Application of Gas Chromatography–Mass Spectroscopy (GC-MS) and Electronic Noses for Detection of Aflatoxin Contamination of Maize

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ABSTRACT

Maize is the main staple food for much of the population in developing countries. It is however, highly susceptible to infection by fungi and subsequently to contamination with mycotoxins. Mycotoxins are toxic secondary metabolites produced by filamentous fungi, mainly *Aspergillus*, *Fusarium,* and *Penicillium.* Among these, aflatoxins, produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus,* pose the greatest threat for agriculture, trade and human health in developing countries. Consumption of highly contaminated grains results in acute aflatoxicosis which can be fatal, while chronic aflatoxicosis as a result of long term low level exposure is more prevalent and is highly correlated with increased incidences of liver cancer, malnutrition, immunosuppression, and impaired growth in children. Contamination also results in economic losses from reduced grain quality, loss of animal productivity and reduced accessibility to international markets.

Management of aflatoxin remains problematic, particularly in developing countries where production and storage conditions favour contamination, coupled with a lack of well-established regulatory systems to frequently monitor food samples prior to trade or human consumption. The problem is further compounded by limited analytical capacity. Available screening and analytical techniques are fraught with numerous challenges, including high cost, laborious and time consuming procedures and lack of necessary infrastructure. There is therefore a need to develop a suitable system for detecting the presence of the toxigenic fungi and their toxins in order to provide real time monitoring data that would facilitate removal of contaminated lots. The use of volatile organic compounds (VOCs) produced by mycotoxigenic fungi upon plant infection has been identified as a potential novel diagnostic technique for detection of mycotoxins, to circumvent the drawbacks associated with current techniques.

The first objective of this thesis was to evaluate the potential for Gas chromatography - Mass spectroscopy (GC-MS) based analysis of VOCs for initial screening of maize to detect aflatoxin contamination using Australian and Kenyan maize varieties infected with *A. flavus* under laboratory and field conditions. The study also aimed to identify unique VOCs as markers for both *A. flavus* infection and aflatoxin contamination of maize. The results show the potential for GC-MS to

discriminate between maize inoculated with 2 % Tween 20 as control, non-aflatoxigenic and aflatoxigenic *A. flavus* with accuracies that ranged from 81 % to 100 % (n = 15 to 30 samples per class). The classification accuracies achieved for maize varieties naturally infected with *A. flavus* were, however, much lower than for the artificially inoculated samples, ranging from only 48 % (n = 34 control vs 31 contaminated) to 80 % (n = 41 control vs 11 contaminated). The VOCs effective in discriminating maize infected and not infected with *A. flavus* were variety dependent. Tetramethyl pyrazine was significantly and consistently effective in discriminating controls from all maize infected with aflatoxigenic *A. flavus* for Australian maize variety DK703w. The compound was not identified as important in discriminating controls from maize infected with the non-aflatoxigenic *A. flavus* isolate, indicating it could be unique to aflatoxigenic *A. flavus*. A similar pattern was observed for p-xylene which was effective in discriminating between Kenyan maize varieties that were infected and not infected with *A. flavus*.

The second objective was to evaluate the potential for electronic nose to detect aflatoxin contamination in maize. The performances of three electronic nose instruments based on different sensor technologies were compared using an Australian maize variety artificially inoculated with *A. flavus*. Electronic noses with metal oxide semiconductor sensors were more effective than conducting polymer sensors based electronic nose in discriminating between maize infected and not infected with *A. flavus*. Based on the marginally higher classification accuracies achieved, field portability and lower capital cost, the electronic nose equipped with metal oxide semiconductor sensors and thermocycling (DiagNose) was selected for further evaluation, using Kenyan maize varieties artificially and naturally infected with *A. flavus*. The DiagNose was able to discriminate between controls and maize samples artificially inoculated with *A. flavus* for two Kenyan varieties, Duma 43 and Pioneer, with accuracies that ranged from 72 % to 88 % (n = 30 samples per class). Classification accuracies for maize varieties that were naturally infected with *A. flavus* ranged from 61 % (n = 34 control vs 31 contaminated) to 86 % (n = 41 control vs 11 contaminated).

This study demonstrates the potential use of GC-MS based analysis of VOCs and electronic noses for detection of *A. flavus* infection and aflatoxin contamination in artificially and naturally contaminated maize samples. The classification accuracies achieved for both techniques are however, at this stage too low to justify deployment for practical field use at this stage. There is therefore need for further research to improve on their performance before they can be deployed in the maize industry.

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SYMBOLS AND ABBREVIATIONS

AFB1: Aflatoxin B1

AFB2: Aflatoxin B1

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

AFM1: Aflatoxin M1

AIDS: Acquired Immunodeficiency Syndrome

BecA-ILRI Hub: Bioscience eastern and central Africa- International Livestock Research Institute Hub

cm: centimetre

CDC: Centre for Disease Control and Prevention

CTO: Chromium Titanium Oxide Sensors

CSIRO: Commonwealth Scientific and Industrial Research Organization

CP: Conducting Polymer Sensors

ELISA: Enzyme Linked Immunosorbent Assay

FAO: Food and Agriculture Organization

GC: Gas Chromatography

GC-MS: Gas Chromatography- Mass Spectroscopy

GPS: Geographical Positioning System

g: Grams

HACP: Hazard Analysis Critical Control Point

Ha: Hectare

HPLC: High Performance Liquid Chromatography

HIV: Human immunodeficiency Syndrome

Int.: International

IARC: International Agency for Research on Cancer

JKUAT: Jommo Kenyatta University of Agriculture and Technology

Kg: Kilogram

KNN: *k*-nearest neighbour

LC-MS: Liquid Chromatography - Mass Spectroscopy

BGYF: Bright greenishyellow fluorescence

MOS: Metal Oxide Semiconductor Sensors

MOSFET: Metal Oxide Semiconductor Field Effect Transistor

m: Metre

µg: Microgram

µm: Micrometre

mL: Millilitre

mm: Millimetre

MI: Mutual Information

ng: Nanogram

nm: Nanometre

NIST: National Institute for Standards and Technology

OA: Ochratoxin

ppb: Parts per billion

PACA: Partnership for Aflatoxin Reduction in Africa

PDA: Potato Dextrose Agar

PCA: Principal Components Analysis

RMF: Relative Match Factor

SPME: Solid Phase Micro Extraction

SVM: Support Vector Machines

TLC: Thin Layer Chromatography

SnO2: Tin dioxide sensors

WO_3: Tungsten trioxide sensors

UPLC: Ultra-High Performance Liquid chromatography

UV: Ultra violet light

$: United States dollar

USAID: United States International Agency for Development

vs: Versus

VOCs: Volatile Organic Compounds

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To you all may God Bless.

DEDICATION

This thesis is dedicated to my best friend Dr Matthew Mutinda, our son Ethan and the entire Machungo family.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE ROLE OF MAIZE IN FOOD SECURITY

Contamination of food crops by mycotoxins is a global problem and has been a recurrent problem in Kenya. It is widely associated with increased post-harvest losses as well as being a major public health concern. Maize, which is the main staple food for over 96 % of the Kenyan population (Otunge *et al.*, 2010) is highly susceptible to degradation by mycotoxigenic fungi. These fungi not only reduce quality through discolouration and reduction of nutritional value, but also potentially cause mycotoxin contamination (Wagacha and Muthomi, 2008; Ngeno *et al.*, 2011; Schroeder *et al.*, 2013). Availability of sufficient supplies of maize to meet the domestic demand is normally equated to household and national food security. Until recently, Kenya has been relatively self-sufficient in maize production, but as a result of changing climatic conditions and increased population, imbalances between production and demand have been reported with a negative effect on national food security (Mati, 2000; Onono *et al.*, 2013; Kiunga, 2015). The annual growth rate in production, estimated at 2 %, has not been in tandem with the growth in population and industrial demand rate, estimated at 4.7 % (Nyoro *et al.*, 2007). As a result Kenya is projected to have a deficit of up to 1.2 million metric tonnes by 2020 (Nyoro *et al.*, 2007; Achieng *et al.*, 2013).

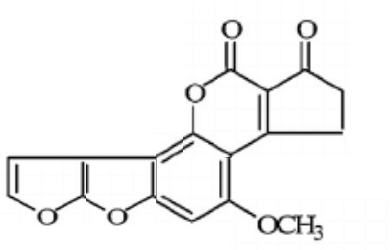
Several factors, including increased pre- and post-harvest losses, can result in very poor farm yields, with losses estimated at 1.5 to 2.6 tonnes against a potential of six tonnes/hectare (Ha) (Schroeder *et al.*, 2013). A significant amount of grain is lost after harvest, as a result of decay caused by mycotoxigenic fungi (Kimatu *et al.*, 2012). According to the Food and Agriculture Organization (FAO), up to a fifth of grain produced in sub-Saharan Africa is lost to pest and

decay caused by mycotoxigenic fungi, a concept commonly known as the "*Missing Food*", at an estimated cost of $ 4 billion. This accounts for half of the annual grain imports to Africa (WorldBank *et al.,* 2011). Therefore, early detection of the mycotoxigenic fungi in grain with an effort to reduce post-harvest losses would be a more cost effective and environmentally sustainable strategy to bridge the yield gap, rather than a corresponding increase in area under production (Anklama *et al.,* 2002; Kimatu *et al.,* 2012).

