has highlighted a composite sensor incorporating indium oxide/tin dioxide that gives high sensitivity to a range of vapours under 100% relative humidity conditions. This and other sensors from this study have been incorporated into a practical sensing device that is operated under 100% humidity at high flow rates. The prototype has been in operation with the same sensors for a number of months and has been used to determine wheat quality in laboratory trials.

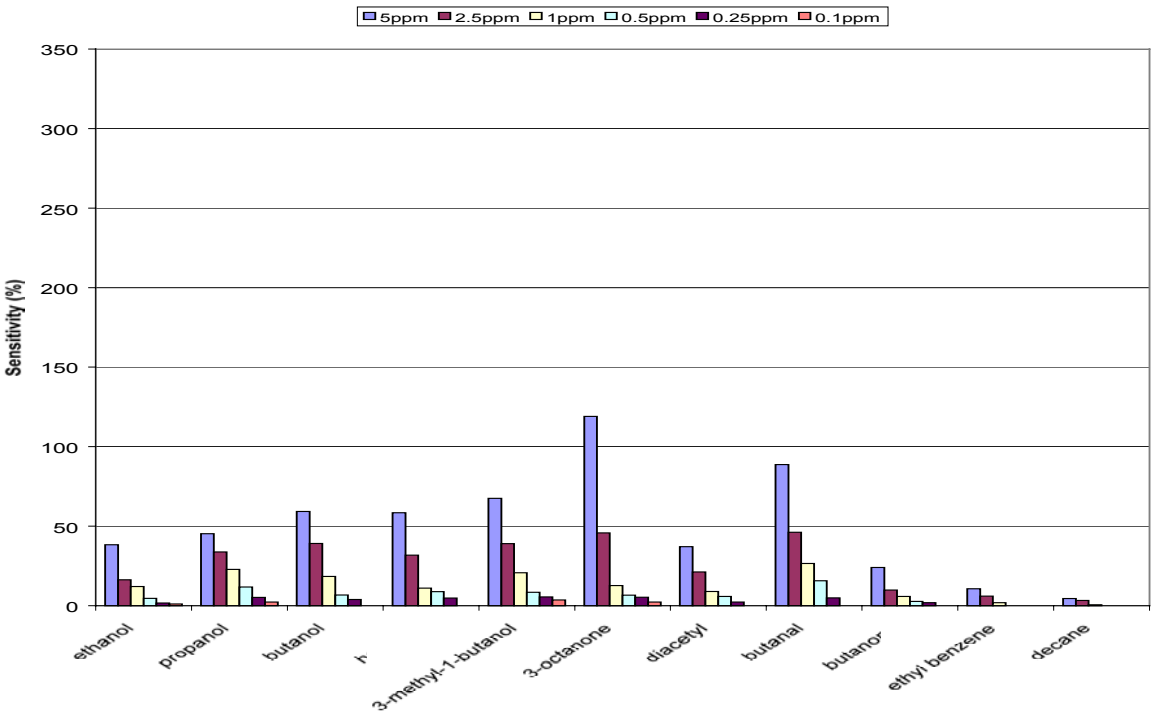


Figure 7: The Response Profile of the Tin Dioxide/Zinc Oxide Composite Sensor when Exposed to 11 Vapours at Six Different Concentrations under a Constant Flow of Humidified Air (100% RH) at 1 litre/min

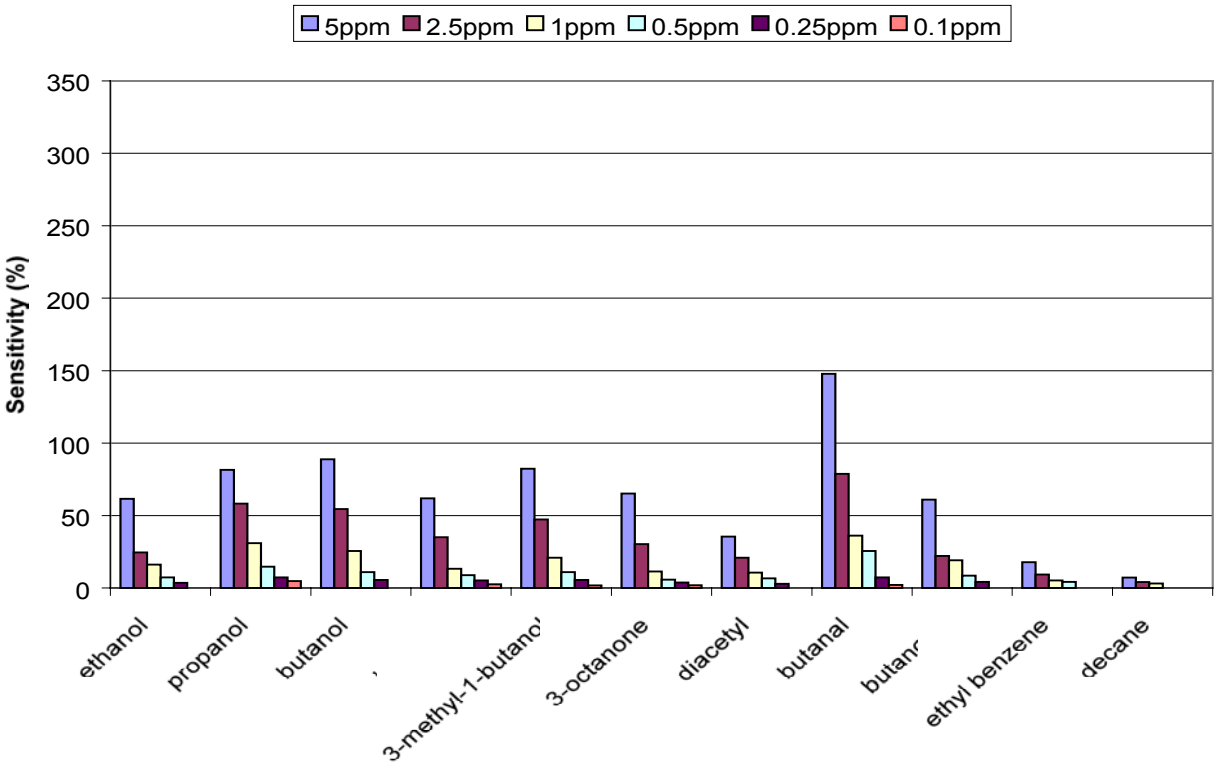


Figure 8: The Response Profile of the Tin Dioxide/Indium Oxide Composite Sensor when Exposed to 11 Vapours at Six Different Concentrations under a Constant Flow of humidified air (100% RH) at 1 litre/min

4.5 CONCLUSIONS

A range of sensors have been identified which exhibit high sensitivity to the volatiles emitted by fungi colonising wheat grain. The study has shown that composite sensors give additional advantages over single oxide sensors as well as the previously described enhancement in sensitivity. A composite sensor incorporating tin dioxide and indium oxide exhibited a high sensitivity to organic vapours, particularly alcohols, when operated under high humidity conditions. However, a composite sensor based on tin dioxide and zinc oxide which exhibited the highest sensitivity to organic vapours when operated under dry conditions suffered a significant loss of sensitivity when operated subsequently at high humidities.

The study gave some insight into the complex reactions occurring on metal oxide surfaces when exposed to organic vapours, particularly in the presence of water vapour. When a range of sensors were exposed to alcohol vapours in the presence of high humidity, a significant reduction in sensitivity (typically 50%) was observed compared with the results obtained under dry conditions, suggesting a strongly competitive effect with water. Conversely, when the same sensors were exposed to carbonyl-containing compounds and non-polar alkanes/aromatics in the presence of water vapour, only a relatively small reduction in sensitivity was observed. This may provide some evidence to suggest that different classes of organic compounds react at different sites on the metal oxide surface.

4.6 REFERENCES

Barsan, N. and Ionescu, R. (1993) Sensors and Actuators B **12**, 71.

de Lacy Costello, B.P.J., Ewen, R.J., Jones, P.R.H., Ratcliffe, N.M. and Wat, R.K.M. (1999) Sensors and Actuators B **61** (1-3), 199-207.

Gopel, W. and Schierbaum, K.D. (1995) Sensors and Actuators B **26-27**, 1.

Ippommatsu, M., Ohnishi, H., Hirokazu, H. and Matsumoto, T. (1991) Journal of Applied Physics **69**, 8368.

Magan, N. and Evans, P. (1999) Journal of Stored Products Research **36**, 319-340.

Reddy, C.V.G., Manorama, S.V. and Rao, V.J. (1999) Sensors and Actuators B **55**, 90.

Xu, C., Tamaki, J., Miura, N. and Yamazoe, N. (1992) Journal of Materials Science **27**, 963.

Yu, J.H. and Choi, G.M. (1998) Sensors and Actuators **52**, 251.

PAPER 5

APPROACHES TO A SENSOR SYSTEM FOR THE EARLY DETECTION OF MICROBIALLY LINKED SPOILAGE IN STORED WHEAT GRAIN

B.P.J. de Lacy Costello, R.J. Ewen, H. Gunson[1], N.M. Ratcliffe,
P.S. Sivanand and P.T.N. Spencer-Phillips[1]
Centre for Research in Analytical Material and Sensor Sciences, Faculty of Applied Sciences,
University of the West of England, Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY

[1]Centre for Research in Plant Science, Faculty of Applied Sciences, University of the West of England,
Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY

5.1 ABSTRACT

Sensors based on composites of metal oxides were fabricated and tested extensively under high humidity and high flow conditions to vapours reported to increase in the headspace of wheat grain (*Triticum aestivum* cv. Hereward) colonised by fungi. The sensors that exhibited high sensitivity to target vapours combined with high stability were selected for inclusion in a four-sensor array prototype system.

A sampling protocol aligned to parallel GC-MS and human olfactory assessment studies was established for use with the sensor system. The sensor system was utilised to assess irradiated wheat samples that had been conditioned to 25% moisture content and inoculated with pathogens known to cause spoilage of grain in storage. These included the fungi *Penicillium aurantiogriseum, Penicillium vulpinum, Penicillium verrucosum, Fusarium culmorum, Aspergillus niger* and *Aspergillus flavus* and the actinomycete*, Streptomyces griseus.* The sensor system successfully tracked the progress of the infections from a very early stage and the results were compared with human olfactory assessment panels run concurrently.

A series of dilution studies were undertaken using previously infected grain mixed with sound grain, to improve sensitivity and maximise differentiation of the sensor system. An optimum set of conditions including incubation temperature, incubation time, sampling time and flow rate were ascertained utilising this method. The sensor system differentiated samples of sound grain from samples of sound grain with 1 % (w/w) fungal infected grain added.

The previous study was focused on a laboratory-based sensor device that may be used in an intake laboratory environment to assess grain quality. However, to establish the versatility of the current prototype system an experimental grain silo was constructed. The silo, which permitted the sampling of air from multiple points , was filled with sound grain after a mesh bag containing *P. aurantiogriseum* inoculated grain (1% of the total

mass of grain) had been placed at its base. After sampling for two weeks the results showed that the system could monitor changes in the environment of the grain silo. However, at sampling points adjacent to the inoculated grain the sensor outputs were reduced; the reasons for this are unclear.

5.2 INTRODUCTION

There is a need for instrumentation to provide an early indication of microbial spoilage of grain (see previous papers in this report). A GC-MS study (Paper 3) tracked the formation of volatile organic compounds produced from the point of inoculation of grain with known fungal species. A large range of simple and branched alcohols were observed to increase in the headspace above the infected grain. Whilst few compounds were found to be specific to a certain infection, the concentrations of the compounds were markedly different when different infecting organisms predominated. For example, *Penicillium* infections gave large increases in the concentrations of many volatile organic compounds, whereas *Aspergillus* infections generally gave lower concentrations, although the broad spectrum of volatiles was comparable.

Chemical sensors constructed from metal oxides and heated to elevated temperatures exhibit high sensitivity to organic vapours, particularly alcohols (Gong *et al*., 1999). Other classes of chemical sensors, such as conducting polymers based on polypyrrole, have also been shown to exhibit good sensitivity to a range of polar compounds (Partridge *et al*., 1996). However, the selectivity of these sensors is poor, and therefore they are commonly combined to produce large arrays which incorporate sensors with slightly different sensitivities and selectivities (Gardner and Bartlett, 1993). Arrays of this type, particularly those based on conducting polymers, have been utilised extensively in the analysis of food quality (Di Natale *et al*., 1998). This has included a number of applications where the aim was the differentiation of sound produce from produce with odour taints due to microbial spoilage (Vernat Rossi *et al*., 1996; Pearce *et al*., 1993; Mistra *et al*., 1998). A number of groups have focused on the problems of wheat spoilage and arrays have been adapted to classify wheat grains into distinct classes (Borjesson *et al*., 1996; Stetter *et al*., 1993). However, many of the devices utilised to date suffered from a lack of sensitivity, and some humidity related problems were also identified in commercial trials (Zannoni, 1995).

Previous work has focused on the production of highly sensitive sensors capable of operation over a wide range of environmental conditions. A sensor capable of detecting low ppb levels of alcohols such as 1-butanol has been developed based on a novel composite of two metal oxides (de Lacy Costello *et al*., 1999). Sensors of this type have been used in combination with conventionally produced sensors in an array to monitor the quality of potato tubers in store (de Lacy Costello *et al*., 2000). This application involved the detection of very low concentrations of volatile metabolites released due to the microbial spoilage of stored produce.

The aim of the present work was to produce an extended range of composite type sensors targeted to compounds identified in the headspace above spoilt wheat grain samples. This would then enable an array of these sensors to be produced in order to address the problem of the early detection of spoilage in grain stores, and the detection of spoiled grain destined for processing.

5.3 MATERIALS AND METHODS

5.3.1 Testing of Gas Sensors to Organic Vapours

Sensors (produced as described in Paper 4) were assessed in an in-house designed test chamber (de Lacy Costello *et al*., 1999). The sensors were tested with low concentrations (1 and 5ppm) of an extended range of organic compounds (30 in number) which had been identified as being associated with fungal contamination. Sensors from the preliminary investigations were taken forward to the next phase and tested with a reduced set of organic volatiles (ethanol, 1-propanol, 1-butanol, 1-hexanol, 3-methyl-1-butanol, 3-octanone, diacetyl, butanal, 2-butanone, ethyl benzene and decane) at six different concentrations ranging from 0.1ppm to 5ppm. The tests were performed under a flow of 1 litre/min blended air because in any prototype device the air from grain samples would be passed over the sensors to aid reversibility and reduce sampling times. The sensors were tested at a range of relative humidities to assess the effects on sensitivity and stability. The best sensors were selected for inclusion in a four-sensor array prototype.

5.3.2 Sensor Array Specifications for Prototype System

A four-sensor prototype system was fabricated to incorporate a tin oxide:zinc oxide composite sensor, an indium oxide: tin oxide sensor, an indium oxide sensor and an indium oxide:tin oxide:zinc oxide composite sensor. Each sensor was mounted in an individual stainless steel chamber of $10cm^3$ volume. A constant flow of 1 litre/min humidified air was maintained across the sensors using blended air from a cylinder (BOC Gases, Guilford, Surrey) passed through a Dreschel bottle of distilled water. When a sample of air from grain was to be introduced, a series of manually controlled valves diverted the humidified air through the grain sample and then directly to the sensor system. To reduce errors, an auto-sampler system was constructed incorporating computer-controlled valves. Software written in-house enabled the programming of a complex series of valve operations to enable a controlled sampling and purge regime.

The system itself was portable and could be powered by a sealed lead-acid battery. The resistance of the sensors and the operating temperature of each sensor were displayed on a LCD mounted on the outer casing of the prototype. The sensor system could be interfaced to a personal computer, allowing the heater temperatures as well as the pass/fail and system settling criteria to be set and downloaded to the system.

Once the values had been downloaded it was not necessary to maintain the computer link to operate the system. However, when the system was being used in a laboratory-based environment it was interfaced via a RS-232 (com) port to a PC. Software written in-house allowed real time traces of sensor resistance and temperature to be observed and recorded. The valve operation software for the auto-sampler was integrated with the sensor software so that events such as purge and sample times were marked automatically on the traces. Data from the sensor runs could be converted directly into a spreadsheet to allow statistical analysis of the data.