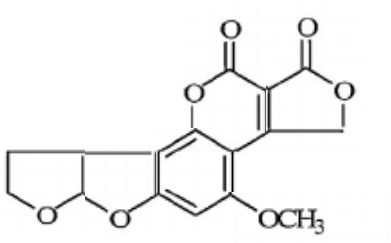
1.2 AFLATOXIN CONTAMINATION OF MAIZE

Mycotoxins are secondary metabolites produced by fungi on various agricultural commodities during pre- and post-harvest stages (Muthomi *et al.,* 2009). According to FAO, some 25 % to 50 % of the world's food is heavily contaminated with mycotoxins and more so among rural communities in developing countries, where there is general lack of food safety control measures before consumption or during trade (Bii *et al.*, 2012; Okoth and Kola, 2012). Mycotoxins have gained considerable worldwide attention due to their significant effect on human health, animal productivity and trade. However, the presence of mycotoxins in Africa remains overlooked due to public ignorance about their existence, lack of enforcement of regulatory mechanisms, dumping of food products, and the introduction of contaminated commodities into the human food chain during chronic food shortage due to drought, wars and political and economic instability (Wagacha and Muthomi, 2008). Among the more than 300 known mycotoxins (Hussein and Brasel, 2001), the food borne mycotoxins of greatest significance for human health, trade and agriculture in developing countries are the aflatoxins and fumonisins (Murphy *et al.*, 2006; Kumar *et al.*, 2008).

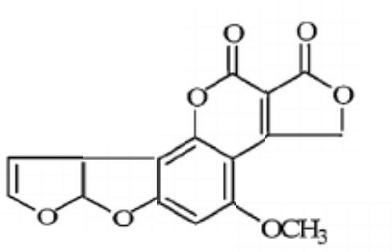
Aflatoxins are natural secondary metabolites produced mostly by *Aspergillus flavus* and *Aspergillus parasiticus* and, to a lesser extent, *Aspergillus nomius*, on varied substrates including soil, agricultural products and organic matter when conditions are favourable for their growth. Optimum conditions for fungal development are temperatures between 25 ^{o}C and 42 ^{o}C with relative humidity above 85 %. Optimal toxin production occurs at 25 ^{o}C to 35 ^{o}C (Schroeder and Boller, 1973; Kurtzman *et al.*, 1987; Klich, 2007; Muthomi *et al.*, 2009; Odhiambo *et al.*, 2013). Aflatoxins are a wide spread problem in Africa due to favourable environmental conditions and their occurrence in major cereal crops has been documented (Shepard, 2003; Bankole *et al.*, 2006; Wagacha and Muthomi, 2008). There are up to 18 different types of toxins identified in the aflatoxin group and among these the four main types are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (Yu, 2012; Filazi and Sireli, 2013). Metabolism of AFB1 results in hydroxylated derivatives, mainly aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2), which are found in animal products such as milk from animals fed on aflatoxin contaminated feed as well as in human breast milk (Samson *et al.*, 2006; Kangethe and Langa, 2009). AFB1 is the most potent and has been classified as a Group I human carcinogen whereas AFM1 is classified as Group 2B and a probable human carcinogen (IARC, 1993). Their names are derived from their fluorescence colours when they are separated by chromatography under long wavelength ultraviolet (UV) light. B type aflatoxins exhibit blue fluorescence and G type green fluorescence. This property is commonly used to assist in isolation, purification and analytical procedures (Wogan, 1966). AFB1 and AFB2 are produced by *A. flavus* while *A. parasiticus* produces AFG1 and AFG2 along with AFB1 and AFB2 (Dorner *et al.*, 1984; Klich and Pitt, 1988). Structurally, aflatoxins are low molecular weight organic compounds with a shared carbon chain length of seventeen but differing in the number of hydrogen and oxygen atoms (Asao *et al.*, 1965; Wogan, 1966). The chemical structures of the major aflatoxins are shown in Figure 1.1.



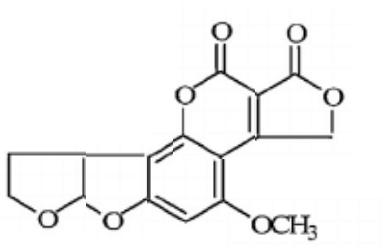
Aflatoxin B1 ($C_{17}H_{12}O_6$)



Aflatoxin B2 ($C_{17}H_{14}O_6$)



Aflatoxin G1 ($C_{17}H_{12}O_7$)



Aflatoxin G2 ($C_{17}H_{14}O_7$)

Figure 1.1: Molecular structures of the four primary aflatoxins (adapted from Wogan (1966)).

Infection of maize by aflatoxin-producing fungi and subsequent production of aflatoxin occurs at the pre- and post-harvest stages and at all points along the value chain. Severity of infection is influenced by several factors, among them environmental factors, agronomic practices, post-harvest and storage practices as well as biotic factors (Rodriguez-del-Bosque, 1996; Cotty and Jaime-Garcia, 2007; Hell *et al.*, 2008). Contaminations occur more frequently during wet seasons after prolonged periods of drought with incidence rates varying across different geographical locations and seasons (Philip *et al.*, 1994). Although most contamination originates at the pre-harvest stage, much of the problem is associated with less than optimal post-harvest handling and storage conditions. Growth of the fungus and subsequent contamination of the grains with aflatoxins during storage is mainly influenced by grain moisture and temperature. Excessive heat, high relative humidity, poor aeration and insect and rodent damage proliferate the spread of fungal spores in storage (Hell and Mutegi, 2011; Mutiga

et al., 2014). Human and animal exposure results from consumption of contaminated maize, contact with contaminated products as well as through milk and meat contaminated with AFM1 and AFM2 metabolites (Wagacha and Muthomi, 2008). Vulnerability is high among poor communities, especially during periods of drought, where food security and nutritional needs overshadow considerations of food safety (Daniel *et al.*, 2011). Consumption of highly contaminated products is associated with frequent aflatoxicosis outbreaks, characterized by vomiting, jaundice, abdominal pain, liver failure and in some cases death (Azziz-Baumgartner *et al.*, 2004). Chronic aflatoxicosis results from long term low level exposure to aflatoxin. This condition is highly correlated with increased incidence of liver cancer, malnutrition, immunosuppression, impaired growth, human immunodeficiency virus /acquired immuno deficiency syndrome (HIV/AIDS) and malaria in sub-Saharan Africa (Williams *et al.*, 2004; Jolly *et al.*, 2007). In agriculture, contamination of main staples (mainly maize and groundnuts) affects the four pillars of food security, namely availability, access, utilization and stability (PACA, 2013). Economic loss results from yield losses, reduced value, loss of animal productivity, costs of managing rejected lots and reduced accessibility to international markets (Wu and Guclu, 2012).

In Kenya, frequent outbreaks of acute aflatoxicosis are a serious recurrent health problem. The first recorded major incidence was in 1981 when 12 people died in Machakos district as a result of consuming maize that was contaminated with aflatoxins at a concentration of 150 to 12000 ng/g (Nangindu *et al.*, 1982). The most severe outbreak was in 2004 in the Makueni district, Eastern Province, when 317 cases and 125 deaths were reported (CDC, 2004). The deaths were traced to consumption of maize heavily contaminated with aflatoxin at concentrations as high as 46400 ng/g (CDC, 2004; Lewis *et al.*, 2005; Probst *et al.*, 2007). The most recent incidence was in 2010 when approximately 2.3 million bags of maize, estimated to be worth 6.9 million

US dollars, were found to be contaminated with aflatoxin and unfit for human and animal consumption. This was a big loss for the small scale farmers who depend on the crop for food and as a source of income (Okoth and Kola, 2012). The outbreaks indicate high aflatoxin exposure levels are prevalent in Kenya and emphasise the need to develop and evaluate potential control measures. Furthermore, adaptability and appropriateness of these measures for the rural populations should be validated.

Various strategies to reduce aflatoxin exposure have been reported to be effective in developed countries and in commercial environments but these are not necessarily suitable for subsistence farmers in developing countries. The majority of these farmers sell their maize through informal marketing channels, which makes it difficult to enforce marketing regulations such as Hazard Analysis and Critical Points (HACCP), tolerance limits and even testing the produce for aflatoxin contamination (Aldred and Magan, 2004; USAID, 2010; Wu and Khlangwiset, 2010). Additionally, contamination is not often evident to the farmer and hence requires robust sampling and analysis technologies. In Kenya, responses to aflatoxicosis outbreaks have been through short-term measures such as awareness raising campaigns and replacement of contaminated maize (CDC, 2005). Attention to acute aflatoxicosis outbreaks has overshadowed the chronic exposure that is more prevalent and potentially detrimental to human health. This can partly be attributed to lack of a well-established regulatory mechanism to monitor food samples frequently at different points along the value chain. This deficiency is further compounded by limited analytical capacity (Owaga *et al.*, 2011).

Recommended strategies for management of aflatoxin at the pre- and post-harvest stages include proper field management, good agricultural practices, insect control, proper drying,

sorting and storage and, more recently, biological control with non-aflatoxigenic *A. flavus*. However, these are not effective at the subsistence farm level (Hell *et al.*, 2008). Therefore, when aflatoxin contamination occurs, regulatory control to prevent entry of contaminated produce into the supply chain is of paramount importance. Regulatory limits established for aflatoxins vary from country to country and are based on prevalence of occurrence, distribution within a sample, method of analysis and available legislation. Internationally, aflatoxin tolerance levels are based on Codex Alimentarius guidance (van Egmond and Jonker, 2004; Codex, 2010) and are generally set at lower limits for developed countries as compared to developing countries, a trend that has a significant impact on African exports to lucrative European markets (Dors *et al.*, 2011). In Kenya, tolerance limits for total aflatoxin were recently reviewed downwards from 20 μg/kg to 5 μg/kg for AFB1 and 10 μg/kg for total aflatoxin (Mutegi *et al.*, 2013). Implementation of these standards is, however, dependant on the availability of analytical capacity to enable detection and quantification of aflatoxins at tolerable limits both for monitoring purposes and compliance with international standards.

1.3 CURRENT ANALYTICAL METHODS FOR DETECTION OF AFLATOXINS

Analysis and quantitative determination of aflatoxins in food samples is commonly carried out using chromatographic and immunochemical techniques. The latter are applied for rapid screening, to detect the presence of toxins, while the chromatographic techniques serve as reference methods to confirm and accurately determine the concentration of toxin present (Manetta, 2011).

1.3.1 Chromatographic techniques

Chromatographic methods used include thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography/ mass spectroscopy (GC-MS), liquid chromatography/ mass spectroscopy (LC-MS) and ultra-high performance liquid chromatography (UPLC) (Pascale, 2009; Rai *et al.*, 2012). Analyses are preceded by sample preparation that involves extraction with an organic solvent such as methanol and clean up to eliminate matrices that may interfere with the analysis. TLC based on fluoro densitometry and visual procedures is the oldest technique for determination and quantification of aflatoxins. Though the method is simple, cost-effective and rapid for screening a large number of samples, it has been replaced by HPLC (de Castro and Vargas, 2001; Manetta, 2011). HPLC can be used to analyse a wide variety of compounds, is easily adapted to confirmatory procedures, has potential for automation and can employ short and high resolution reversed columns, coupled with highly sensitive fluorescence and electrochemical detectors (Holcomb *et al.*, 1992). As a result, HPLC coupled with either detection by UV absorption, fluorescence, mass spectrometry or amperometric detectors is the most widely used chromatographic technique for analysis of mycotoxins (Turner *et al.*, 2009). However, separation of target analytes by HPLC is time consuming in addition to the tedious pre- or post-column derivatization required to improve the limits of detection (Wacoo *et al.*, 2014). UPLC coupled with either mass spectrometry or

fluorescence detection has been developed to overcome the above challenges associated with HPLC. Reduction of column material particle size from 5 µm used for HPLC to less than 2 µm used for UPLC gives the latter improved chromatographic performance (Taleuzzaman *et al.*, 2015). Improved chromatographic resolution, sensitivity, reduction in the analysis cycle and cost has been demonstrated with UPLC. However, the variable matrix effects limit its application in routine analysis (Cui *et al.*, 2017).

Analysis of mycotoxins by GC-MS is based on differential portioning of analytes between the mobile and stationary phase. The mobile phase is a carrier gas, while the stationary phase consists of inert particles coated with a layer of liquid normally confined to a long stainless steel or glass tube called the column, maintained at an appropriate temperature. The sample to be analysed is vaporized into a gaseous phase and carried by a carrier gas through the stationery phase /column. The components of the sample move through the column at different speeds based on their molecular weight where they are separated into individual components (Wacoo *et al.*, 2014). After separation, detection of the volatile products is achieved by a flame ionization detector, an electron capture detector, mass spectrometer or fourier transform infrared spectrometer (Turner *et al.*, 2009; Roseanu *et al.*, 2010). Although GC-MS is cheaper compared to other chromatographic techniques such as HPLC and LC-MS, it is less widely used commercially for direct determination and analysis of aflatoxins. The major limitations on the use of GC-MS for direct analysis of aflatoxins include non-volatility of aflatoxins (hence need for pre-column derivatization), non-linearity of calibration curves, drifting responses, carry-over or memory effects from previous samples, and high variation in terms of reproducibility and repeatability (Pascale, 2009; Rahmani *et al.*, 2009). In general the chromatographic methods, though accurate, sensitive and capable of medium throughput with a low limit of detection, are characterized by very tedious, time consuming and expensive

sample extraction and clean up procedures as well as requirements for specialized equipment, skills and laboratory facilities. These limit their use to the laboratory environment and thus are inapplicable in field situations (Wilson, 1989; Berthiller *et al.,* 2007; Jinap and Soleimany, 2009).

1.3.2 Immunochemical techniques

A number of immunochemical assays that rely on antigen- antibody reactions to detect aflatoxins have been developed. The enzyme-linked immunosorbent assay (ELISA) is the most established. Commercial formats based on monoclonal and polyclonal antibodies have been developed for qualitative and semi-quantitative analysis of aflatoxins (Li *et al.,* 2009; Waliyar *et al.,* 2009). Immunochromatographic test strips, commonly referred to as lateral flow devices, such as dipsticks, are more adaptable to on-site field analysis (Pascale, 2009; Mostafa *et al.,* 2012). They consist of immobilized antibodies and antibody-coated dye receptors on a membrane strip which react with any aflatoxin present in the sample to form a coloured band on the strip that indicates if the aflatoxin concentration is above a pre-determined threshold (Prieto-Simo *et al.,* 2007). Compared to ELISA, lateral flow devices are more expensive and are designed to measure contamination levels up to set regulatory limits which can vary between commodities and countries. Additionally, determining the cut-off value is based on visual inspection of a colour change on the strip, which may lead to subjective interpretation of results (Manetta, 2011). Generally, immunological techniques are relatively rapid, simpler and more suited for on-site analysis compared to chromatographic techniques. However, their main limitations are cross-reactivity and matrix interference that can lead to overestimation of contamination levels, commonly referred to as false positives (Anfossi *et al.,* 2013). Though false negatives are rare, false positives are more frequent. These can result from inhibition of enzyme activity, denaturation of the antibody or hindered interaction between the antigen and

the antibody due to presence of substances structurally related to aflatoxins in the assay and other factors such as temperature, sample viscosity, pH, and ionic strength which influence the results (Lee *et al.*, 2004; Kralj and Prosen, 2009).

While chromatographic and immunochemical methods have been found to be effective for detection of aflatoxins (Kralj and Prosen, 2009; Rai *et al.*, 2012), their application in developing countries is limited by availability of resources and necessary infrastructure. Though sensitive enough to detect aflatoxins below legal regulatory limits, these methods are characterized by time consuming extraction and clean up procedures. They are also expensive and require trained personnel to operate them. Furthermore their use is constrained by the nature of farming systems in Africa where up to 90 % of the farmers are small scale (Mbithi and VanHuylenbroeck, 2000; USAID, 2010) and located in remote villages. The farmers have limited or no access to laboratory facilities located in major cities. The long distances between the laboratories and the villages, coupled with a lack of well-coordinated information flow between the laboratories and the extension systems, further complicates sampling and access to the analysis results (Strosnider *et al.*, 2006). There is, therefore, a need to develop simple, rapid, reliable, non-destructive, cost effective and field portable diagnostic techniques which are well adapted to the regulatory, research and surveillance requirements of developing countries where aflatoxin contamination is more prevalent. These would allow for quick screening of produce at the market entry level, thus reducing potential exposure within a reasonable time and with little cost (Daniel *et al.*, 2011). Though the methods may not quantify the level of contamination, they could be useful as an initial screening where indication of the presence or absence of contamination is sufficient for decision making (Stroka and Anklam, 2002). This would facilitate movement of goods along marketing channels and help to reduce the cost and capital investments associated with aflatoxin analysis (Hassanshahian *et al.*, 2012).

1.3.3 Other rapid cost-effective approaches

A number of rapid and cost-effective approaches have been proposed including bright greenishyellow fluorescence (BGYF) test, fluorescence polarization immunoassays, near infrared spectroscopy, sensors and indirect screening methods such as detection of volatile metabolites of the fungi (Magan and Evans, 2000; Kralj and Prosen, 2009; Pascale, 2009; Cheli *et al.*, 2012). The BGYF test is widely used for the detection of aflatoxin contamination in food items. The produce is inspected under long-wave UV light (365 µm.) for a characteristic bright greenish yellow fluorescence (Fennell *et al.*, 1973; Bothast and Hesseltine, 1975; Shotwell and Hesseltine, 1981).The fluorescence is produced by a reaction of heat labile peroxidase present in the living plant tissues with kojic acid, which is synthesised as a secondary product by *Aspergillus* species and other fungi. The test indicates the presence of fungal growth that may have resulted in aflatoxin contamination and the number of BGYF particles found in samples is related to the aflatoxin contamination level (Kalkana *et al.*, 2014). However, application of the test for detection of aflatoxin contamination in maize is limited by low capability of maize kernels to exhibit BGYF due to the insufficient amount of peroxidase in kernels (Wilson, 1989). Additionally, the observed fluorescence results from kojic acid which is also synthesised by other fungi that do not produce aflatoxins resulting in high incidence of false positives and false negative samples (Pearson *et al.*, 2001).