5.3.3 Assessment of Grain Quality Using Chemical Sensors

Spoilage of grain was induced and monitored using the approaches described in earlier papers in this report.

The apparatus in Figure 1 was utilised for the assessment of grain by the sensor system. Grain was incubated at 50°C for 1 hour and then humidified air was passed through the sample and then directly to the sensor system via a tube connected to the filter in Figure 1. The sampling time used was 15 seconds and the sensors were left to recover fully (reach previous baseline value) before exposure to subsequent samples. As with the human sniffing trials, grain was assessed over a number of weeks to track the progress of any infection.

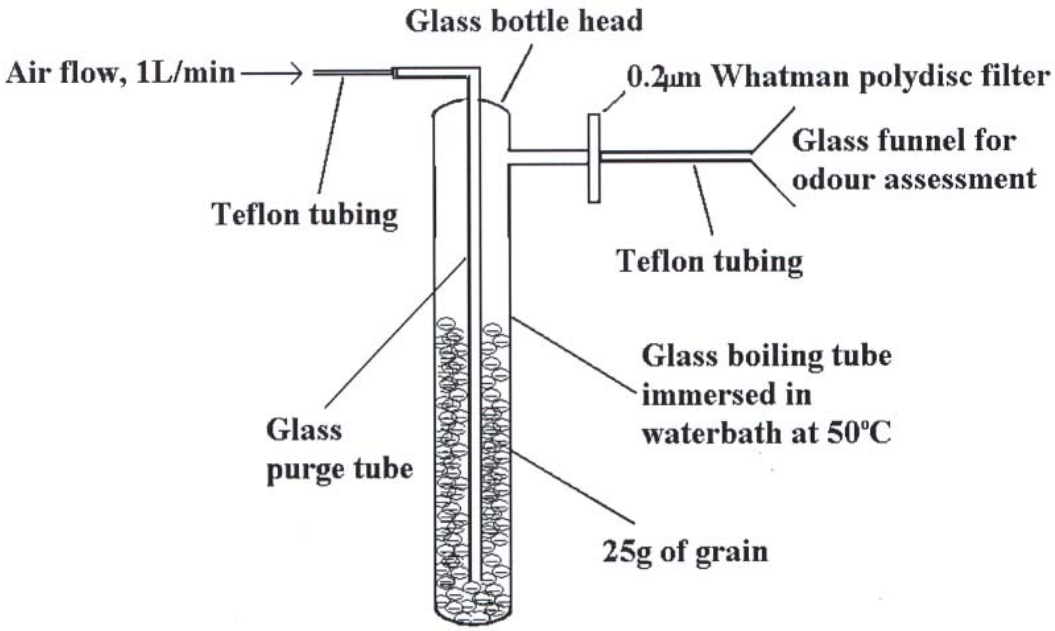


Figure 1: Schematic of Apparatus Used for Human Assessment Panels and with Minor Modifications for Analysis with the Sensor System

5.3.4 Dilution Studies to Determine System Sensitivity

A grain sample inoculated with *P. aurantiogriseum* which had developed characteristic musty odours and visible signs of fungal infection 42 days after inoculation was used in a series of dilution studies. The inoculated grain (irradiated, at 25°C moisture content) was mixed with sound, dry, non-irradiated grain (13% moisture content) at a number of different concentrations ranging from 0.5% to 10% w/w. These were then assessed against uninfected grain as a control using the sensor system as in 5.3.3.

5.3.5 Optimisation of Sampling Protocol

5.3.5.1 Altering Sampling Method and Sensor Set-up

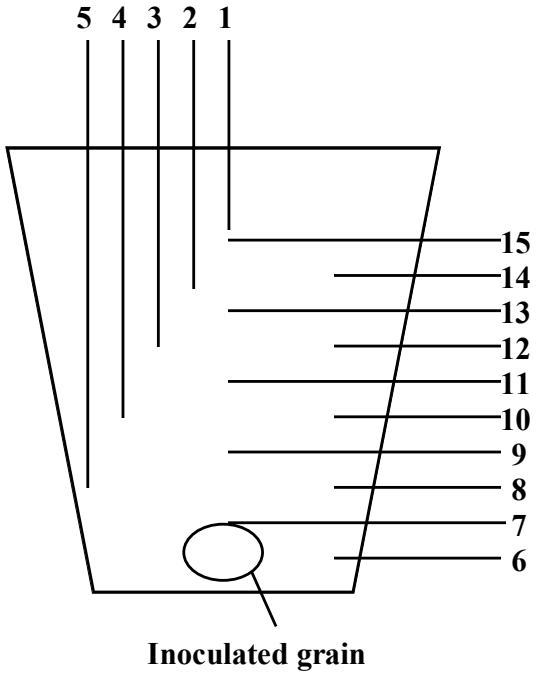
Parameters such as incubation temperature, incubation time, sensor temperature, flow rate, sampling time and sensor recovery time were varied in order to enhance the ability of the prototype to differentiate between sound and infected grain.

5.3.5.2 Grinding Studies

A number of control and spoilt grain samples were ground using a Protimeter Digital Grain Master to determine the effects of this pre-treatment on the differentiation capabilities of the prototype. The ground samples were then incubated at different temperatures (20°C, 30°C, 50°C, 80°C) for an hour prior to sampling by the method described in 5.3.3.

5.3.6 Grain Silo Experiment

An experimental grain silo was constructed to test whether the prototype system could be used for on site monitoring of grain storage facilities. The silo (see Figure 2) had a capacity of 70 litres and could house approximately 70 kg of grain. Fifteen evenly-spaced sampling points (10 at the side and 5 at the top surface) were incorporated where air could be sampled by the sensor system. In this mode the sensor system used a small diaphragm pump (KNF Neuberger GmbH, Freiburg) to sample air. Temperature and humidity sensors were placed at the same points so that these data could be correlated with sensor outputs. An experiment was established whereby 700g of irradiated grain at 25% moisture content and inoculated with *P. aurantiogriseum* (7 days post-inoculation) was put in a mesh bag and placed near to the bottom and exactly in the centre of the silo. The silo was then filled with sound non-irradiated grain and the changes in sensor outputs measured over a two-week period.



Each point had a sensor capable of measuring relative humidity and temperature. There was also a tube positioned for sampling the intergranular air from this point with the four sensor system.

The air was sampled for 2 minutes at a flow of 200cm^3/minute

Figure 2: A Schematic of the Experimental Grain Silo Showing Sampling Points for Humidity, Temperature and Sensor System Measurements and Their Relative Position with Respect to a Pocket of Inoculated Grain

On day sixteen of the experiment 5ml of air was sampled using a gas tight syringe from point seven of the silo and this was injected into a GC system set up to measure carbon dioxide concentrations. The instrument used was a Pye series 104 chromatographic oven, with a PE Nelson 900 series interface linked to a personal computer. The column used was a Porapak Q 20-100 mesh with helium as the carrier gas. The analysis was performed three times over the space of a day to ascertain the carbon dioxide levels and the levels at point six were also measured using the same method.

5.4 RESULTS AND DISCUSSION

5.4.1 Sensor Responses to Organic Vapours

The sensors tested exhibited high sensitivity to sub-ppm concentrations of a range of organic compounds associated with spoilage infections. Whilst the sensitivity was reduced at high humidity, it was considered that the sensors should be operated under these conditions to eliminate any effects from changes in moisture content of the grain samples. Thus the best sensors were selected for inclusion in the prototype system on the basis of sensitivity and stability when operated under a constant flow of humidified air. The sensors selected were indium oxide, tin oxide:zinc oxide composite, indium oxide:tin oxide composite and an indium oxide: tin oxide:zinc oxide composite. More details on the sensitivity and humidity effects on these and other sensors has been reported elsewhere (de Lacy Costello *et al*., in press).

5.4.2 Assessment of Grain Quality Using a Four-sensor Array

5.4.2.1 Analysis of Grain Inoculated with Known Spoilage Organisms

Grain was inoculated with a range of spores from various microorganisms and the growth-vapour response profiles with time were studied using vapour sensors. Figures 3 to 5 show the changes in sensor system output against incubation time after inoculation. Data for day 1 was not included as there were large variations in the sensor outputs, thought to be due to free water which had not been fully adsorbed by the grain during moisture conditioning. All the graphs, except that for *Asp. flavus*, exhibited a rise in sensor system output over the initial period of incubation. This then reached a peak but subsequently decreased in each case. Both the time at which the peak was reached and the overall size of the peak were found to be indicative of the infecting organism. For example, *Aspergillus* species gave far lower sensor outputs than the other spoilage species studied whilst also reaching a peak after a shorter time period. Visually it could be seen that the *Aspergillus* species produced a proliferation of spores at a far earlier stage than the other species studied. The results correlate well with a parallel GC-MS study conducted by CCFRA, where the overall concentrations of volatiles detected above grain inoculated with *Aspergillus* species were far lower than the concentrations detected above grain inoculated with *Penicillium* species.

Figure 6 shows that the changes in sensor outputs when exposed to a SDW inoculated control over the same time period mirrored those of the inoculated samples. After some time these controls were found to have become infected and therefore the sensor system was giving an early warning of this infection process. The results show that wheat at moisture contents of 25% is very prone to infection. Repetition of this work showed that lower responses were obtained for a set of control samples; however, some infection was still present as indicated by microbiological investigations.

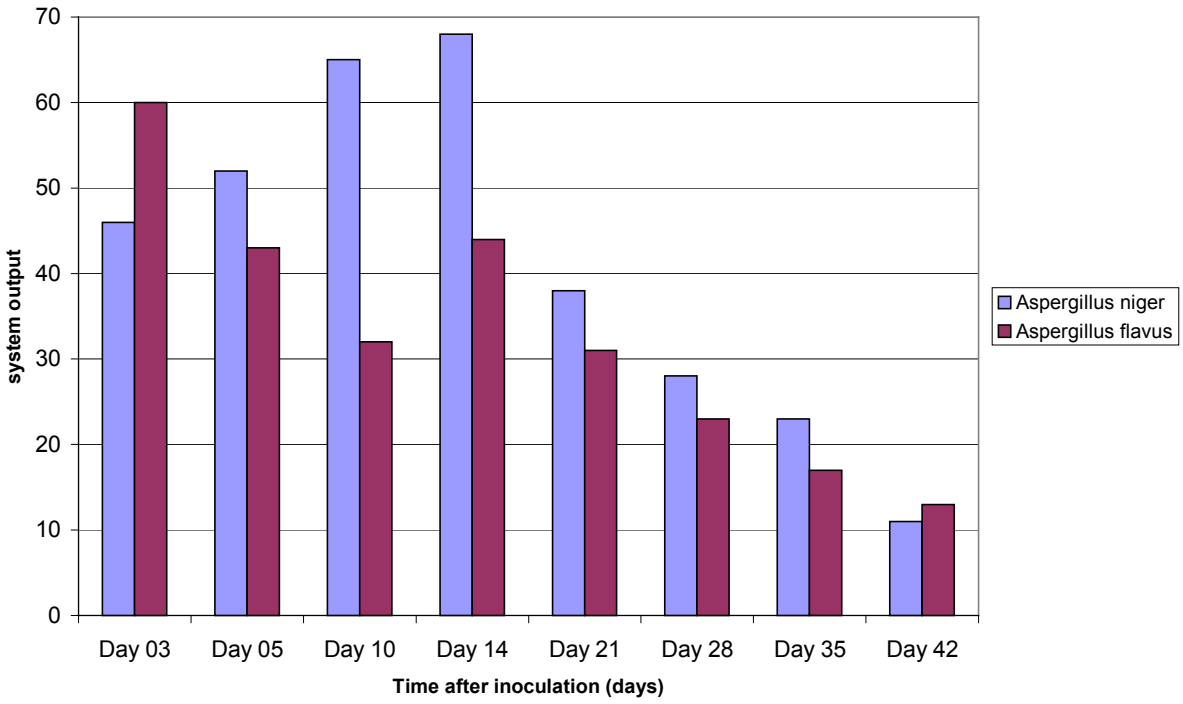


Figure 3: Prototype Sensor System Output for Irradiated Wheat Grain Inoculated with *Aspergillus niger* and *Aspergillus flavus* over a 42-Day Period

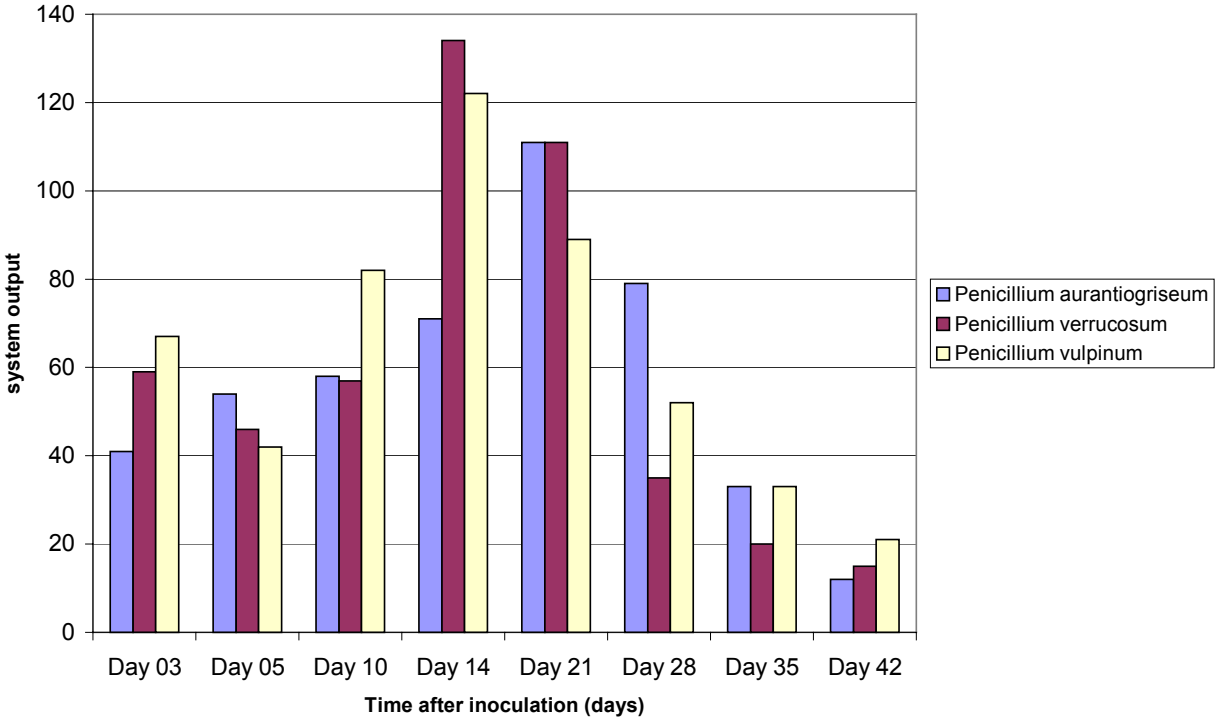


Figure 4: Prototype Sensor System Output for Irradiated Wheat Grain Inoculated with *Penicillium aurantiogriseum*, *Penicillium vulpinum* and *Penicillium verrucosum* over a 42-Day Period