Fluorescence polarization immunoassay is a single step homogenous assay with potential for rapid analysis of a large number of samples (Nielsen *et al.*, 2000). Available formats for analysis of mycotoxins are based on polarization of fluorescently labelled antigen (tracer) and a specific antibody in solution (Smith and Eremin, 2008; Maragos, 2009). Compared to heterogeneous assays such as ELISA, the separation and washing stages are eliminated hence offering potential for a rapid assay (Manetta, 2011). However, commercially available assays

have shown low sensitivity and accuracy when used for analysis of cereal samples (Pascale, 2009). Near infrared spectrometry has been proposed as a potentially rapid, non-destructive technique for analysis of aflatoxins in maize kernels and milled flour (Berardo *et al.,* 2005; Harvey *et al.,* 2013). Additional advantages over conventional analytical techniques include ease of equipment operation, improved accuracy and precision and ability to simultaneously determine several components from a single spectrum (Pascale, 2009; Espinosa-Calder *et al.,* 2011). However, application of the technique is dependent on development and validation of calibration models to allow for classification of analysis samples. Additionally their ability to detect aflatoxins at the set regulatory limits for food and feed has not been proven (Berardo *et al.,* 2005; Cheli *et al.,* 2012; Harvey *et al.,* 2013).

1.4 EMERGING METHODS FOR DETECTION OF AFLATOXINS BASED ON DETECTION OF VOLATILE METABOLITES OF FUNGI

Microbial species produce volatile organic compounds as intermediate and end products during their primary and secondary metabolic processes. A number of volatile organic compounds (VOCs) occurring as mixtures of hydrocarbons, heterocyclics, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives have been identified from fungi (Kuske *et al.*, 2005; Korpi *et al.*, 2009; Morath *et al.*, 2012; Hung *et al.*, 2015; Schulz-Bohm *et al.*, 2017). The compounds are involved in different biological processes such as biocontrol, communication between microorganisms and their environment, mediation of defence against predators and to facilitate reproduction (Insam and Seewald, 2010; Stoppacher *et al.*, 2010; Morath *et al.*, 2012; Hung *et al.*, 2015). Additionally, the compounds can be used as a practical and rapid tool for detection of food spoilage caused by microorganisms (Schnürer *et al.*, 1999). Food spoilage fungi produce volatiles that are species and sometimes strain specific as they colonize nutrient rich substrates (Kershi *et al.*, 1988; Borjesson *et al.*, 1992; Magan and Evans, 2000; Casalinuovo *et al.*, 2006). Therefore monitoring of volatiles could be used as an early indicator of quality loss and contamination of grains with mycotoxins. The volatiles are characteristic markers of the fungal presence and can be used to discriminate between contaminated and un-contaminated grains (Jeleń and Wasowicz, 1998).

The metabolic pathways for the production of volatiles are an important indicator of relationships between volatile and non-volatile compounds and the process of mycotoxin production. Studies conducted with a wide range of fungal species including *Fusarium, Aspergillus* and *Penicillium* show that similar volatile compounds may be produced by the different fungal species regardless of the type of grain substrate (Keshri and Magan, 2000; Demyttenaere *et al.*, 2004; Karlshoi and Larsen, 2005; Jurjevic *et al.*, 2008; De Lucca *et al.*,

2010; Jelen´ *et al.*, 1995). However, fungal species also produce volatiles that are species and sometimes strain specific (Kershi *et al.*, 1988; Magan and Evans, 2000; Casalinuovo *et al.*, 2006). Therefore the use of volatiles as an indicator of fungal presence and contamination should be based on a combination of volatiles unique to the species rather than individual compounds (Magan and Evans, 2000). Among the volatiles produced by fungi, aliphatic alcohols, carbonyls, esters, lactones, aromatics and terpenoids are the most abundant (Korpi *et al.*, 2009; Morath *et al.*, 2012; Hung *et al.*, 2015). The breakdown of lipids through fungal lipase activity results in free fatty acids that are oxidised to β-keto acids, which are subsequently decarboxylated to methyl ketones. The methyl ketones undergo reduction to form alcohols. Esters are formed through enzyme catalysed reactions between alcohols and acyl-CoA compounds. Pyrazines are thought to be synthesised by the condensation reaction between acetoin and ammonia whereas terpenoids are derived from the mevalonic acid pathway (Jeleń and Wasowicz, 1998; Magan and Evans, 2000). The substrates involved in biosynthesis of the main volatiles are shown on Figure 1.2.

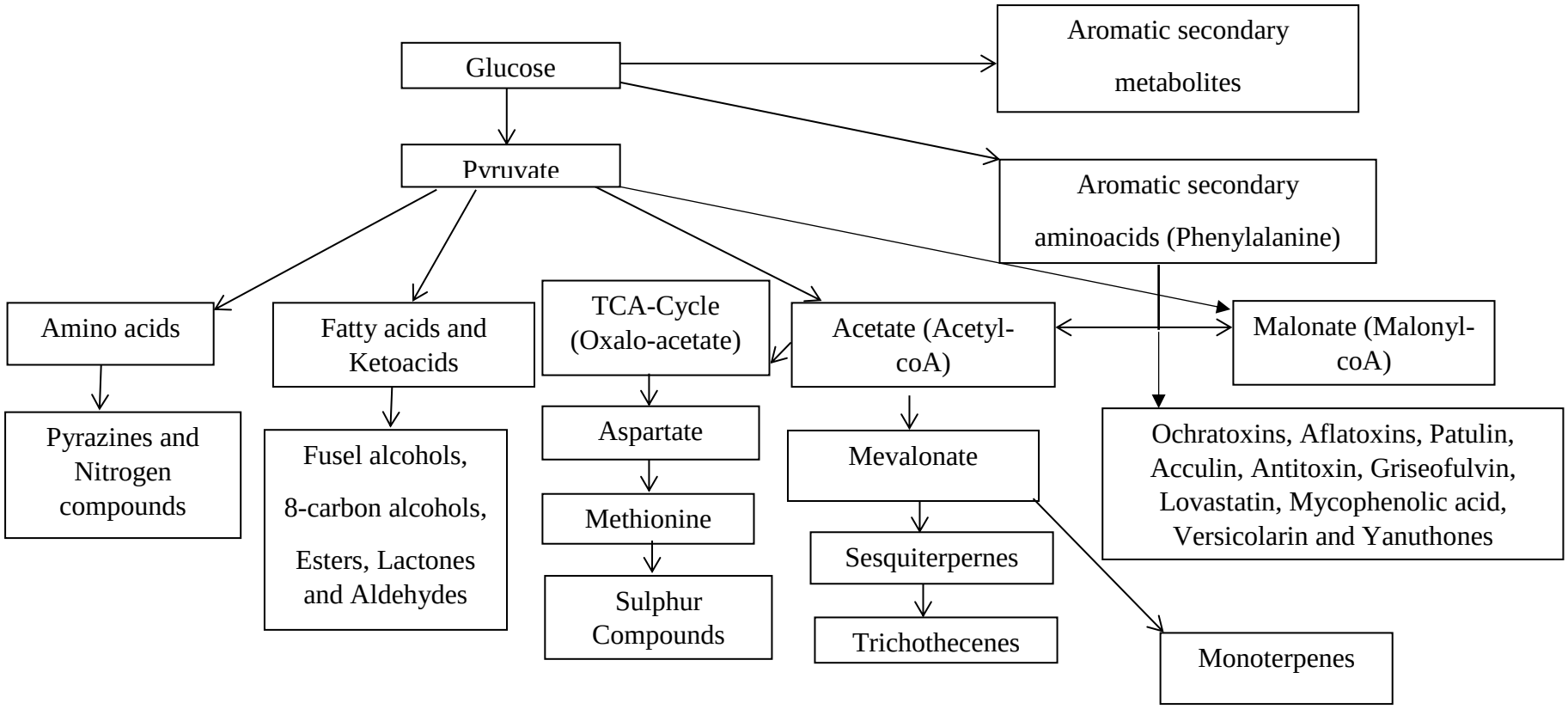


Figure1.2: Metabolic pathways for the biosynthesis of the main groups of fungal volatile compounds (adapted from Jeleń and Wasowicz (1998); Magan and Evans (2000); Vadlapudi et al. (2017)). TCA- Tricarboxylic acid cycle.

1.5 ANALYTICAL METHODS FOR DETECTION OF VOCs

1.5.1 GC-MS

Separation and detection of VOCs is normally performed with a GC in tandem with a mass spectrometer (MS). A number of techniques that include liquid extraction, steam distillation, simultaneous distillation extraction, purge and trap, supercritical fluid extraction and more recently solid phase micro extraction (SPME) have been used for sampling VOCs (Zhang and Li, 2010). Among these, the SPME has become increasingly popular because it reduces preparation time by combining extraction, concentration and introduction into one step while increasing sensitivity over other extraction methods (Zhang *et al.*, 1994; Kataoka *et al.*, 2000). The VOCs are adsorbed onto a fibre-coating mounted to the plunger of a syringe-like holder and subsequently desorbed from the coating into the heated inlet of a GC. This allows for immediate thermal desorption and rapid transfer of compounds to the GC column for separation (Eisert and Pawliszyn, 1997). Using a MS, the molecular mass and typical fragmentation pattern of unknown volatiles are obtained. Structural characterisation and confirmation of identity is usually achieved by comparison of mass spectra with established library spectra, the determination of chromatographic retention indices and/or the determination of authentic standards in parallel (Zhang and Li, 2010).

Numerous *in vitro* studies have been conducted to determine the types of volatiles produced by crop spoilage fungi. The studies have been based on growth of individual fungi on cereal based substrates including sterile wheat, maize, barley, and whole bread and, the use of GC or GC-MS for detection and identification of volatiles produced (Kaminski *et al.*, 1972; Jelen and Wasowicz, 2000; Magan and Evans, 2000; Olsson *et al.*, 2002; De Lucca *et al.*, 2012). However, differences in volatile metabolites have been documented by different researchers.