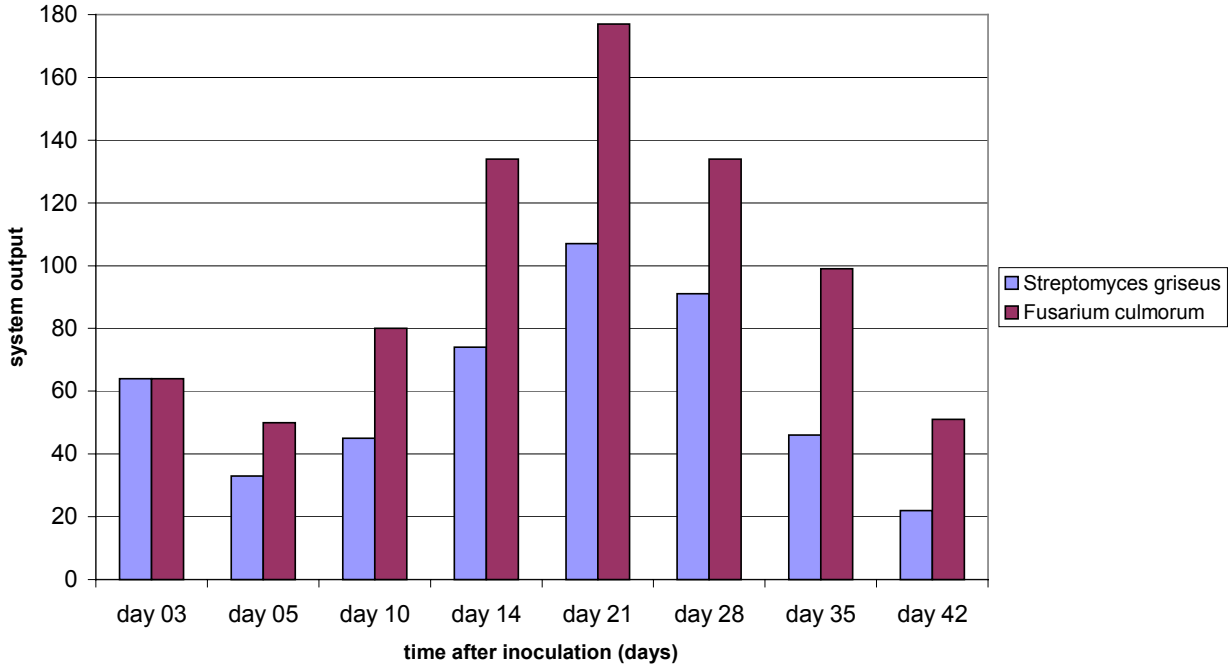


Figure 5: Prototype Sensor System Output for Irradiated Wheat Grain Inoculated with *Streptomyces griseus* and *Fusarium culmorum* over a 42-Day Period

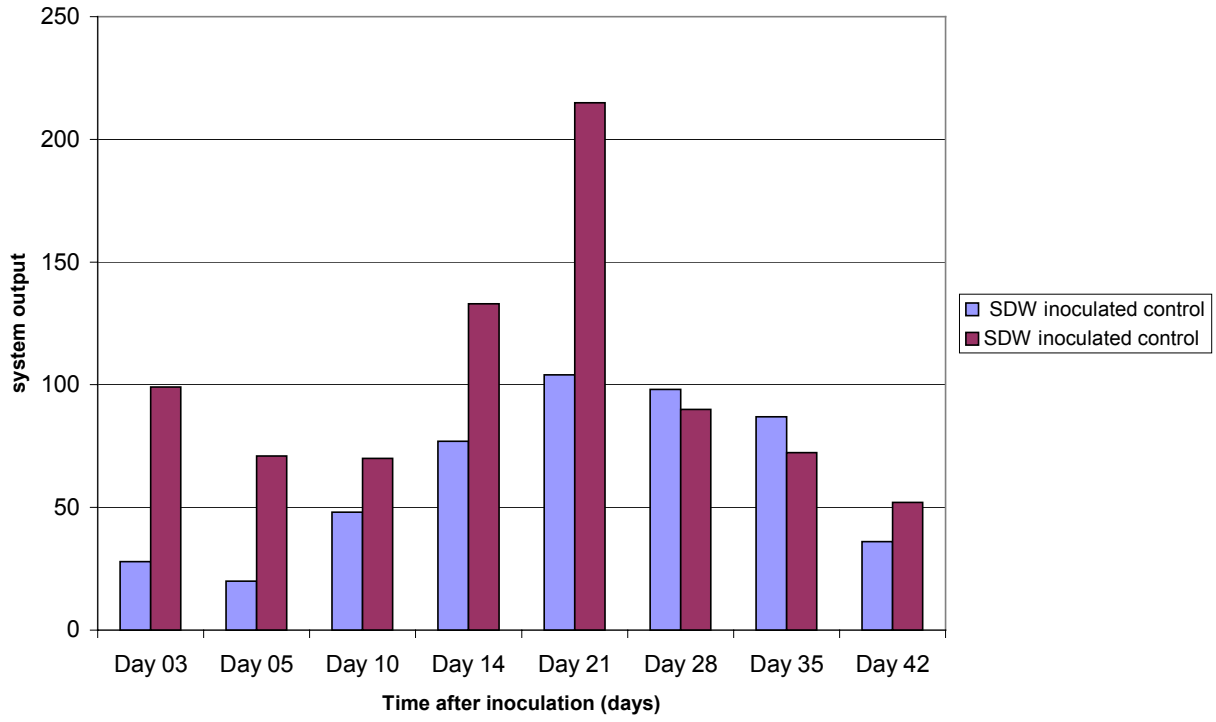


Figure 6: Prototype Sensor System Output for Irradiated Wheat Grain Inoculated with Sterile Distilled Water over a 42-Day Period

The results show that the sensor system was able to detect differences in the volatile profiles above inoculated grain samples. The system was able to track the infection process as it progressed and the rises observed in sensor outputs over time mirror a detected rise in volatile organic compounds above inoculated wheat samples, determined by a parallel GC-MS study carried out by CCFRA.

5.4.2.2 Dilution Studies to Ascertain System Sensitivity

Previously the sensor system had been utilised for the detection of grain samples with high levels of microbial infection, but these levels of infection and thus vapour concentrations are likely to be far lower in commercial samples. Therefore, this experiment was devised to test the sensitivity of the system to volatiles emitted by grain with associated musty odours. It is also possible that it could mirror the commercial situation in another way as pockets of highly infected grain forming in a store could be mixed with sound grain when emptying and distributing the store's contents.

Figure 7 shows that the system gives a continual increase in sensor system output with increased percentage of microbially infected grain and could, with confidence, detect 1% (w/w) levels of the infected grain when it had been mixed with sound dry grain. It was possible to detect lower percentages than 1% but with less certainty, due to the natural variability likely to be experienced within a number of control samples combined with expected errors in the repeatability of the measurement process and small effects from sensor drift. Note that the sensitivity values for Figures 3-6 should not be compared with those in Figures 7-9 as changes in system set up and sampling protocol had been made in the latter work to optimise sensitivity and differentiation.

5.4.2.3 Effects of Grinding Grain Samples

It was thought that grinding of control and infected samples and subsequent incubation at elevated temperature prior to analysis with the sensor system would improve the differentiation currently exhibited by the sensor system. However, whilst the differentiation for badly spoilt samples was improved, the differentiation was greatly reduced for samples where the infection process had ceased due to lack of available moisture and for samples which had merely been in contact with spoilt grain. Thus, a dilution effect was observed for samples of this type and, as they are the most likely to be borderline accept/reject samples from a commercial store, it was considered that grinding the samples would not offer any benefits.

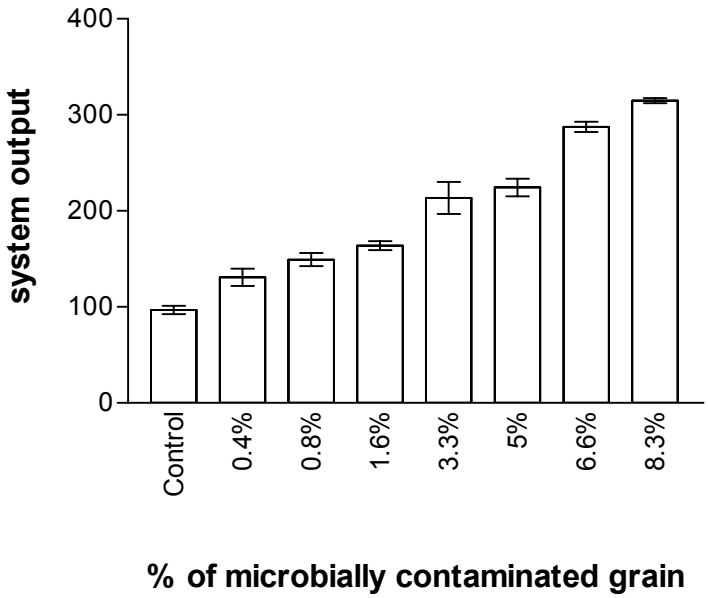


Figure 7: System Output for Irradiated Grain Samples Containing Different Proportions of *Penicillium aurantiogriseum* Infected Grain Incubated at 50°C for 1 Hour and Sampled for 15 Seconds

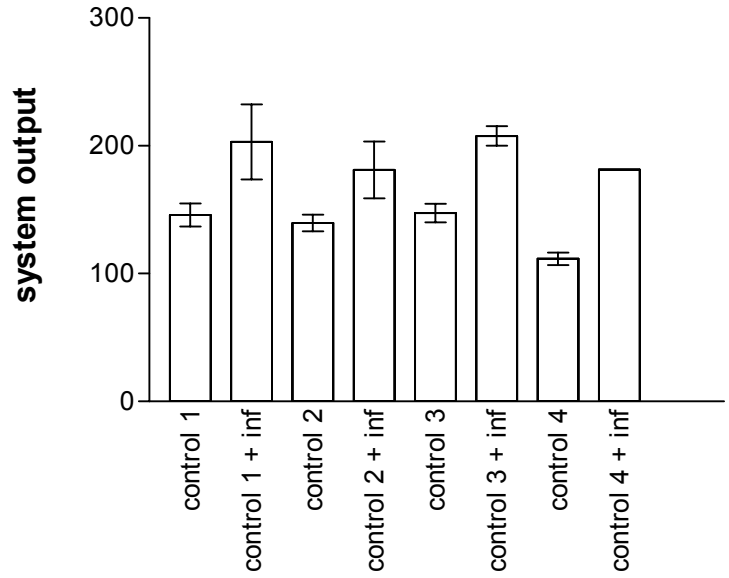


Figure 8: System Output for Sound and Infected (1.5% w/w *P. aurantiogriseum* Infected Grain Mixed with Sound Grain) Grain Samples Incubated at 50°C for 1 Hour and Sampled for 20 Seconds

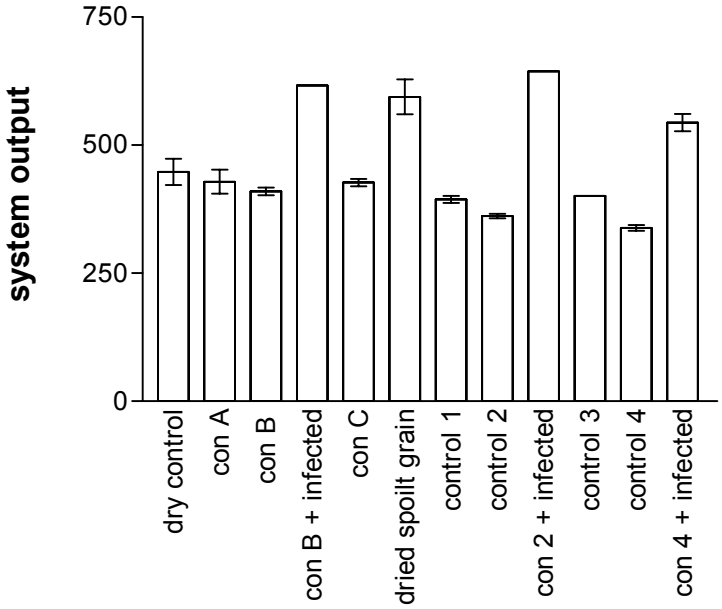


Figure 9: System Output for Sound and Infected Grain (as in Figure 8)
Incubated at 80°C for 15 Minutes and Sampled for 20 Seconds

5.4.2.4 Optimisation of Sampling Protocol

The method for sampling with the sensor system remained unchanged after an initial optimisation study that had aligned it with the parallel GC-MS and human assessment studies. Grain samples (approximately 30 g) were heated for 1 hour in a water bath at 50°C and then the air at a flow of 1 litre/minute was passed through the grain sample and sampled via an auto-sampler by the sensor system for 15 seconds. After each sample, the sensor system was allowed to recover for at least 15 minutes prior to analysis of the next sample. However, the process for measurement of a single grain sample was considered to be too slow, particularly if the device were to be used in grain stores or even in an intake laboratory where an answer is required in a matter of minutes. Therefore, experiments were undertaken using samples of control grain and control grain with 1% infected wheat added to reduce the sampling time, whilst ensuring that the differentiation between samples was maintained at current levels.

Figures 8 and 9 show the results of changing experimental parameters on the sensitivity/differentiation of the sensor system. It was found that heating the grain samples for a shorter period of time (10-15 minutes) at a higher temperature (80°C) gave equivalent results to the previous best method. A high flow rate had to be maintained to ensure that good desorption kinetics were observed for the sensors. It was found that a longer sampling time of 20 seconds and subsequently a longer recovery time for the sensors of 20 minutes gave appreciably better differentiation and improved the repeatability of the measurements. From Figure 9 it can be seen that a suitable sound/infected threshold using this experimental procedure (incubation at a higher

temperature of 80°C for a period of 15 minutes and with a sampling time of 20 seconds) and method of data analysis would be 500. It was considered that the present prototype system would have applications in a grain intake laboratory for checking the odour contamination of incoming samples.

5.4.2.5 Calibration of Prototype System

The sensor system was always calibrated with ethanol prior to and at the end of each day to assess the change in the sensitivity of the sensors. A study was carried out which compared an average sensitivity to ethanol with an average value for a series of control grain samples over a number of days. As this factor remained constant despite small changes in the overall sensitivity of the sensors, it was ascertained that the ethanol sensitivity of the sensors correlated directly with their sensitivity to control grain. Over a period of two weeks the sensitivity of the sensors changed by a few percent; however, the ratio between a control grain sample and a standard ethanol injection remained constant at 2:1. Therefore, it was assumed that ethanol sensitivity could be used to normalise sensor results in order that any set accept/reject thresholds remained viable.

5.4.3 Results of Laboratory Grain-silo Experiment

Figure 10 shows the outputs of the sensor system when sampling from each of the 15 sampling points. The graph shows only measurements made after day 2 when the silo had reached some degree of equilibrium. It can be seen that as sampling was undertaken further away from the inoculated grain, the sensor outputs decreased, apart from where the air was sampled from directly above the inoculated grain (point 7) and the point directly adjacent to the inoculated grain (point 6), when the lowest sensor outputs were obtained.

Experiments were undertaken to explain the low results at these points. Measurement of the relative humidity of the air at points 6 and 7 revealed that it was considerably higher than at other points, approximately 65 and 75%, while all the other sampling points were shown to be surrounded by air of approximately 60% RH, at c. 20°C. Our research has shown that increases in humidity of this magnitude would be expected to produce a drop in sensor system output. It should be noted that no control of humidity was undertaken in the silo experiments. As an example, a sensor system output of 62 would fall to 35 with a change of relative humidity from 0 to 100%. This clearly could explain the results and offers the possibility of normalising the sensor system output, if information on the humidity and temperature is available. However, due to the complex nature of these interactions, this would require further work before any reliable system could be implemented. It highlights the reasons for the chosen protocol where humidity control is routinely undertaken and emphasises the difficulties in making a truly portable device when the control of humidity proves necessary.

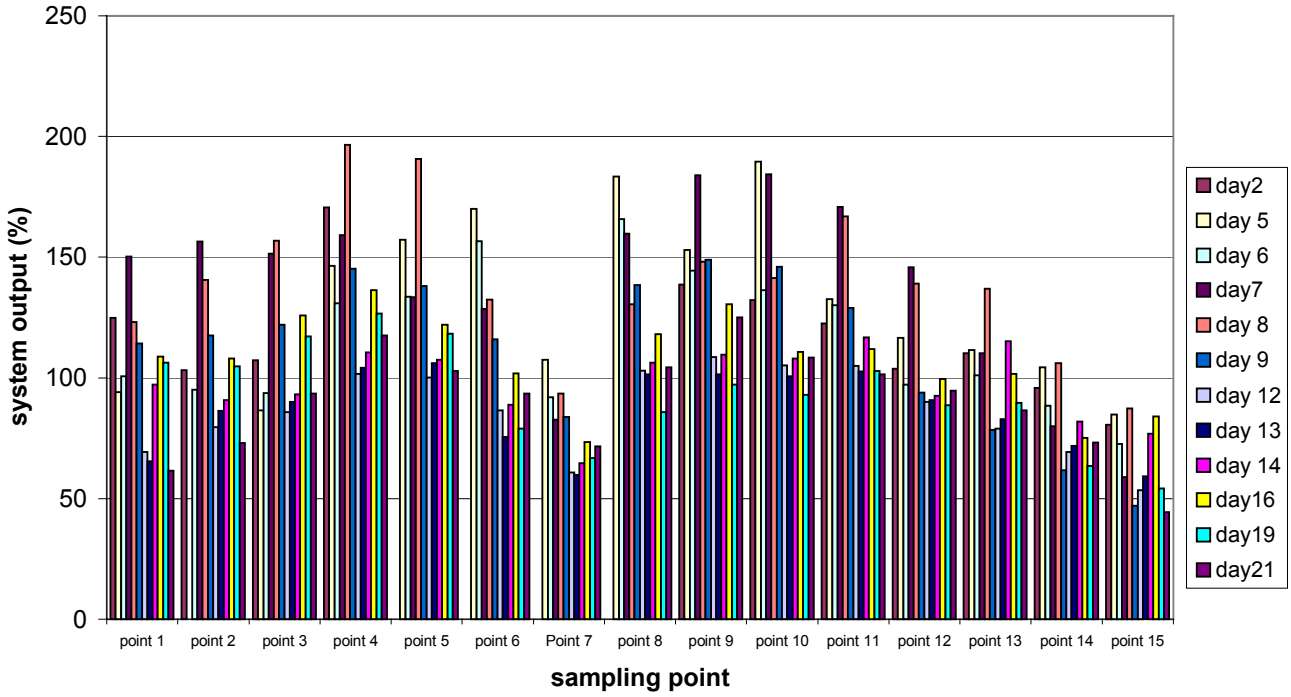


Figure 10: System Output at 15 Separate Sampling Points in a Simulated Silo Containing 70 kg of Non-irradiated Sound Grain with a 700 g Pocket of Irradiated Grain Inoculated with *P. aurantiogriseum* on Day 1 of the Experiment

The carbon dioxide levels were measured and found to be slightly higher than at ambient levels at points 6 and 7. Experiments were then undertaken to assess the responses of the sensors to carbon dioxide and organic vapours. Responses to low concentrations of ethanol (an alcohol given off by infected wheat) in a flow of carbon dioxide at 200cm^3/minute for 2 minutes gave similar results to ethanol in air, therefore this clearly was not the reason for the results.

The silo was emptied after 21 days and the grain inspected at different levels, both visually and by measurement of the moisture content. The experiment did show that there was little spread of the infection within the silo and negligible changes in the moisture content of the grain over time. So the experimental set up modelled well a possible real-life situation where water collects at specific points (usually top and bottom) in a store and leads to discrete pockets of infected grain. The results show that the sensor system could be used to give a measure of infection within a store.

The system in its current form could not be used as a static device to monitor changes in a stores environment with time. However, the work does show that this is a promising area for further research.

5.5 CONCLUSIONS

A prototype sensor system has been shown to be responsive to organic compounds emitted by wheat grain infected by a range of spoilage microorganisms. The system was also sensitive to low levels of inoculated grain mixed with sound grain, and was considered to be able to differentiate sound grain from the same grain with 1% inoculated grain added. The system was operated at a constant humidity and temperature, and under these operating conditions the effects of water vapour on the system are nullified and the decision on grain quality is predominantly based on the release of volatile organic compounds. Even though there is a strong link between moisture content and microbial spoilage it does not necessarily follow that wet grain is already spoilt or that dry grain has not been previously spoilt by fungal contamination. Therefore, any decision on quality must be based on an increase in certain organic volatiles and the prototype system reported here was able to do this reliably under laboratory conditions. As the system performed well under laboratory conditions it is now being assessed in a pilot industrial trial. The grain silo experiments revealed considerable difficulties with this approach, and batch sampling by heating small samples appeared the most promising test approach.

5.6 REFERENCES

Borjesson, T., Eklov, T., Jonsson, A., Sundgren, H. and Schnurer, J. (1996) Cereal Chemistry **73**, 457.

de Lacy Costello, B.P.J., Ewen, R.J., Gunson, H.E., Ratcliffe, N.M. and Spencer Phillips, P.T.N. (2000) Measurement Science and Technology **11**, 1685.

de Lacy Costello, B.P.J., Ewen, R.J., Jones, P.R.H., Ratcliffe, N.M. and Wat, R.K.M. (1999) Sensors and Actuators B **61** (1-3), 199.

de Lacy Costello, B.P.J., Ewen, J.R., Ratcliffe, N.M. and Sivanand, P.S. (in press) Paper 4, HGCA Report 293e.

Di Natale, C., Macagnano, A., Paolesse, R., Mantini, A., Tarizzo, E., D'Amico, A., Sinesio, F., Bucarelli, F.M., Moneta, E. and Quaglia, G.B. (1998) Sensors and Actuators B **50**, 246.

Gardner, J.W. and Bartlett, P.N. (1993) Sensors and Actuators B **18**, 211.

Gong, H., Wang, Y.J., Teo, S.C. and Huang, L. (1999) Sensors and Actuators B **54**, 232.

Magan, N. and Evans, P. (1999) Journal of Stored Products Research **36**, 319-340.

Mistra, S.C.K., Suri, A., Chandra, S., Kumar, N. and Sethi, V.C. (1998) Journal of Scientific and Industrial Research **57**, 897.

Partridge, A.C., Harris, P. and Andrews, M.K. (1996) Analyst **121**, 1348.

Pearce, T.C., Gardner, J.W., Friel, S., Bartlett, P.N. and Blair, N. (1993) Analyst **118**, 371.

Stetter, J.R., Findlay, M.W., Schroeder, K.M., Yue, C. and Penrose, W.R. (1993) Analytica Chimica Acta **284**, 1.

Vernat Rossi, V., Garcia, C., Talon, R., Dennoyer, C. and Berdague, J.L. (1996) Sensors and Actuators B **37**, 43.

Williams, J.S. and Brown, H.M. (in press). Paper 3, HGCA Report 293e.

Zannoni, M. (1995) Scienza e Technico Lattiero-Casearia **46**, 277.

PAPER 6

TRIAL OF A PROTOTYPE SENSOR SYSTEM FOR THE DETECTION OF FUNGAL SPOILAGE IN A COMMERCIAL WHEAT GRAIN INTAKE LABORATORY

B.P.J. de Lacy Costello, R.J. Ewen, N.M. Ratcliffe, P.S. Sivanand and P.T.N. Spencer Phillips[1]
Centre for Research in Analytical Material and Sensor Sciences, Faculty of Applied Sciences, University of the West of England, Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY

[1]Centre for Research in Plant Science, Faculty of Applied Sciences, University of the West of England, Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY

6.1 ABSTRACT

A prototype electronic sensor system developed for detecting volatile organic compounds produced by spoilage fungi was evaluated in a commercial wheat grain intake facility. Thresholds calculated from laboratory tests were used to differentiate between sound and infected samples collected routinely from trucks delivering grain for use in food manufacture. All samples identified as having an odour related problem by the intake laboratory gave a total system output above the set threshold and were therefore rejected by the prototype system. A number of samples passed by the intake laboratory were rejected by the prototype system, resulting in what appeared to be "false positive" results. However, the sensor system, which was designed to detect incipient spoilage, may detect compounds associated with spoilage that can not be detected by the human assessors. Nevertheless, the thresholds used were selected on the basis of a limited number of samples and may need to be adjusted. The output from the sensor system was also compared with moisture content values for the wheat (where available) to demonstrate that the system was not measuring differences in moisture.

A separate study assessed 37 newly-harvested wheat samples of different varieties and from different geographic locations within the UK. These samples were analysed by the sensor system, using the same thresholds as before. Six samples rejected by the system were then assessed by the wheat intake laboratory, where only one sample was rejected. This rejected sample had given the highest output when exposed to the sensor system. This experiment also suggests that the value of the threshold should be raised to avoid "false positive" results.

The trial highlights the promise of this prototype for the detection of spoilage in wheat grain and a larger trial should ascertain the reliability and long term stability of the device and therefore confirm its usefulness to the industry.

6.2 INTRODUCTION

A prototype sensor system for detecting fungal spoilage of wheat grain has been developed. It was found to work effectively under laboratory conditions and could differentiate between sound and infected grain. However, for successful operation of the prototype system in a commercial environment it must be tolerant of factors such as the background volatile profile (likely to already possess overtones of wheat associated volatiles), irregular and frequent changes in the background volatile profile and possible contaminant vapours such as diesel. Other problems such as long system down time and irregular sampling protocols are also likely to occur. The device must therefore be robust enough to work under these conditions and produce reliable decisions on the relative quality of grain samples if it is to be deployed in the commercial environment. This paper describes the trial of the prototype device in the intake laboratory of a major processor of British wheat grain.

6.3 MATERIALS AND METHODS

The prototype system was in place for two working weeks and during that time over 100 grain samples taken for routine testing from trucks delivering to the processing plant were analysed. A number of odour-tainted samples collected on previous occasions were also assessed to give some indication of the effectiveness of the thresholds. The thresholds were set using laboratory tests whereby a number of sound wheat-grain samples could be easily differentiated from other samples of the same controls with 1.5% by weight of *Penicillium aurantiogriseum* infected grain added.

A further set of 37 samples supplied by CCFRA were analysed. These samples consisted of grain from a broad range of wheat varieties and geographical locations. The quality of these samples had not been assessed for odours, but moisture content and location to the field where the grain was grown were known.

6.4 RESULTS AND DISCUSSION

6.4.1 Threshold Values

When using the dedicated test rig for testing sensors (Paper 4), the current flowing through each sensor was measured. Sensitivity values for each sensor were calculated using the following relationship:

(Ip-Ib)/Ib x 100

where **Ip** is the maximum current of the sensor when exposed to the vapour, and **Ib** is the stable baseline current.

However, the prototype sensor system gives a measurement of the sensor resistance, and therefore the following relationships were used to calculate the sensitivity and to ensure ready comparison with values of sensitivity calculated from sensors in the test rig.

(Rb-Rp)/Rp x 100 ≡ (1/Rp-1/Rb)/(1/Rb) x 100

where **Rb** is the stable baseline resistance and **Rp** is the lowest value of the resistance in the test gas.

When using this formula a sound to infected threshold of 500 was calculated for the prototype (Paper 5, section 5.4.2.4).

However, an alternative method of calculation is to use the following relationship.

(Rb-Rp)/Rb x 100

Using this method the maximum change of any sensor must be less than 100%. This method of calculation is particularly useful where one or two sensors have high sensitivities but do not differentiate most effectively between sound and infected grain and therefore when using this method of calculation their input towards the final system output is limited.

When using this formula, a sound to infected threshold of 180 was calculated for the prototype system.

6.4.2 Samples from Commercial Intake Laboratory

Figure 1 shows a plot of sensitivity with the threshold set at 500 on the basis of laboratory experiments for the 106 samples received during the trial and which had been assessed by trained wheat grain intake laboratory technicians. Figure 2 shows the same data but using the alternative method of calculation and where the threshold was set at 180. This emphasises the difference that data analysis can have on the final decision-making process. It shows that in this case the second method of calculation is more suitable with the current prototype set-up whereby one sensor exhibits high sensitivity but does not exhibit the most effective sample differentiation. The results serve to emphasise the potential for weighting the input of each sensor to the final decision-making process, or alternatively the reduction of sensor numbers in any future device.

It should be noted that there are many reasons why wheat samples may be rejected at the intake laboratory stage and the majority of these are not odour-related. Therefore, it is only imperative that the system rejects samples that would have been rejected on the grounds of associated odour taints. It should also be remembered that the sensor device was designed to detect compounds indicating the early stages of spoilage, which may be prior to the point that they can be detected by the human nose. Therefore, it is to be expected that the system will reject some samples that the odour assessors will accept. Such samples will fall into the category of "false positives".

Figure 1 shows that the use of the pre-calculated threshold of 500 provides reasonable differentiation between sound and infected wheat samples from a commercial intake laboratory. Using this method of calculation there are a number of false positives (13 in total) which may suggest that practically the threshold is set too low. Five false negatives occur, although the first of these was rejected as having an odour related problem linked to the grain drying process and not microbial contamination. Of the four other false negatives none were rejected for odour related reasons; one was rejected for having too many broken grains, one was infested by insects and two contained *Sclerotia purpurea* (ergot). Therefore, using this analysis all but one of the samples with an odour-related problem were rejected. However, there is a problem with the low frequency of spoilt samples *per se* and the problems this causes in reliably calibrating the instrument. Many of the odour-tainted samples had been held back from previous intake runs, whereas all the sound samples tested were from intake grain received during the trial. If Figure 2 is considered (threshold 180), it can be seen that it significantly reduces the number of false positives from 13 to 5 and removes one of the false negatives which was the sample rejected due to a dryer-related odour taint. Therefore, for this sensor configuration this method of data analysis/interpretation was favoured and was used for subsequent data collected on other batches of samples.

If the rejected samples are considered, the majority were rejected by the intake laboratory for odour-related reasons which may be linked to microbial action. The exception was one sample rejected due to high moisture content, and two rejected due to ergot. The samples with ergot may have had odour-related problems in addition to the reason for their rejection, which precludes the need for any further testing as they were rejected on a purely visual basis.

If the remaining false positives are considered, then the only feedback from the company was to re-affirm that with their normal test procedures these samples passed on odour and had been used in the production line. If the samples are analysed for their moisture content, then no trend is observed. For instance, the false positives range from 13-15.2%, but were all still rejected by the prototype. It may be considered that the threshold level could be raised to 185; the majority of the odour-tainted samples would still be rejected, but the problem of false positives would be completely overcome.

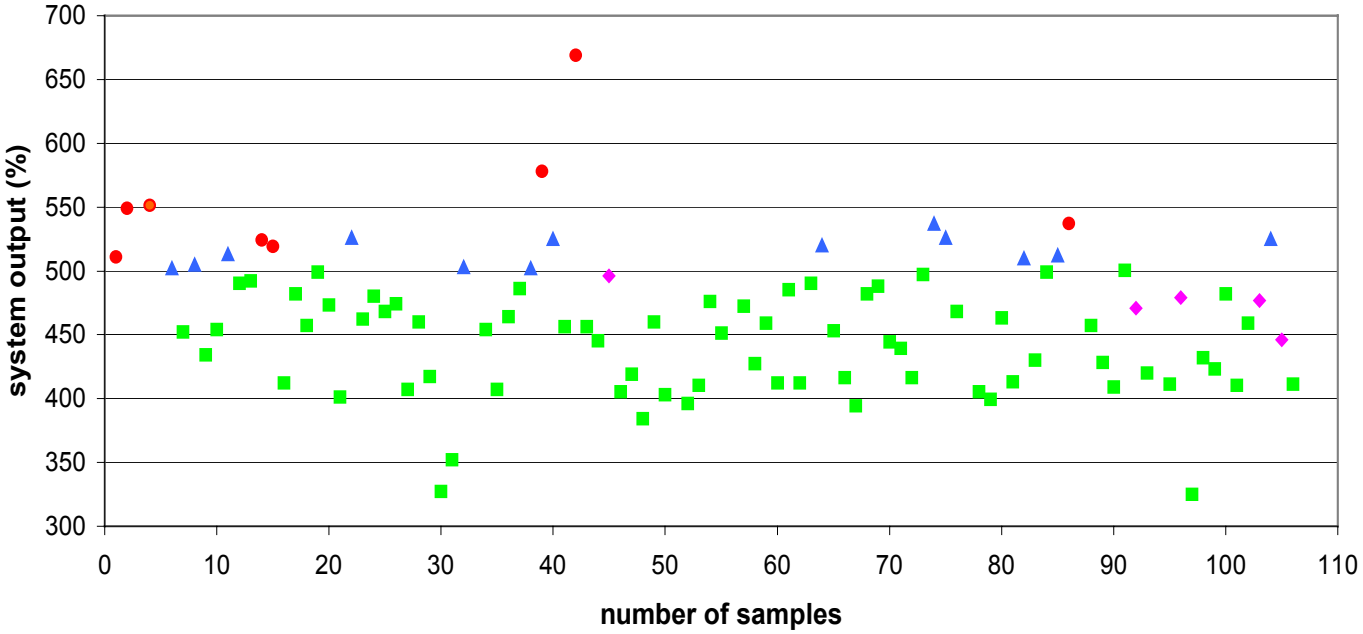


Figure 1: Output from Prototype Sensor System, with a Threshold Set at 500 for 106 Samples from a Commercial Wheat Grain Intake Laboratory