Kaminski *et al.* (1972) identified a number of volatile compounds produced by *A. flavus* grown on a slurry of wheat meal. The volatiles were extracted by distillation with dichloromethane and analysed by packed-column GC. Identified compounds were: 3-methyl-butanol, 3-octanone, 3-octanol, 1-octen-3-ol and *cis*-2-octen-1-ol. However, the toxigenic status of the *A. flavus* strain was not documented. Jelen and Wasowicz (2000) documented 3-octanone, 1-octen-3-ol, p-xylene and limonene when monitoring growth of *Aspergillus ocharaceous* on autoclaved wheat. Production of 3- methyl-1-butanol, 1-octen-3-ol and other 8-carbon ketones and alcohols was recorded by Magan and Evans (2000) due to the activity of toxigenic and food spoilage fungi including *Aspergillus*, *Eurotium*, *Alternaria* and *Fusarium* species. There was however, no attempt to correlate specific volatiles with mycotoxins or measure the concentration of the mycotoxin. Zeringue *et al.* (1993) demonstrated correlation between the release of β-caryophyllene ($C_{15}H_{24}$) and the initiation and decline of aflatoxin synthesis unique to aflatoxigenic *A. flavus* grown on potato dextrose agar (PDA). Terpenoid compounds identified were different from those produced by aflatoxigenic *A. flavus* on intact maize cobs. De Lucca *et al.* (2012) observed production of a wider range of volatile compounds when *A. flavus* was grown on rewetted cracked corn compared to *A. flavus* grown on PDA. The non-aflatoxigenic *A. flavus* strain produced more volatiles than the aflatoxigenic strain and there were few volatiles unique to the aflatoxigenic strain. In this study, the headspace was not sampled after drying the contaminated maize and neither was there an attempt to simulate natural processes by which grains are contaminated at the pre-harvest stage. Jurjevic *et al.* (2008) determined metabolites associated with active fungal cultures of aflatoxigenic and non aflatoxigenic *A. flavus* strains using autoclaved corn and peanut samples. Volatiles were collected by trapping the head space with thermal desorption tubes. Volatiles found to be unique for the non-aflatoxigenic strain after 25 days growth at 25 ^{o}C were hexanal, 1- hexanol, 1-octene-3-ol and 2-pentyl furan while dimethyl disulfide and nonanal were unique for the

aflatoxigenic strain. However, production of volatiles differing between non-aflatoxigenic and aflatoxigenic strains was inconsistent for the corn and peanut substrates. Olsson *et al.* (2002), working on Swedish barley samples containing different levels of ochratoxin (OA), showed that samples with OA levels below 5 mg/kg had higher concentration of aldehydes (nonanal, 2-hexanal) and alcohols (1-penten-3-ol, 1-octanol) while samples with OA levels above 5 mg/kg had higher concentration of ketones (2-hexanone, 3-octanone) and pentane, methyl pyrazine, 3-pentanone, 3-octene-2-ol and isooctyacetate. They indicated that these differences could be exploited using multivariate statistical modelling to predict whether barley samples met the legal regulatory limits.

These studies show the potential of GC-MS analysis of VOCs for differentiation of toxigenic and non-toxigenic strains of mycotoxigenic fungi as well as contaminated and un-contaminated grains. However, most of the studies have been based on *in vitro* procedures with fungus cultured on artificial media or sterile grains. The volatile profiles of fungi grown on artificial media or autoclaved grains are likely to differ considerably from profiles of live plants infected with the fungus under simulated laboratory or field conditions. Additionally, unique diagnostic volatiles specific for detection of mycotoxin contamination in cereals have so far not been identified.

1.5.2 Electronic nose

The electronic nose offers potential as a rapid, cost-effective, non-invasive, high throughput and field portable diagnostic technique, for initial screening of commodities to detect mycotoxin contamination (Falasconi *et al.*, 2012; Alam and Saeed, 2013). Detection is based on changes in the composition of volatile compounds produced by mycotoxigenic fungi during their growth and biochemical processes (Cheli *et al.*, 2009). The volatiles are indicators of fungal activity and could be used as potential taxonomic markers to differentiate between fungal species as well as between contaminated and un-contaminated produce (Keshri and Magan, 2000; Magan and Evans, 2000). An electronic nose is a mimic of the human sensory apparatus and utilizes an array of chemical sensors that detect volatile compounds and generate an electrical signal that can be recognized and analysed as a signature for the sample (Persaud and Dodd, 1982). It consists of a sampling unit for capturing volatiles from the headspace of a sample, a sensory unit and a processing unit for analysis of sensor responses (Figure 1.3) (Di Natale *et al.*, 2001; Sliwinska *et al.*, 2014).

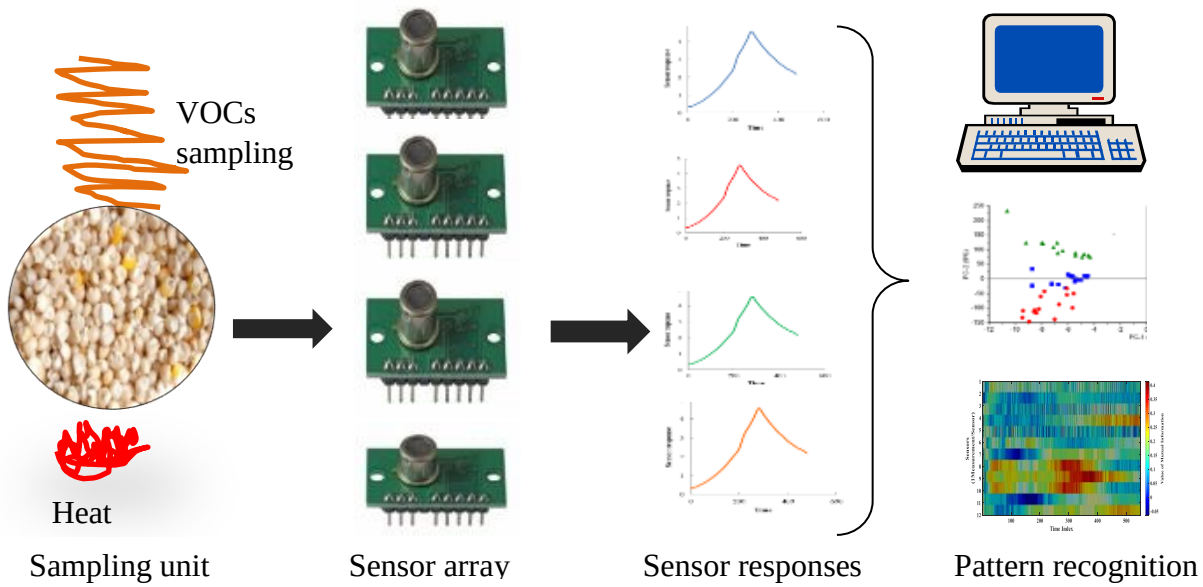


Figure 1.3: Schematic representation of an electronic nose system

The sensory unit can contain a single sensing element or an array of several different sensing elements with broad sensitivity and cross-reactivity or partially-overlapping selectivity (Wilson, 2013). The volatiles are drawn into the electronic nose sensory unit by either direct injection of sampled headspace or by carrying the headspace with a gas stream across the sensory elements (Arshak *et al.*, 2004). Interaction of volatile compounds and the sensory elements induces a reversible change in electrical properties, such as conductivity of the sensing materials, that is recorded and processed by a pattern recognition system which displays an output used for statistical analysis (Harsanyi, 2000; Peris and Escuder-Gilabert, 2016). In practice, the process involves three distinct stages. The measuring chambers and sensors are purged with clean air (zero air grade) to remove remnant compounds from previous measurements. The sensors are then exposed to the volatile compounds present in the sample headspace followed by a second air purge to clean the system for the next sample and stabilize the sensors back to their baseline (Macías *et al.*, 2014).

1.5.2.1 Electronic nose sensor types and characteristics

Electronic noses normally incorporate an array of sensors with varied sensitivities to different volatile compounds present in the sample headspace (Albert and Lewis, 2000; Rock *et al.*, 2008). Therefore, ideal sensors to be integrated into an electronic nose should show high sensitivity towards different compounds present in the headspace, high selectivity to allow detection of a wide number of different compounds, low sensitivity to variable environmental parameters especially humidity and temperature, stability and reproducibility as well as short reaction and recovery times between runs (Schaller *et al.*, 1998; Wilson and Baietto, 2009). In the past decade advances in sensory technology have resulted in the development of a range of sensor formats and microarray devices with varied applications. Several types of sensing technologies are available and have been incorporated in electronic nose models in the market. Sensing technologies commonly used in commercialized electronic noses include metal oxide semiconductors (MOS); conducting organic polymers (CP); piezoelectric crystals and most recently the mass detection-based electronic noses (Schaller *et al.*, 1998; Ampuero and Bosset, 2003; Rock *et al.*, 2008; Berna, 2010; Loutfi *et al.*, 2015; Peris and Escuder-Gilabert, 2016).