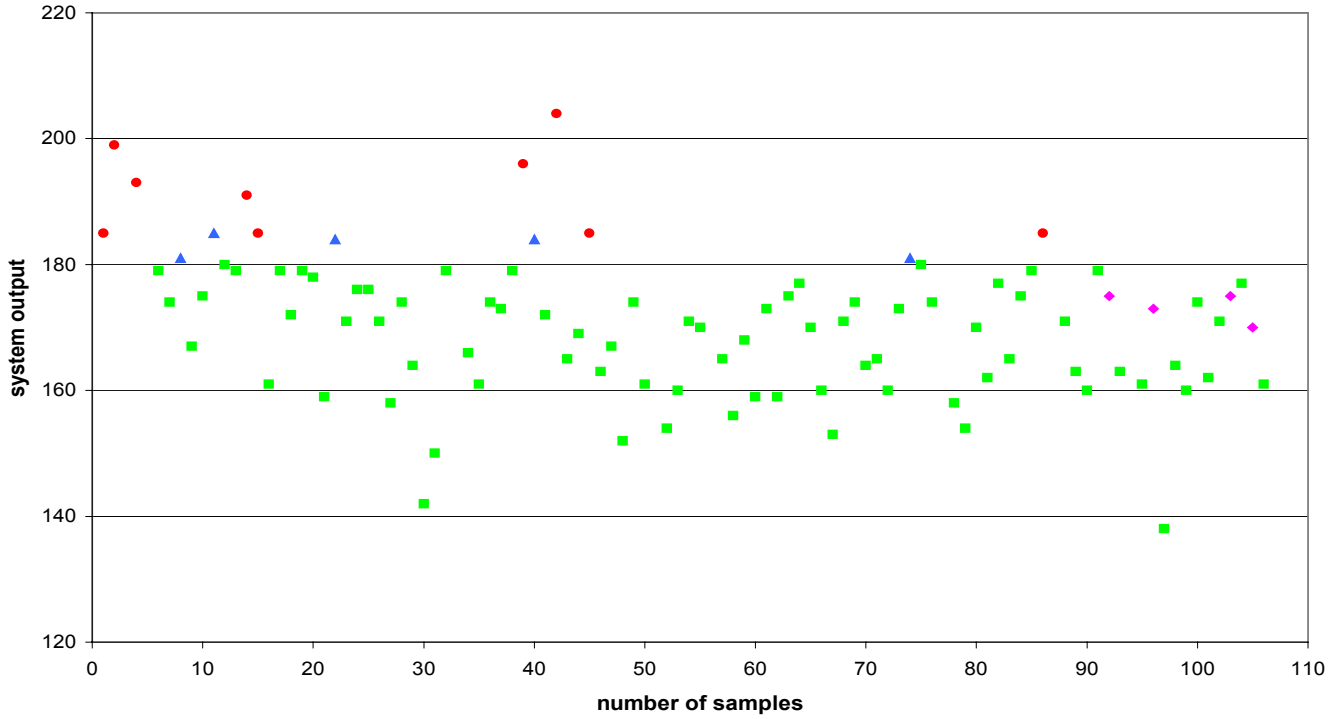


Figure 2: Output from Sensor System for the Grain Samples in Figure 1 but with a Set Threshold of 180

Squares denote samples passed by the intake laboratory which fall beneath the set threshold. Circles denote samples rejected by the intake laboratory and which were rejected by the prototype system, i.e. were above the set thresholds. Triangles denote samples passed by the intake laboratory but rejected by the prototype system, i.e. false positives. Diamonds denote samples rejected by the intake laboratory but accepted by the prototype, i.e. false negatives. Note: Samples can be rejected by the intake laboratory for reasons other than odour.

Figure 3 shows the moisture content of the samples against their classification by the UWE prototype system. The high correlation between sensor system outputs and moisture content of the grain has always been a criticism levelled at other devices in this type of application. Indeed, a high ratio of musty samples are associated with high moisture contents, but ideally all other samples with odour taints (specifically microbially related) must be rejected regardless of their moisture content, and all samples without associated odour taints must be accepted regardless of their moisture content. Figure 3 goes some way towards proving that the UWE prototype system was not responding primarily to moisture content, but to organic volatiles associated with odour taints, as there is no apparent correlation between moisture content and system decision. For instance, if the rejected samples which are on the borderline are taken then these can be seen to vary in moisture content between 13.8% and 16.5% (the highest moisture content measured amongst these intake samples). In contrast the infected sample giving the highest system output had a moisture content of 14.8%, which was not much above the mean moisture content and certainly below the moisture contents of a number of sound samples which exhibited very low system outputs well below the set threshold of 180. These moisture content data coupled with the preliminary assessments by this prototype device in a commercial environment leads to the conclusion that this sensor technology shows a high degree of promise in this specific application.

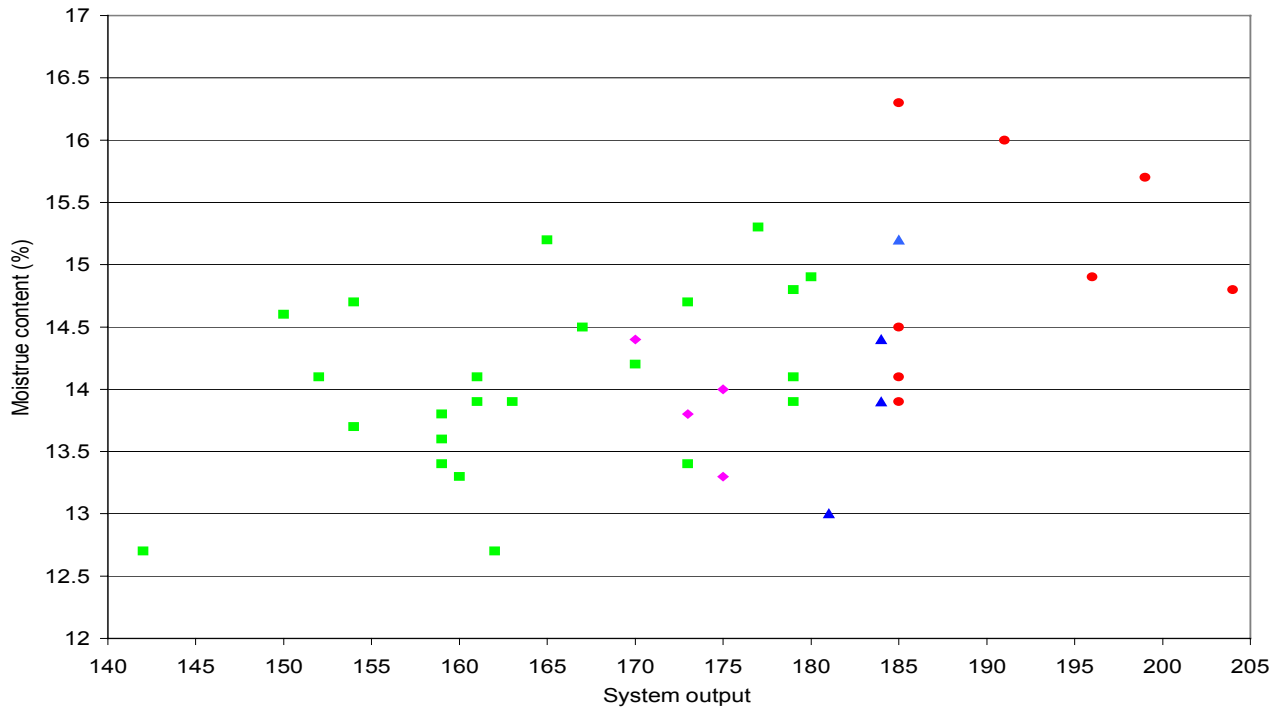


Figure 3: Moisture Content of the Grain Samples in Figures 1 and 2 Related to the Sensor System Output for Each Sample

Squares denote samples passed by the intake laboratory which fall beneath the set threshold of 180. Circles denote samples rejected by the intake laboratory and which were rejected by the prototype system, i.e. were above the set thresholds. Triangles denote samples passed by the intake laboratory but rejected by the prototype system, i.e. false positives. Diamonds denote samples rejected by the intake laboratory but accepted by the prototype, i.e. false negatives.

6.4.3 Results for Samples Supplied by CCFRA

Over a two-week period, additional samples to those from the intake line were analysed. These samples consisted of a cross section of British-grown grain varieties from different geographic locations and were supplied with the intention of providing a broad range of control (sound) samples. As sound factory intake samples were likely to be from a fairly limited geographic location, it was considered that this study would be beneficial to test for any trends related to variety or geographic location where the wheat was grown. The samples had been cleaned and their moisture contents measured. However, they had not been analysed for odour, and so the test procedure was carried out completely blind. Figure 4 shows the results of the analysis

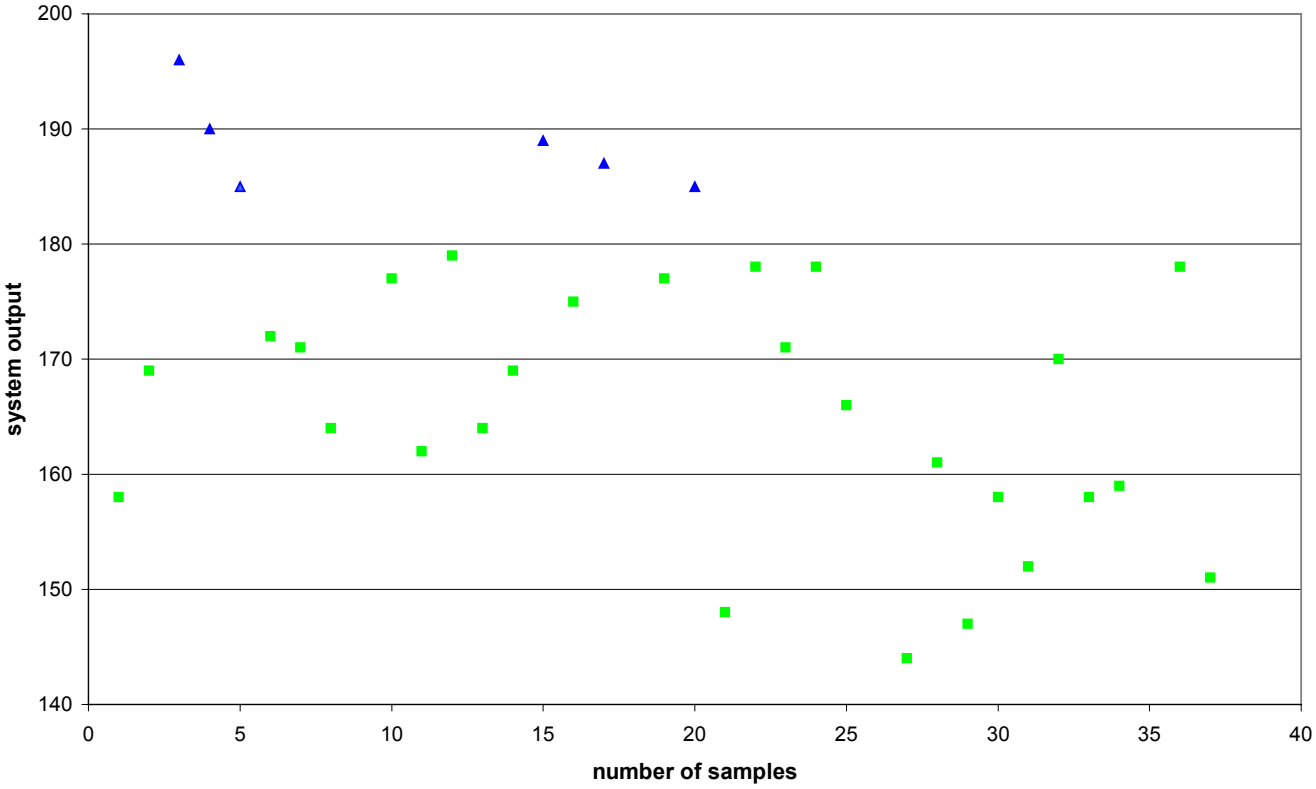


Figure 4: Output from Prototype System, with a Threshold Set at 180 for 37 Samples Supplied by CCFRA

Any sample that crossed the set threshold of 180 is denoted as a triangle. Samples falling below this are denoted as squares.

with system output plotted against sample number. Any sample which crossed the set threshold of 180 was denoted as a triangle whereas samples falling below this threshold were displayed as squares. The analysis shows that, of 37 samples only 6 were above the set threshold of 180. These samples were subsequently analysed by the intake laboratory to ascertain the associated odour intensity. Of the 6 samples only one would have been rejected by the intake laboratory on the basis of an odour-related problem. Encouragingly, the sample that would have been rejected by the intake laboratory was the one that gave the highest system

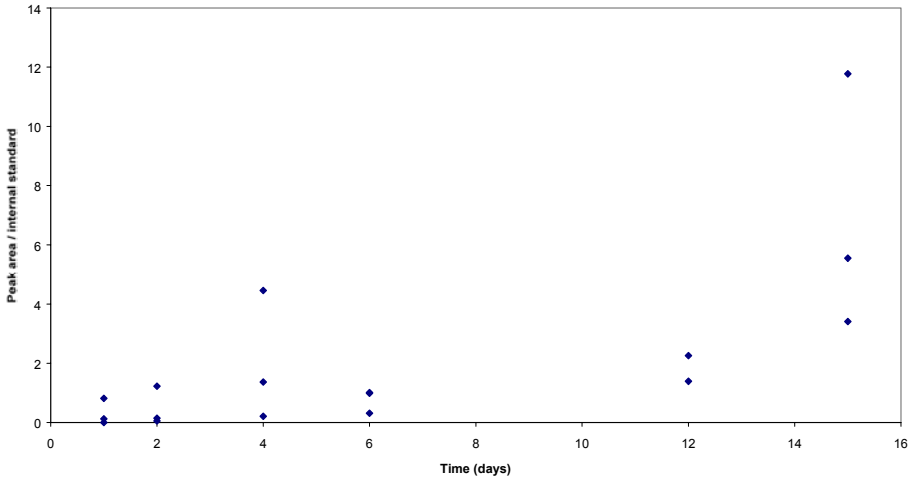
output of 196. Therefore, the conclusion must be, as with the previous analysis, that the set threshold may need to be adjusted to a higher value. This analysis indicates that this should be 190 if the problem of false positives is to be removed. Re-analysis of the intake sample batch using a threshold of 190 does eliminate the false positives, but introduces a number of false negatives. It is clear that a larger throughput of samples is required to gauge the exact value for an effective threshold. However, the prototype shows a high degree of promise to a diverse range of grain samples, even when utilising the provisional threshold of 180. Again, the moisture content of the grain had been analysed and there was no obvious correlation between system output and moisture content.

6.5 CONCLUSIONS

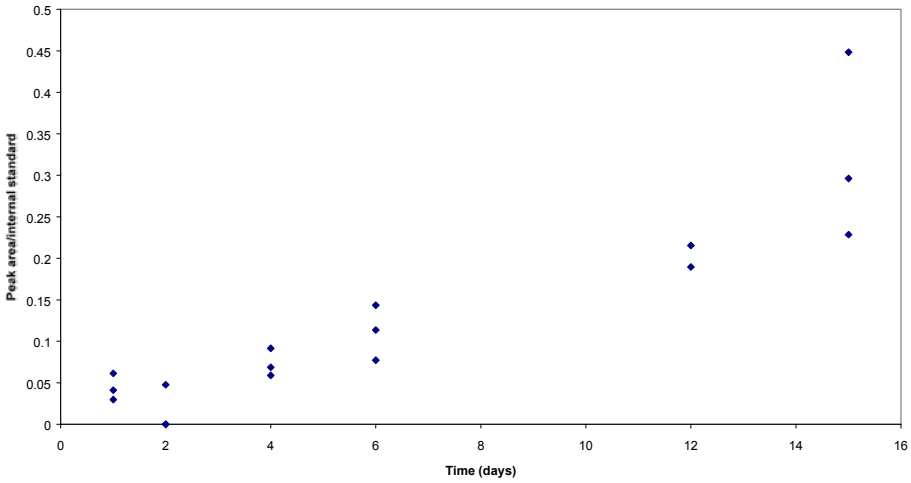
On the basis of previous experiments with a number of sound samples and diluted spoilt grain samples, a sound/odour tainted threshold of 180 (500 previous method) had been set. In this preliminary trial, the device and this threshold were tested against a number of intake wheat samples from a commercial laboratory. The results show a high degree of promise with very few misclassified samples from a total data-set of close to 150 samples (including CCFRA samples). The system output of the prototype showed little correlation with the moisture content of the grain, allowing some confidence that grain samples were either rejected on the grounds of odour, or that they were wet and would have been rejected on moisture alone. The experiments carried out did suggest that there may be a need to raise the value of the threshold to avoid the problems of false positives or, more accurately, to bring the threshold into line with what commercially is considered to be an acceptable sample. However, it would be a mistake to raise the threshold on the basis of these results to eliminate false positives and then find that a number of false negatives had been introduced, which commercially is far less desirable. Therefore, it is necessary to test the prototype further to an extended range of commercial intake samples to ascertain a firm value for the threshold. It would be more applicable then to re-test the device at a period in the calendar when odour tainted samples are commercially more of a problem.