1.5.2.1.1 Metal oxide semiconductor sensors (MOS)

Gas sensors based on semiconducting metal oxides have been more widely used in electronic nose platforms compared to other types of sensors due to their low cost, flexibility of production, simplicity of use and potential applications in a large number of fields (Barsan *et al.*, 2007). They consist of a metal-oxide semiconducting film coated onto a ceramic substrate most often with a heating element included (Williams, 1999). The principle of operation relies on changes in electrical resistance induced by the adsorption of gases and subsequent combustion reactions occurring within the lattice oxygen species on the surface of metal oxide particles (Schaller *et al.*, 1998; Arshak *et al.*, 2004). Volatile molecules are adsorbed at the

surface of the semiconductor where they react with the dissolved gases (mostly oxygen) causing a modification of the device resistance/conductivity which is recorded as a response of the system to the particular sample (Ampuero and Bosset, 2003; James *et al.*, 2005). The sign of the response can either be an increase or decrease in resistance, depending on whether the oxides are *n* or *p* type, hence the gas can be classified as oxidizing or reducing and subsequently classification of the oxides as *n* or *p* type. The n-type oxides (mainly zinc oxide (ZnO), tin dioxide (SnO_2), titanium dioxide (TiO_2), iron (III) oxide (Fe_2O_3) or tungsten oxide (WO_3)) respond to oxidizing compounds while the p-type oxides (mainly nickel oxide or cobalt oxide) respond to reducing compounds (Williams, 1999; Berna, 2010; Kim and Lee, 2014). In addition to the MOS sensor material composition, metal oxide film deposition is an important variable affecting sensor sensitivity and selectivity. The MOS sensor films are classified as thin (10 nm-5 µm) or thick (10–300 µm), based on the film deposition techniques that include physical or chemical vapour deposition, evaporation or spraying and screen printing or painting. Though the thin film devices offer faster response and high sensitivities, they are difficult to manufacture and thus commercially available MOS sensors are based on thick film technologies (Dermarne and Sanjine, 1992; James *et al.*, 2005). They respond to a wide range of volatile compounds with a higher affinity for aldehydes, alcohols and ketones. Sensitivity is however low for terpenes, aromatic compounds and organic acids. The sensors operate at high temperature (200-600 ^oC) hence are less sensitive to changes in relative humidity compared to other sensor types (Dickinson *et al.*, 1998; Ampuero and Bosset, 2003; Wilson and Baietto, 2009). Through experimentation, the performance of these sensors has been improved by doping the semiconductor with metal catalysts such as palladium or platinum, modulation of the operational temperature and introduction of thermal gradients/cycles (Heilig *et al.*, 1997; Albert and Lewis, 2000; James *et al.*, 2005; Zhang and Xie, 2015). Gutiérrez *et al.* (1998) reported increased sensitivity of tin oxide sensors to benzene and toluene when doped with

lower concentrations of platinum and palladium. Corcoran *et al.* (1998) also reported increased sensitivity of these sensors with use of a thermal cycling technique. Using an eight sensor array with a temperature cycle between 250 and 500 °C in conjunction with a novel genetic algorithm for feature extraction techniques to classify tea, a classification rate of 90 % was achieved with the thermally cycled array compared to 69 % with the same array at a fixed temperature. Improved sensitivity of a tungsten oxide (WO_3) gas sensor through temperature modulation has been reported by Zhang and Xie (2015). Among the metal oxides, the most widely used is SnO_2 doped with palladium and platinum (Wilson and Baietto, 2009). Commercially available formats of electronic noses based on MOS include i-Pen, PEN2 and PEN3 (Airsense Analytics, GmbH Schwerin, Germany), Fox 2000, Fox 3000, Fox 4000 (Alpha-M.O.S., Toulouse, France), EOS507C (Sacmi, Imola, Italy) and DiagNose (C-it BV, Zutphen, The Netherlands) (Wilson and Baietto, 2009; Bruins *et al.*, 2013; Wilson, 2013; Cellini *et al.*, 2017).

1.5.2.1.2 Conducting polymers sensors (CP)

Conducting electroactive polymers have attracted attention as potential sensing elements for use in electronic noses. Compared to MOS, they have higher sensitivity and selectivity, short response time and more importantly they operate at room temperature (Yoon, 2013). Their chain structure can be selectively modified to incorporate diverse functional groups, such as enzymes and antibodies, giving the sensors a wide variety of functions (Dickinson *et al.*, 1998; Bai *et al.*, 2007). The sensor is made of a substrate (normally silicon or fibre-glass), a pair of gold-plated electrodes and a conducting organic polymer coating as the sensing element. Polypyrrole, polyaniline, polythiophene, polyindole, and polyacetylene are some of the most widely used polymer coating materials for the sensors (Janata and Josowicz, 2003). More recently, sensors based on electrically insulating polymers loaded with carbon black as a conducting filler have been developed (Doleman *et al.*, 1998). Interactions between VOCs and

the polymer surface result in an expansion of the polymer film which causes a change in the electrical resistance. The maximum change in resistance recorded for each sensor is considered as the sensitivity of the system to the particular sample (Dickinson *et al.*, 1998; Arshak *et al.*, 2004; Turner and Magan, 2004). While the sensors are sensitive and selective to polar organic compounds, their sensitivity to non-polar compounds can be improved by the use of different polymer structures and functional groups and doping with ions such as copper and palladium (Ampuero and Bosset, 2003). The sensors are easy to synthesize through chemical or electrical processes and have good mechanical properties that allow miniaturization into small devices (Mielle, 1996). However due to low operating temperatures they are extremely sensitive to moisture. The polymers readily absorb water vapour which reduces the available binding sites for volatiles, hence there is reduced sensor sensitivity at high humidity levels (Schaller *et al.*, 1998; Wilson and Baietto, 2009). Commercially available formats of CP based electronic noses include the AromaScan A32S (Osmetech Inc., Wobum, MA, USA) and Cyranose 320 (Sensigent, Inc. Balfwin Park, CA, USA) (Wilson and Baietto, 2009; Wilson, 2013; Cellini *et al.*, 2017).

1.5.2.2 Processing and analysis of electronic nose data

Electronic nose sensors collect data and convert it into an electrical signal pattern that can be subjected to computer analysis. Unlike traditional analytical instruments such as GC-MS, the signal has no direct connection to an individual chemical compound. Pattern recognition methods are therefore required to analyse the fingerprint signal generated by the sensor arrays and to classify the data (Loutfi *et al.*, 2015). Analysis of electronic nose data can be divided into four sequential stages that include signal pre-processing, dimensionality reduction, pattern classification and validation (Figure 1.4) (Gutierrez-Osuna, 2002).

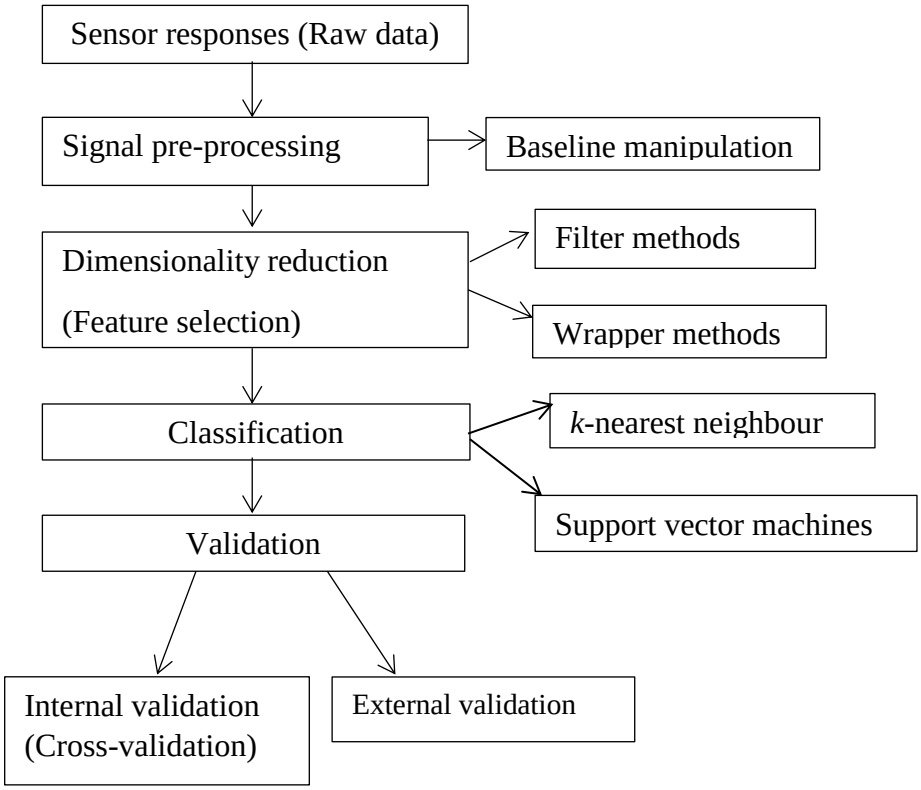


Figure 1.4: Sequential stages for analysis of electronic nose data.

1.5.2.2 1 Signal pre-processing

The objective of the signal pre-processing stage is to compensate for sensor drift and extract descriptive parameters from the sensor array responses to use for further analysis. Electronic nose sensor signals tend to exhibit significant temporal variation when exposed to identical volatile compounds over a long time which is normally referred to as 'drift'. The cause is unknown though is closely associated with factors like sensor poisoning, ageing or gradual changes in the environment conditions; mostly temperature and humidity (Scott *et al.*, 2007). These factors modify both the baseline and the sensitivity of the sensors in the array in different ways, depending on the sensor technology used. Sensor drift when not corrected compromises the pattern recognition ability of most multivariate techniques as the characteristics and limitations of the transducer may limit or distort the available information (Hines *et al.*, 1999).