APPENDIX I: *Penicillium aurantiogriseum*

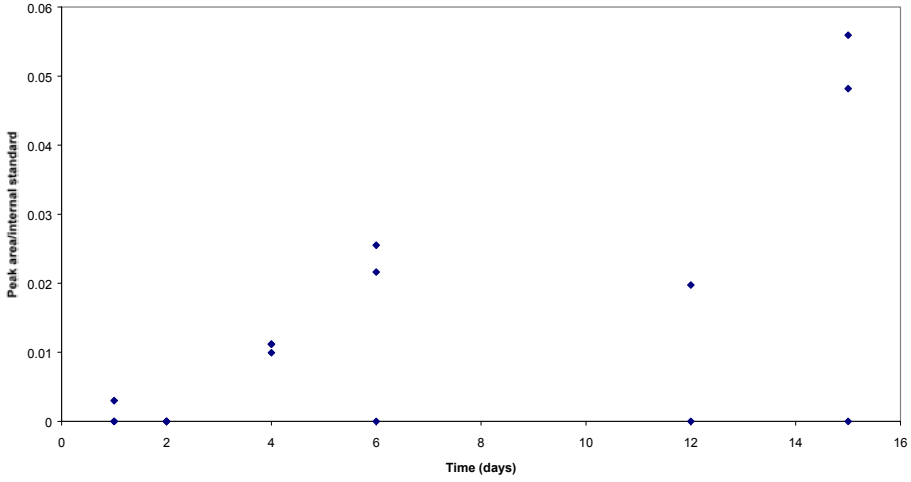
Ethanol



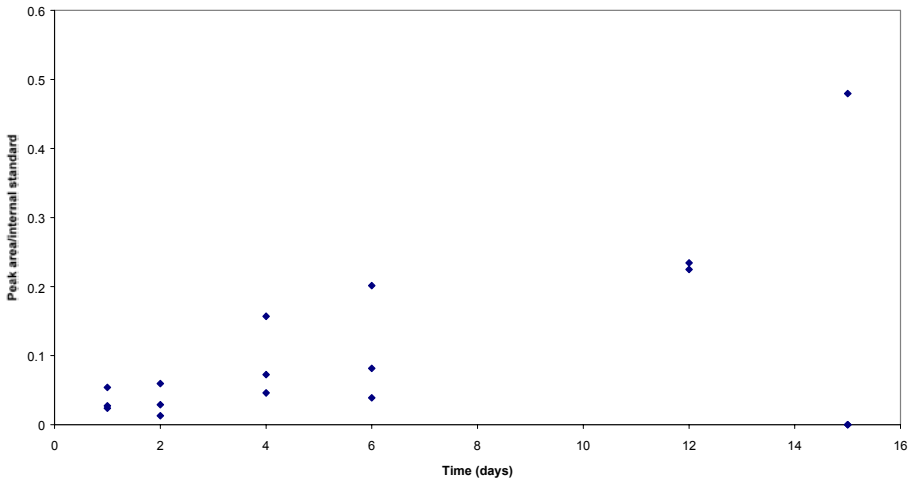
1-butanol



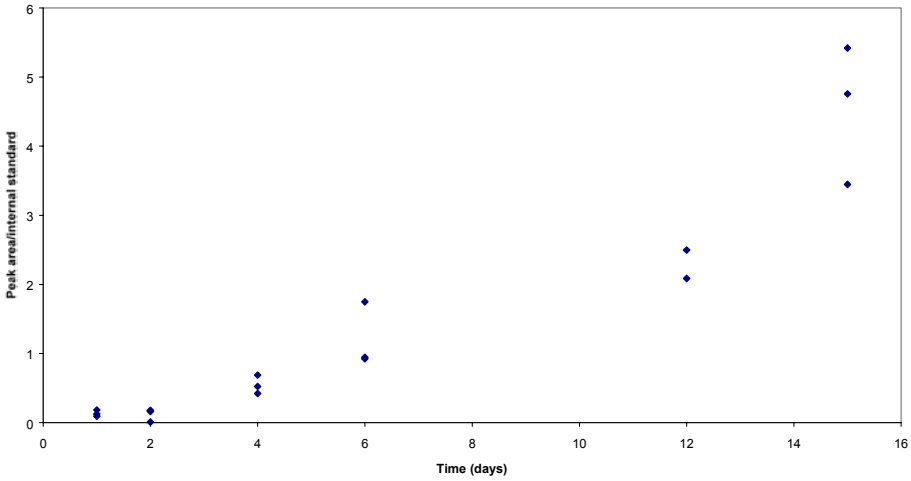
2-buten-1-ol



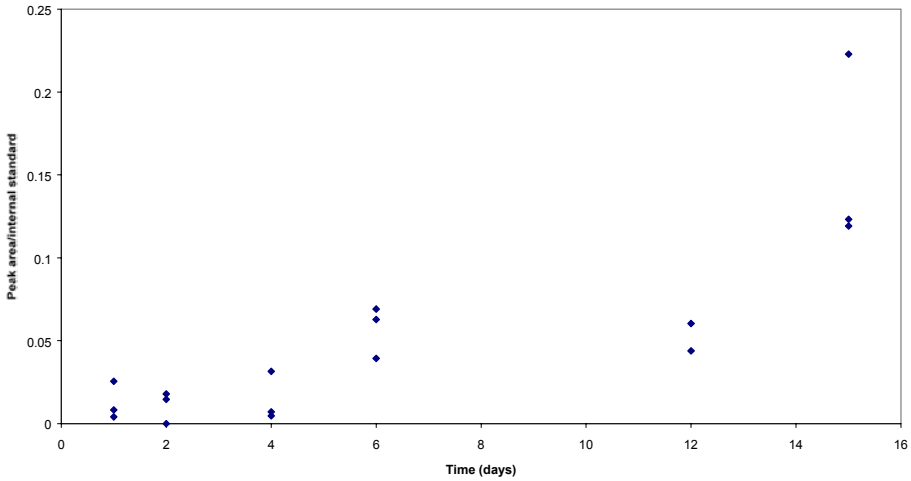
2-pentanol



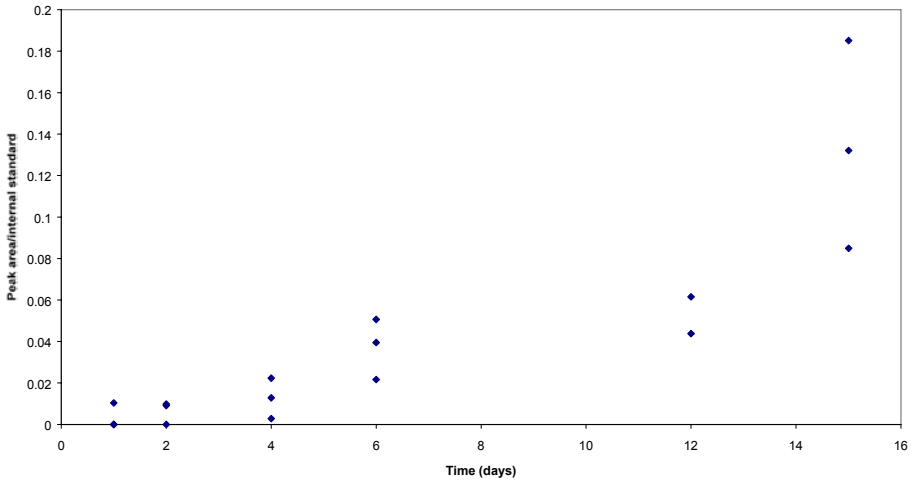
3-methyl-1-butanol



Cyclopentanol

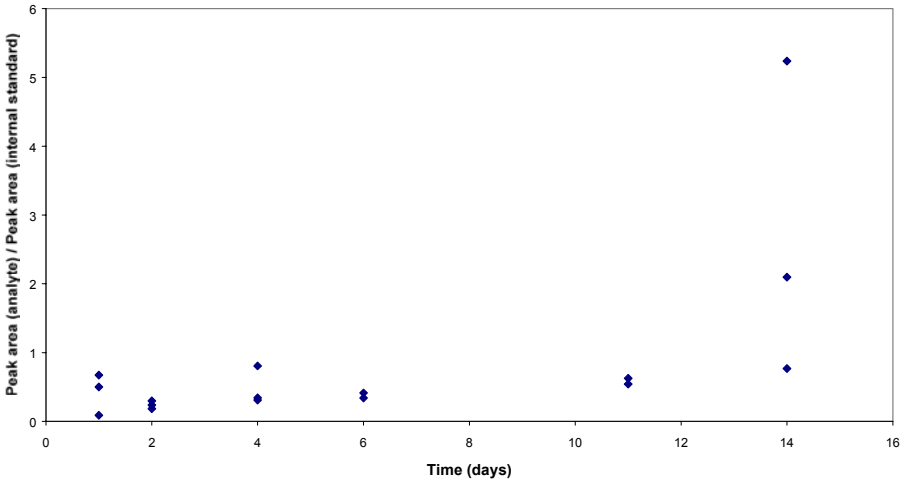


3-methyl-2-buten-1-ol

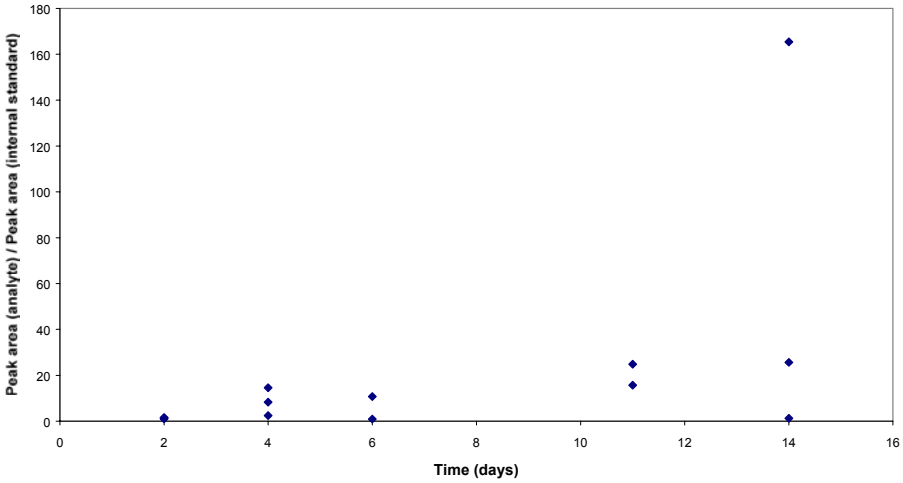


APPENDIX II: *Fusarium culmorum*

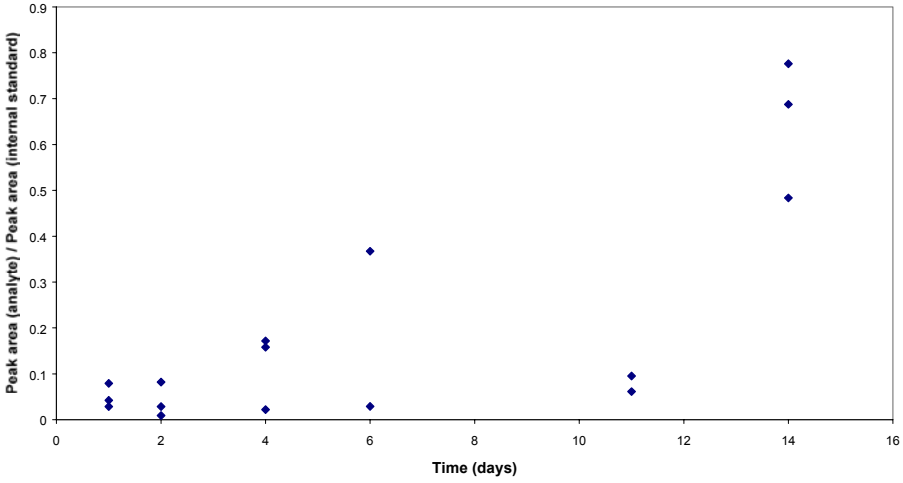
1-Butanol



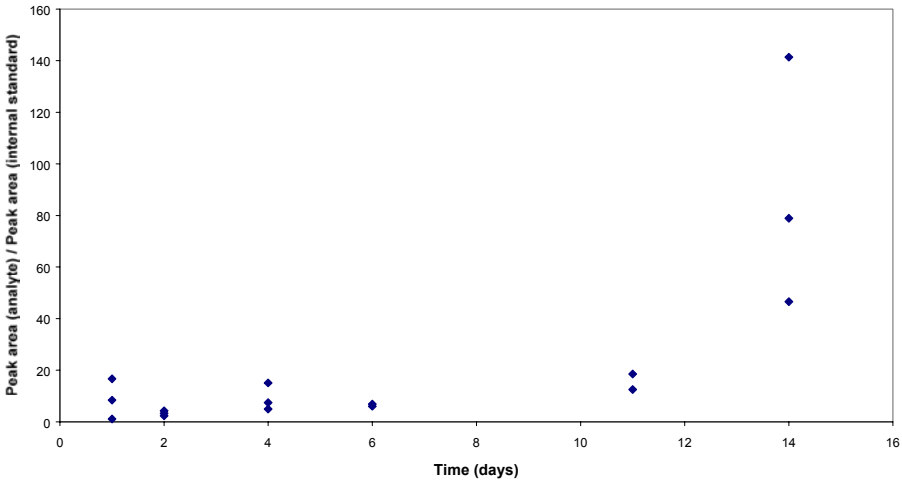
2-Butanol



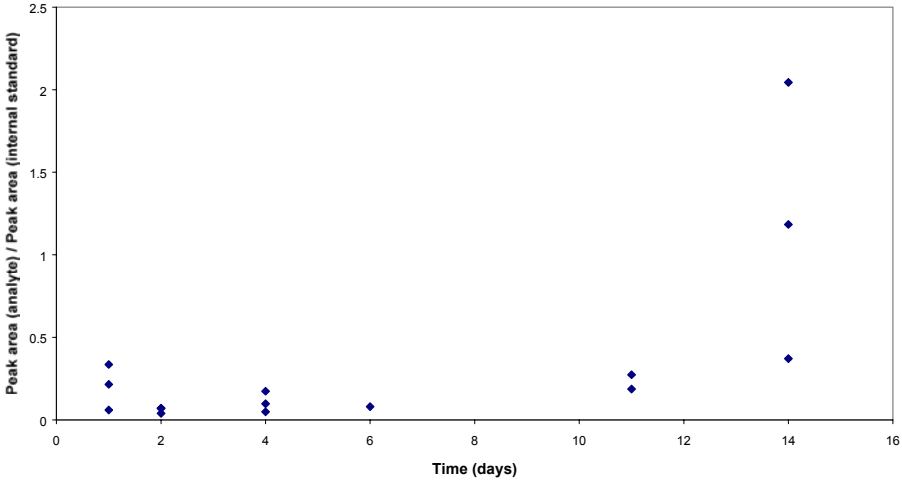
2-Ethyl-1-hexanol



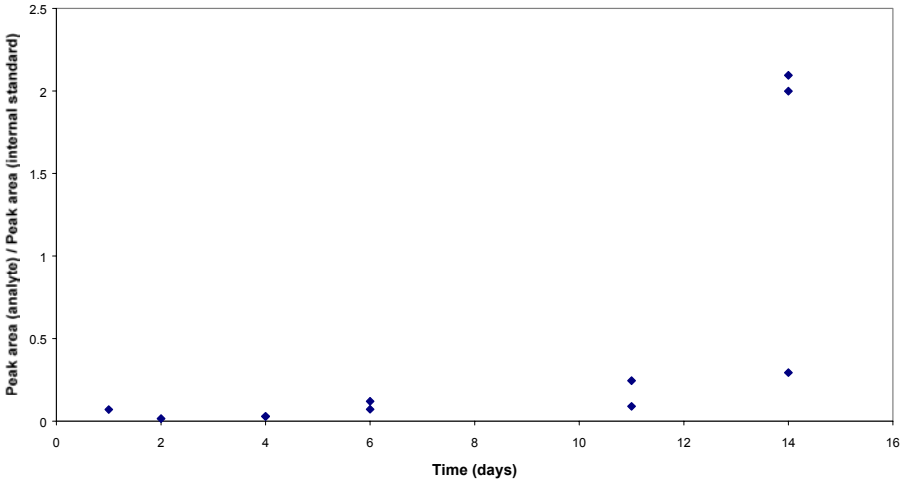
3-Methyl-1-butanol



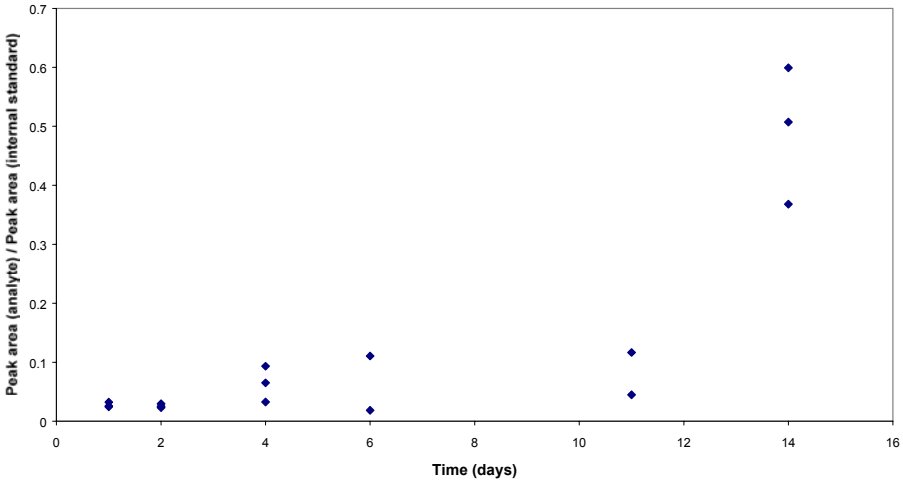
3-Methyl-3-buten-1-ol



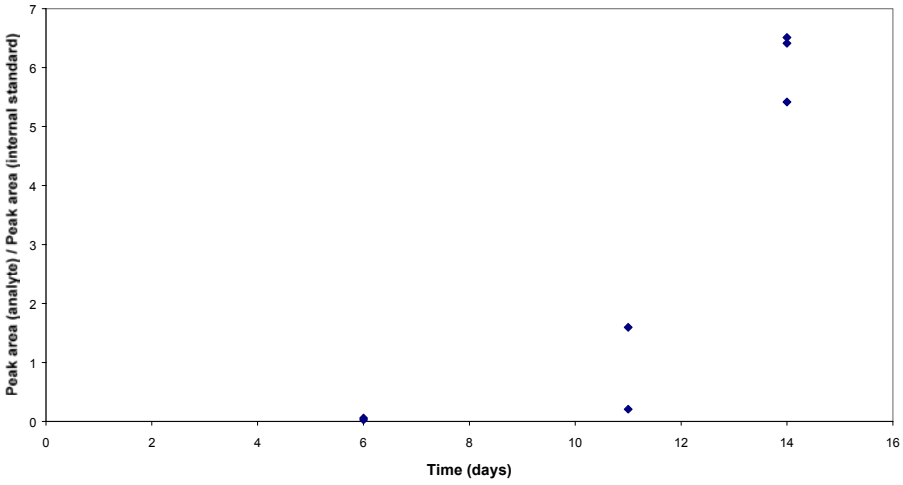
2-Heptanol



Ethylphenylacetate

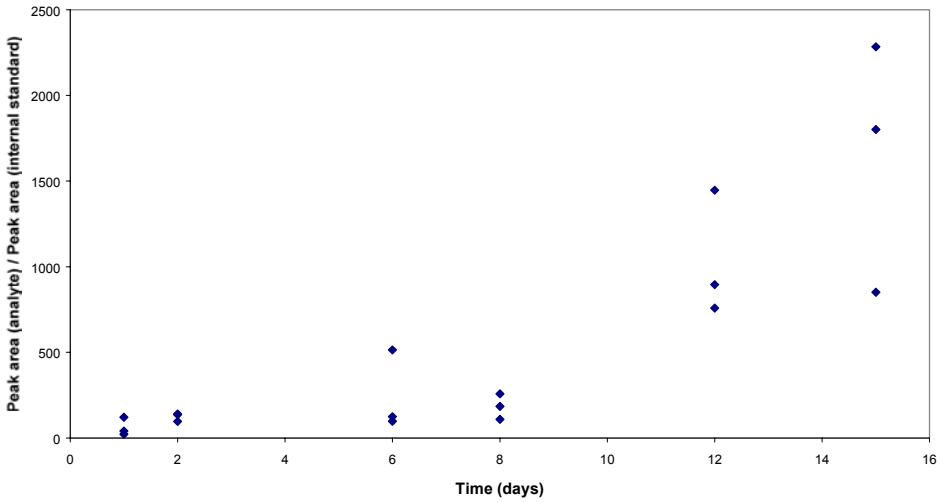


4-Ethyl-2-methoxyphenol

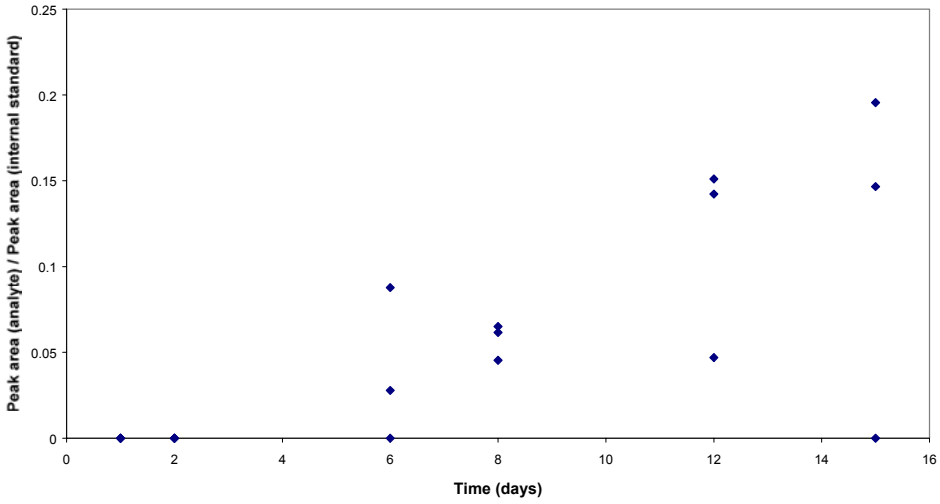


APPENDIX III: *Aspergillus niger*

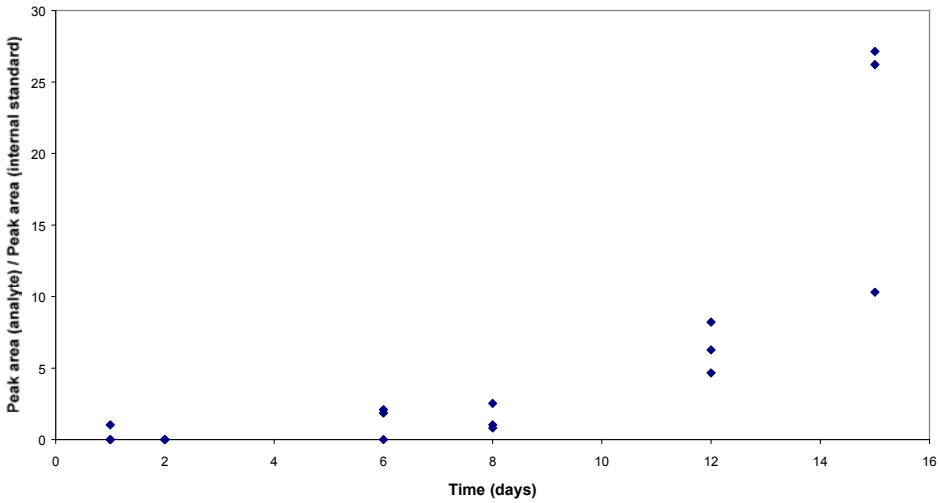
Ethanol



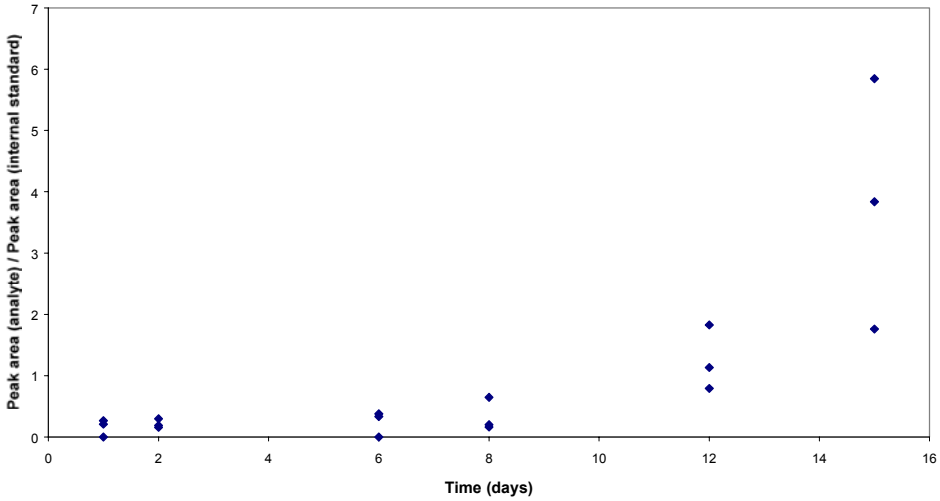
3-Methylbutanoic acid, ethyl ester



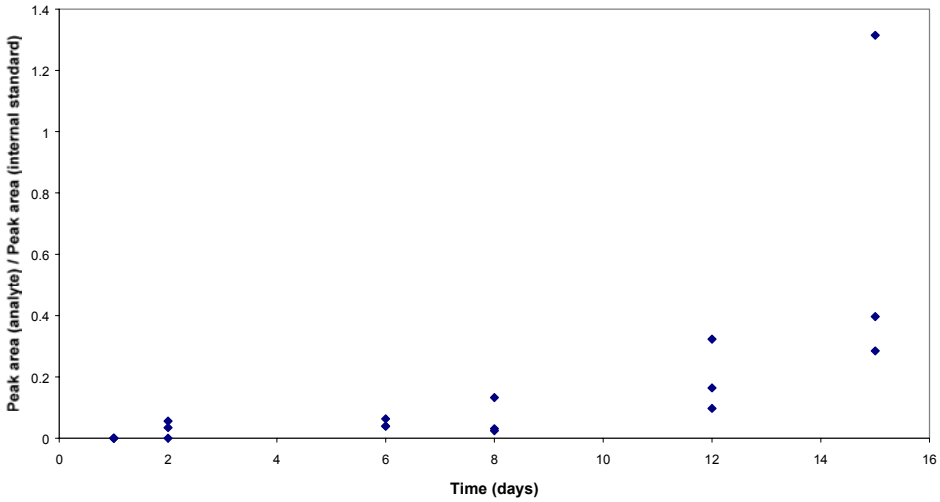
2-Methyl-1-propanol



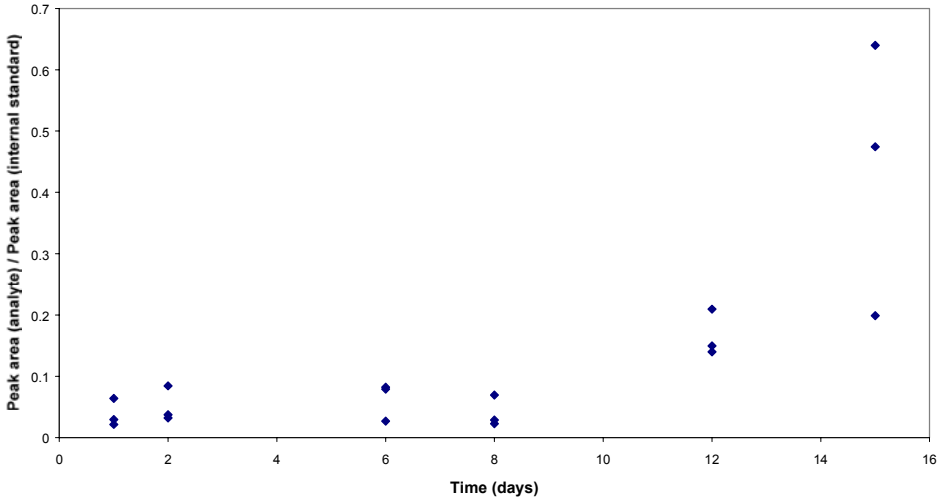
2-Pentanol



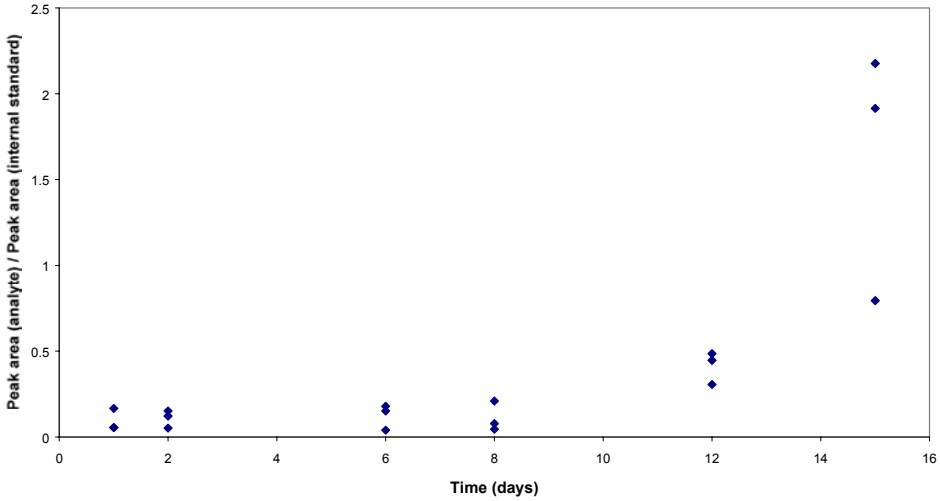
3-Pentanol



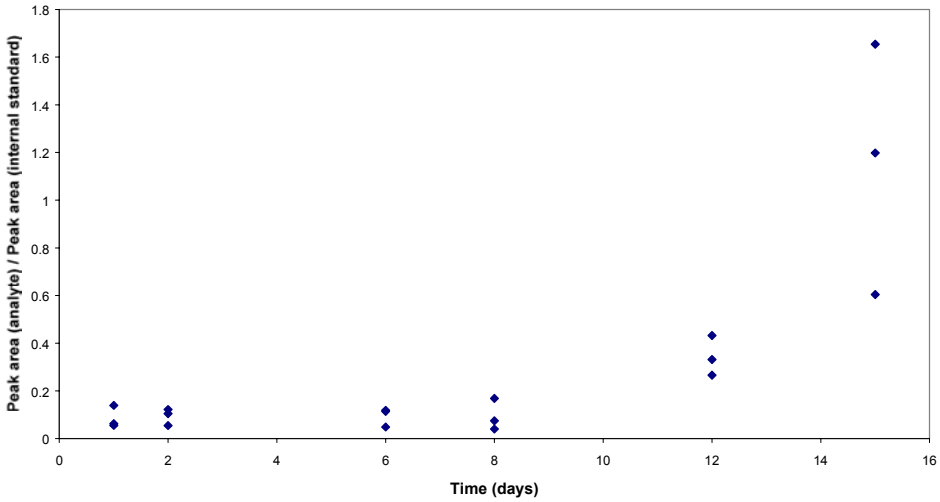
2-Octanol



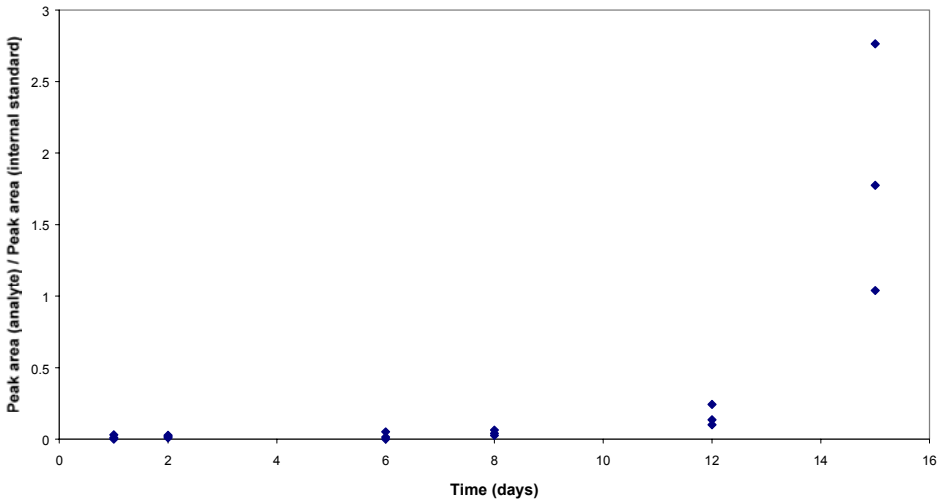
1-Octen-3-ol



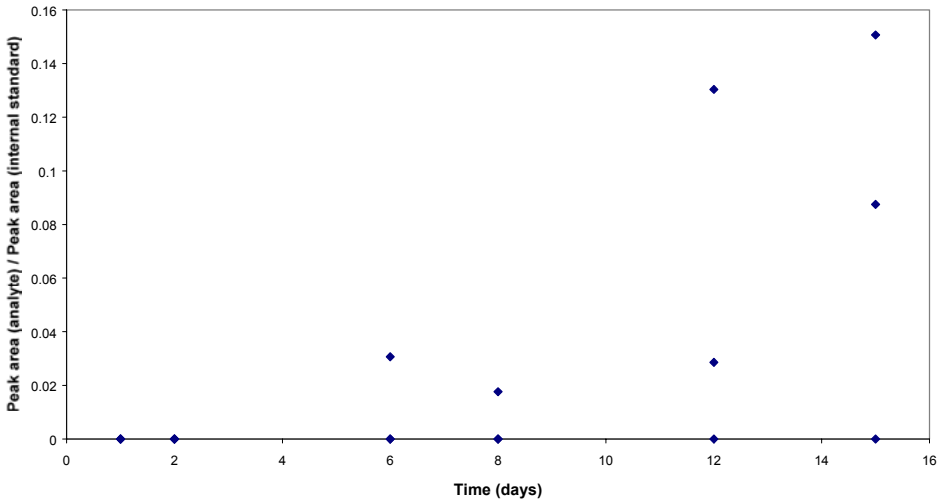
2-Ethyl-1-hexanol



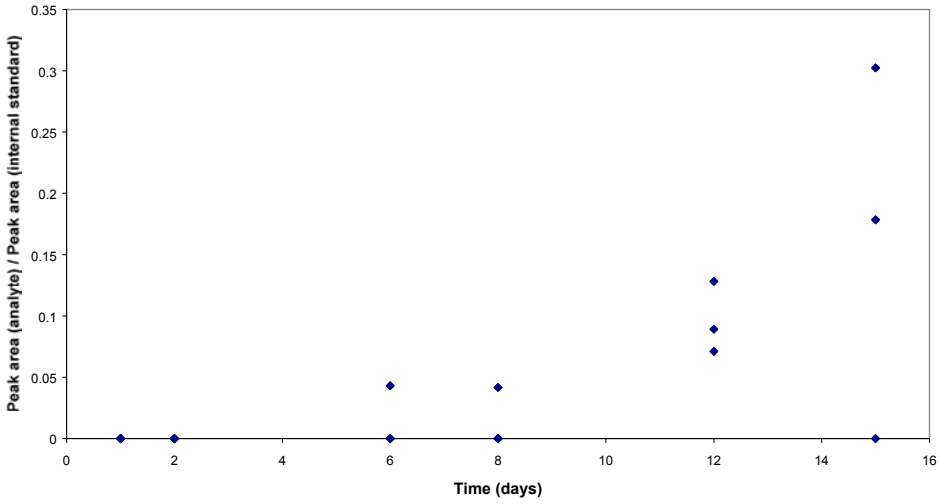
ß-Cubebene



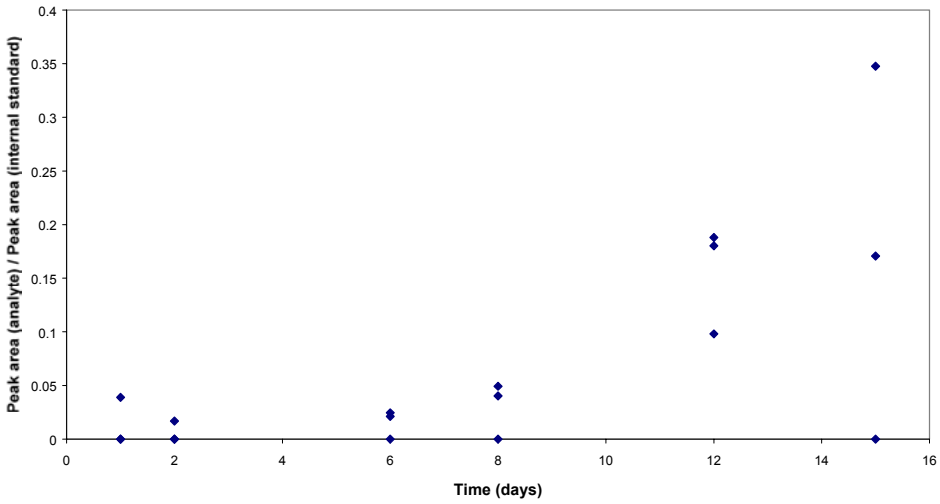
2-Butenoic acid, ethyl ester



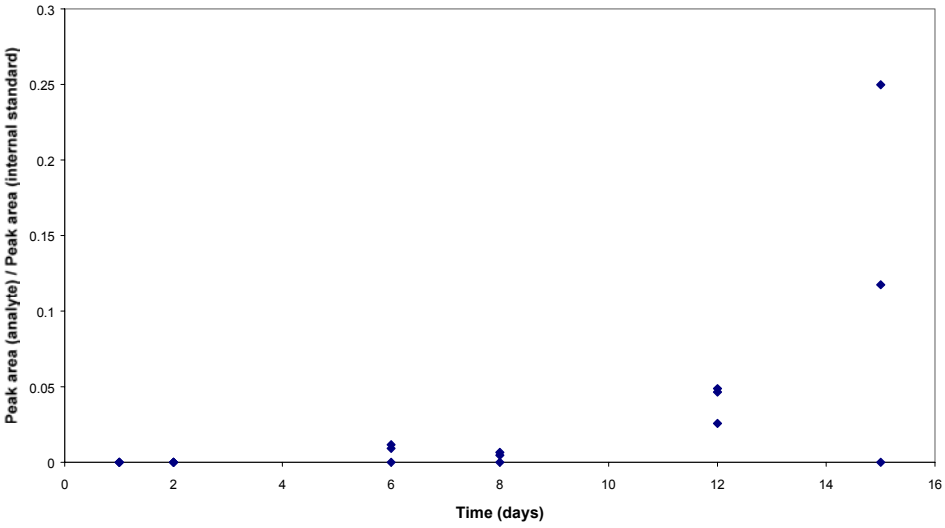
3-Methyl-2-butenoic acid, ethyl ester



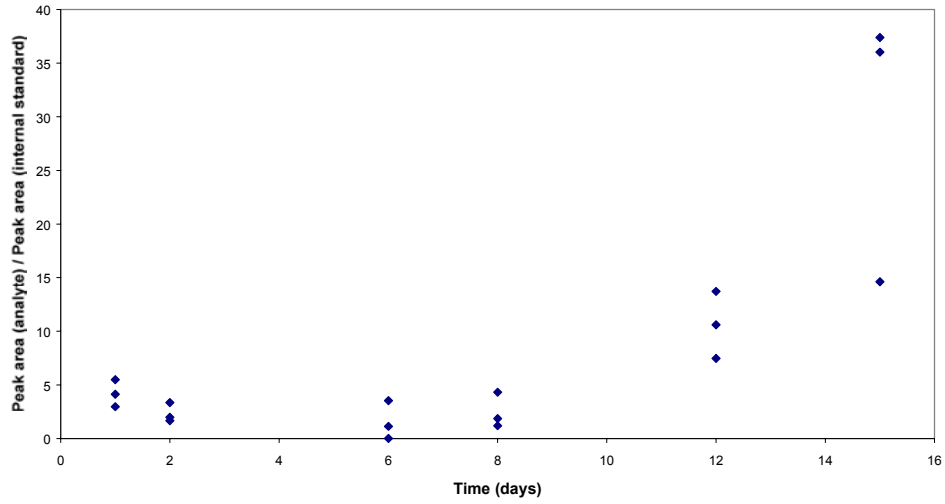
3-Methyl-2-butenal



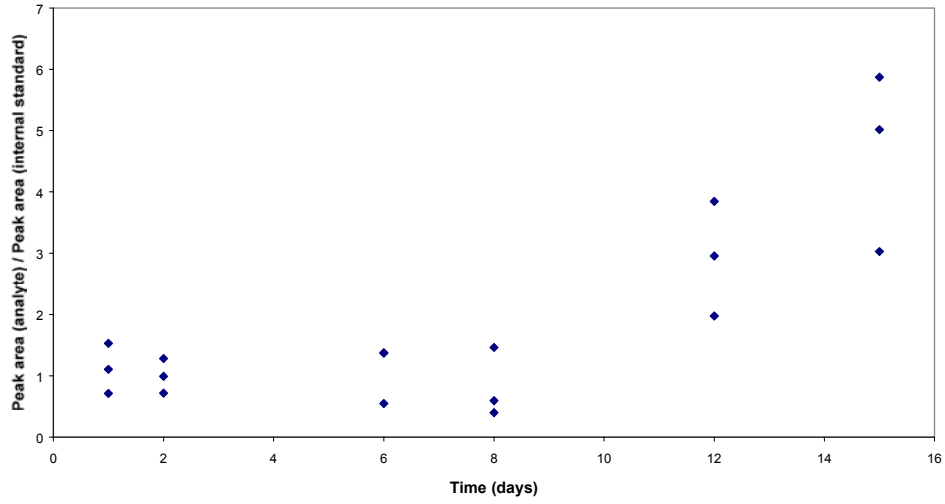
3-Octanone



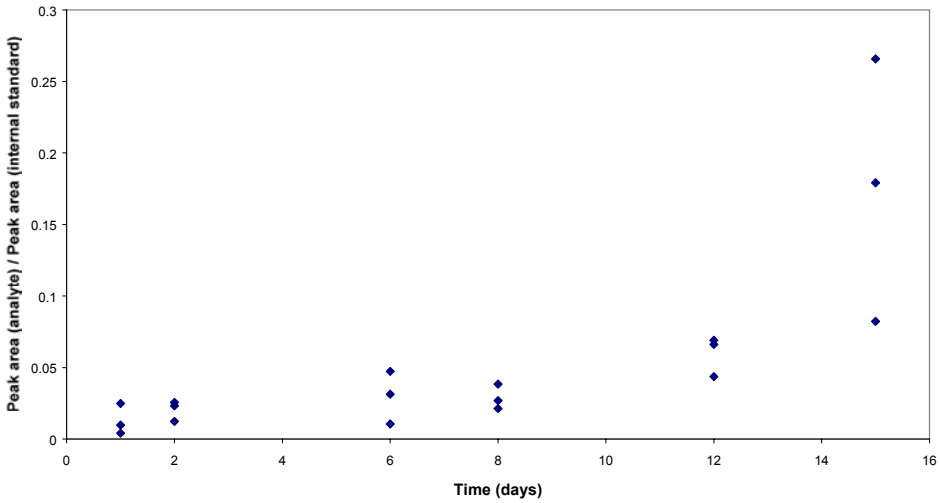
3-Methyl-1-butanol



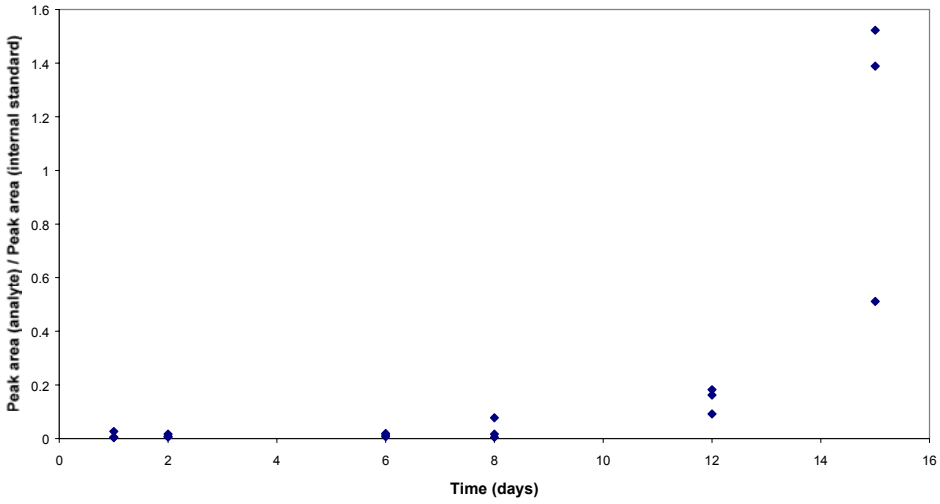
1-Hexanol



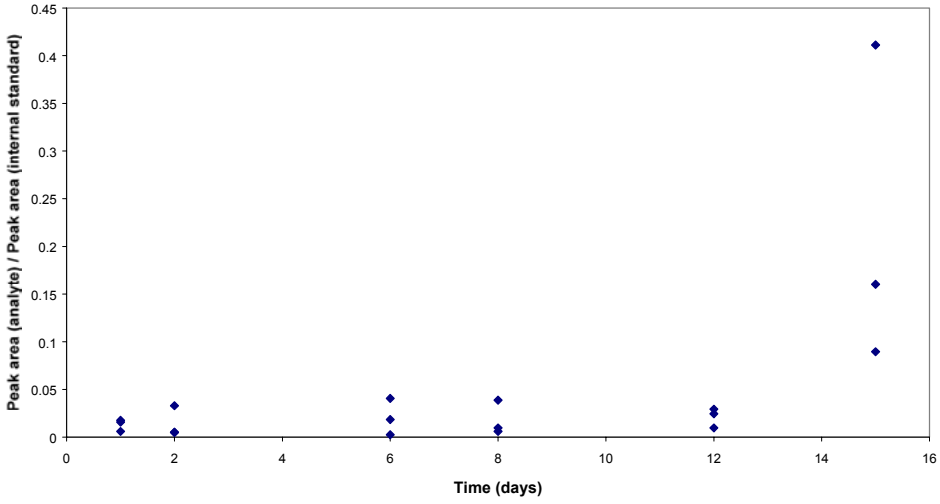
Benzoic acid, ethyl ester



cis-Piperitol



(E,E)-Nona-2,4-dienal



Ethylphenylacetate