Different approaches to correct sensor data that suffer from drift have been developed and include baseline manipulation, compression algorithms and normalization (Artursson *et al.*, 2000; Padilla *et al.*, 2010). Baseline manipulation is the simplest and most widely used drift compensation method. Individual sensor signals are transformed based on the initial value of the transient response referred to as the 'baseline'. Correction is by either subtracting, dividing or subtracting and dividing the baseline from the sensor response to obtain dimensionless and normalized responses (Di Carlo and Falasconi, 2012). A number of compression algorithms that generate descriptive parameters from the sensor transient responses are also applied to compensate for sensor drift. The algorithms extract additional information from the transient response resulting in improved selectivity and increased recognition accuracy (Gutierrez-Osuna, 2002). Normalization procedures are used on a local or global pattern to prepare the sensor responses for the subsequent pattern analysis. The most common local method is vector normalization, in which the feature vector of each individual response is divided by its norm and forced to lie on a hyper-sphere of unit radius. Global methods include sensor auto scaling, in which the mean and standard deviation of each response are set to zero and one, respectively, and sensor normalization in which the range of values for each individual feature is set to [0,1] (Pearce *et al.*, 1998).

1.5.2.2.2 Dimensionality reduction

Feature selection is a fundamental component of electronic nose signal processing and significantly affects the classification ability of the system. Pattern recognition is the process of classifying data by comparison to known patterns. The patterns are described in terms of multidimensional data vectors, where each component is called a 'feature' which represents the sensor parameters. A 'feature' therefore can be defined as a property of a process, measured or constructed from the original input variables (Vergara and Estevez, 2014). Most electronic nose

systems comprise an array of sensors with differing selectivity and sensitivity. Depending on the field application, only a subset of the sensors in the array contributes to the correct classification of the samples of interest. The redundant sensors may produce imprecise, incomplete and inconsistent data that degrades classification accuracy, i.e. "noise" (Bag *et al.*, 2014). Selection of sensor arrays and parameters that can produce the best possible classification of samples is therefore an important intermediate stage in data processing (Boilot *et al.*, 2003). Feature selection is a dimensionality reduction technique aimed at identifying the most effective subset of the original features (sensors and sensor parameters) to use in the classification process that lead to the smallest classification error (Scott *et al.*, 2007).

Multivariate analysis techniques that include principal component analysis (PCA) and clustering algorithms are exploratory and reduce the high dimensionality of electronic nose data allowing the information to be displayed in a smaller number of dimensions while retaining as much of the information present in the original data as possible (Berna, 2010). These multivariate methods are referred to as unsupervised or untrained techniques as they attempt to identify unknown data vectors without prior information on the nature of the samples. The classes are learnt based on some form of similarity measure (Scott *et al.*, 2007). Supervised/trained techniques on the other hand, include principal component regression, linear discriminant analysis, *k*-nearest neighbour algorithm (KNN), partial least squares regression, soft independent modelling class analogy and neural networks. They use prior information about the samples to classify new unknown individuals in one of the known classes on the basis of its pattern of measurements (Hodgins and Sirnmonds, 1995; Berrueta *et al.*, 2007). In general, feature selection methods are categorized into two, namely classifier dependent wrapper methods and classifier independent filter methods (Brown *et al.*, 2012). Selection of the method depends on the type of application and it is therefore important to identify methods

that maximise the value of the information obtained for reliable and acceptably-accurate classification (Wang *et al.*, 2014).

1.5.2.2.2.1 Wrapper methods

In wrapper methods, the selection algorithm conducts a search for a good set of features using the classifier as part of the evaluation function (Scott *et al.*, 2007). They use the training/validation accuracy of a particular classifier as a measure of utility for a candidate feature subset. Therefore the selected features depend on the classification model used (Jayaram *et al.*, 2010; Brown *et al.*, 2012). Wrapper methods give a better predictive accuracy compared to filter methods because features are easily tuned to the particular bias of the learning and classification algorithms. However, they are more computationally expensive in terms of complexity and time since each candidate feature set has to be evaluated with the classifier (Guyon and Elisseeff, 2003). Additionally they may produce feature sets that are overly specific to a classifier, therefore any change in the learning model is likely to reduce the usefulness of the feature set (Brown *et al.*, 2012; Bennasar *et al.*, 2015). As a result, filter methods are more widely used for analysis of electronic nose data (Gutierrez-Osuna, 2002; Vergara and Estevez, 2014).

1.5.2.2.2.2 Filter methods

Filter methods select features on the basis of the information content provided by a combination of sensors. Selection is based on feature separability of samples which is independent of the learning and classification algorithm (Jiacheng *et al.*, 2015). Filter methods evaluate statistics of the data independently of any particular classifier, thereby extracting features that are generic. In contrast to wrapper methods, feature selection is conducted once and selected features can be provided as input for different classifiers. They are therefore more computationally simple, fast and scalable (Jayaram *et al.*, 2010; Brown *et al.*, 2012). A major limitation of filter methods is lack of information on interaction between features and the classifier and selection of redundant and irrelevant features leading to overestimation of the feature significance (Nowotny *et al.*, 2013; Bennasar *et al.*, 2015). To overcome this, the concept of information theory has widely been applied in filter methods, where information measures are used to determine features' relevance and redundancy (Battiti, 1994). The features are ranked according to their individual predictive power based on their relevance to the class label in supervised learning (Fleuret, 2004). The relevance score is calculated using distance, information, correlation and consistency measures with techniques such as Pearson correlation coefficients, Fisher score, Kolmogorov-Smirnov test or mutual information (MI). According to the theory, a measure of uncertainty associated with the data is provided, enabling quantification of information collected by sensors. The measure is used in selecting the features independently from the classification methods to be used (Wang *et al.*, 2014). MI is a measure of statistical independence of the amount of information one random variable contains about another. The MI between two variables shows the reduction in uncertainty of one due to the knowledge of the other. MI is not increased where features contain redundant information about the class. Therefore maximising MI does not involve eliminating all redundancies between the features but ensuring any redundancies in the features about the class are not double-counted,

and any features which carry redundancies are only selected due to unique and synergistic information they provide (Wang *et al.*, 2014). Subsets of features are selected by maximising the MI between earlier selected features, a concept that minimises the uncertainty about the class given the features (Cover and Thomas, 2006).

Successful application of MI as a feature selection method for classification of electronic nose data has been demonstrated. Wang *et al.* (2014), evaluated the performance of mutual information as an efficient filter approach in feature selection for classifying chemicals using an array of metal oxide sensors across a range of classifiers in comparison to a wrapper approach applied earlier on the same data set. The method allowed selection of features that gave consistently better classification performances than randomly selected features across all the classifiers tested. Additionally the classification performance was not significantly different from that previously observed with the wrapper method which is more computationally intensive. The study provides important insights into the potential application of a MI approach for selection of feature subsets from electronic nose data that could be used to discriminate between toxigenic and non-toxigenic strains of fungi as well as between mycotoxin contaminated samples and uncontaminated samples. The electronic noses comprise an array of sensors with broad and overlapping specificities, where some sensors in the array lack sensitivity to the target compounds which increases variance/noise, while others have very similar sensitivities to the target compounds hence provide redundant information. The redundant and irrelevant sensors/features may contain incomplete and inconsistent information which degrades the predictive accuracy of the system (Wilson, 2013; Fang *et al.*, 2015). MI can facilitate selection of features (electronic nose sensors and sensor parameters) that contain the most information for discriminating between the different classes of samples, resulting in a smaller feature subset. This can potentially result in improvement of the predictive accuracy of

the system with a smaller number of features, hence reduced cost and time for data acquisition and management (Gardner and Hines, 2005; El Akadi *et al.*, 2008).

1.5.2.2.3 Classification

The goal of a pattern classifier is to use selected features to generate a model that assigns an unknown sample to one of several pre-specified classes, based on the properties that characterize the given class using supervised learning techniques. The model is trained with available data and used to develop rules for classifying new samples. The ease of classification depends on the variability of feature values for samples in the same class relative to feature values for samples in different classes (Scott *et al.*, 2007). The classification algorithms find a boundary that separates samples into different classes by calculating the distance between them with an appropriate metric. The Euclidean distance and the Mahalanobis distance are the most commonly used distance metrics (De Maesschalck *et al.*, 2000). The nature of the boundary varies and can be linear, quadratic, complex linear or complex non-linear depending on the type of classifier. Linear boundaries are based on Euclidean distance in which a straight line divides the variable space in two regions using classification methods such as linear discriminant analysis and partial least squares discriminant analysis. Complex linear boundaries are obtained with classifiers such as KNN where boundaries between classes consist of a series of straight lines, with the number of lines being a function of the number of samples. Complex non-linear boundaries on the other hand are obtained with Support Vector Machines (SVM) which are able to divide the space into non-linear regions using a kernel function (Dixon and Brereton, 2009). Due to the complex and non-linear nature of electronic nose data, KNN and SVM are the most widely used classifiers (Berrueta *et al.*, 2007).

The KNN algorithms are considered to be the simplest supervised learning algorithms in pattern classification. The algorithm compares the input data with an existing set of training data by computing a distance metric (usually the Euclidean distance). The *k* data points with the lowest distance metric are the neighbours to the input data, and are used for assignment of class membership to the input data. An unknown sample is classified in the class represented by a majority among the *k* neighbours (Gou *et al.*, 2012). The method has several advantages that include simplicity, effectiveness, intuitiveness and competitive classification performance (Berrueta *et al.*, 2007; Weinberger and Saul, 2009). The performance of KNN algorithms is however greatly influenced by selection of the neighbourhood size, *k*. Classification performance of KNN is based on an estimate of the conditional class probabilities from a training set, hence different *k* values yields different conditional class probabilities. A small *k* value results in a poor estimate due to data sparseness and the presence of noisy or mislabelled points, referred to as "overfitting". A large *k* value on the other hand results in over smoothing and degradation of the classification performance due to introduction of the outliers from other classes. The selection of *k* is therefore optimized by calculating the prediction ability with different *k* values (Gou *et al.*, 2012).

SVM represent a relatively new classification method that attempts to classify samples by finding the optimal hyperplane, or decision boundary that separates the samples into different classes (Xu *et al.*, 2006). The algorithm finds the hyperplane by use of support vectors which are the training samples closest to the boundary and the margins which represent the minimal distance from the hyperplane to the closest data points (Berrueta *et al.*, 2007; Entezari-Maleki *et al.*, 2009). Generally SVM exhibit superior classification performance compared to the traditional classification techniques (Shao and Lunetta, 2012). They have an inherent ability to deal with non-linear complex separation problems through application of the kernel function.

This function enables transformation of the vector space to a higher dimensional space where the classes can be separated. Commonly used kernels include the linear, radial basis, polynomial and sigmoid functions (Entezari-Maleki *et al.*, 2009). The linear function is the simplest and is used for data where classes are linearly separable. The polynomial function is used for data where classes are non-linearly separable, whereas the radial function is based on a class conditional Gaussian probability distribution which maps data into a different space where linear separation can occur. The sigmoid function is applied in instances where the structure of the data is unknown (Gromski *et al.*, 2014).

1.5.2.2.4 Validation

Validation is the final stage in analysis of electronic nose data that seeks to select an optimal classification model, define the model parameters and evaluate performance of the model with an unknown dataset. It is the process of demonstrating how good a selected model is in classification of unknown samples through evaluation of its recognition and prediction abilities (Gutierrez-Osuna, 2002). To develop a classification model the data is normally split into two sets namely the *training* set and a *validation (test)* set. The training set is used to define the model parameters whereas the validation set is used to evaluate performance of the trained model. Recognition ability is therefore defined as the percentage of the samples in the training set correctly classified during the modelling step and the prediction ability is the percentage of the samples in the validation set correctly classified using the models developed in the training step. The ideal scenario would be to have a validation set that is completely independent from the training set, what is referred to as external validation. However, since this is rarely the case, cross-validation methods are more widely used, where part of the data is used as a training set to develop the model and part is used as a validation set to evaluate prediction ability of the model (Berrueta *et al.*, 2007). Cross-validation is applied to compensate for the optimistic

apparent error rate, which is the fraction of misclassified samples as a result of training and validating on the same data set (Scott *et al.*, 2007). Commonly used cross-validation techniques include the *k*-fold cross-validation and the leave one-out cross-validation.

In *k*-fold cross-validation, samples are assigned randomly to training and validation sets in a way that allows for a sufficient number of samples in the training set and a representative number of members in the validation set. The data splitting is repeated *k* times with different constitutions of both sets to ensure that all the samples have the probability of being included in the validation set at least once and in the training set *k*-1 times. The leave-one-out cross validation on the other hand removes only one sample at a time from the training set, recalculates the classification function using the remaining data, and then classifies the omitted observation. The number of folds is thus equal to the number of samples in the data set (Baumann, 2003). In both techniques the average classification error rate across the different data partitions is used as the measure of the classifier performance. This represents the percentage of predictions that are correct out of the total presented to the system. In addition, a confusion matrix for the best classification accuracy obtained is usually provided to show the type of misclassified samples (Xiaolong *et al.*, 2015). A confusion matrix summarizes the performance of a classification algorithm by showing the number and type of correct and incorrect actual and predicted classifications (Tarca *et al.*, 2007; Santra and Christy, 2012). The type of misclassified samples can either be false positives (negative subject classified as positive) or false negatives (positive subject classified as negative) as illustrated in Table 1.1. The preference is to have a classifier that yields fewer false negatives compared to false positives as few actual cases are missed (Maxim *et al.*, 2014).

Table 1.1: Confusion matrix for a binary classifier.

		Predicted	
		Negative	**Positive**
Actual	**Negative**	**(a)** True negative	**(b)** False positive
	Positive	**(c)** False negative	**(d)** True positive

(a) - number of correct negative predictions, **(b) -** number of incorrect positive predictions, **(c) -** number of incorrect negative predictions, **(d)** – number of correct positive predictions.

1.5.2.3 Application of electronic nose for detection of mycotoxin contamination

The capability of an electronic nose to discriminate between samples infected with mycotoxigenic and non-mycotoxigenic fungi has been investigated by various researchers. Borjesson *et al.* (1996) attempted to classify wheat, barley and oats samples based on the sensory attributes 'moldy/musty', 'acid/sour', 'burnt' and 'normal' using ten metal oxide semi-conductor field effect transistor sensors (MOSFET) and four different SnO_2 semi-conductors. The electronic nose correctly classified 75 % of samples according to the four descriptors. Ninety percent of the samples were correctly assigned to the 'good' and 'bad' class. These values exceeded the levels of agreement between two human grain inspectors classifying the same samples. Similar results were obtained by Jonsson *et al.* (1997) using the same set of sensors to predict odour attributes: 'good', 'moldy', 'weak musty' and 'strong musty' in oats, rye, barley and wheat with different levels of ergosterol, fungal and bacterial colony forming units. A classification accuracy of 100 % was reported for oats with an artificial neural network (ANN) as the pattern recognition system. In wheat, correlations of 0.99, 0.84 and 0.88 were found between measured and predicted levels of fungal colony-forming unit (cfu), bacterial cfu and ergosterol respectively. In another study Olsson *et al.* (2002) demonstrated the ability of GC-MS and an electronic nose made up of ten MOSFET sensors and six SnO_2-based Taguchi sensors to quantify ochratoxin A and deoxynivalenol in barley grains. Evaluation of the data using multivariate projection methods, principal component analysis (PCA) and partial least

squares (PLS) showed that the GC-MS predicted ochratoxin concentrations with a higher accuracy than the electronic nose, since the GC-MS misclassified only three of 37 samples and the electronic nose seven of 37 samples. Falasconi *et al.* (2005) used a metal oxide based electronic nose to discriminate between a *Fusarium verticillioides* strain producing fumonisin and non-producing strain inoculated into artificial media *in vitro* and coarsely cracked maize kernels. Though discrimination of the different *F. verticillioides* strains was achieved using both the synthetic agar and autoclaved maize kernels maize, specific volatile associations were not investigated. Paolesse *et al.* (2006) evaluated the potential of an electronic nose with eight thickness shear mode resonators coated with various films of metalloporphyrins to discriminate between infected and non-infected wheat seed samples inoculated with *Penicillium chrysogenum* and *F. verticillioides.* Using partial least squares discriminant analysis, a classification accuracy of 85.3 % was achieved. Cheli *et al.* (2009) evaluated the potential of an electronic nose equipped with a Tenax-based enrichment and desorption unit to identify maize samples contaminated with aflatoxins. They did not identify any difference in specific volatiles, but found the system was able to discriminate maize samples according to the presence and absence of aflatoxins. However, the source of contamination (artificial or natural) was not specified. Campagnoli *et al.* (2011) employed an electronic nose with ten metal oxide sensors to discriminate between durum wheat grain samples naturally contaminated with deoxynivalenol and non-contaminated samples. Using PCA and regression trees, the electronic nose was able to classify the samples into three clusters, namely non-contaminated, contaminated below the regulatory limit and contaminated above the regulatory limit, with a classification error rate of 0 to 3.28 %. Gobbi *et al.* (2011) evaluated the ability of an electronic nose equipped with six metal oxide semiconductor chemical sensors to diagnose fungal contamination. It could detect and predict fumonisin concentration in maize cultures inoculated with different species of *Fusarium.* The electronic nose, in combination with univariate and

multivariate exploratory data analysis techniques, was able to discriminate the maize samples according to their fumonisin content. A summary of studies conducted to evaluate the potential application of electronic noses for detection of mycotoxin contamination in cereals are summarised in Table 1.2.

Table 1.2: A summary of studies on the evaluation of electronic nose for detection of mycotoxins in cereal grains.

Grain	**Sensor technology**	**Mycotoxin/ Fungal species**	**Reference**
Wheat, barley & oats	MOSFET & SnO_2	Odour classification	(Borjesson *et al.*, 1996)
Barley & wheat	MOSFET & SnO_2	Odour classification	(Jonsson *et al.*, 1997)
Barley	MOSFET and Taguchi SnO_2	Ochratoxin & deoxynivalenol	(Olsson *et al.*, 2002)
Maize	MOS	Fumonisins & aflatoxins	(Falasconi *et al.*, 2005), (Cheli *et al.*, 2009), (Campagnoli *et al.*, 2009), (Gobbi and Locci, 2006), (Gobbi *et al.*, 2011)
Wheat	Resonators coated with metalloporphyrin films and MOS	Deoxynivalenol, *Penicillium chrysogenum* & *F. verticillioides*,	(Campagnoli *et al.*, 2011), (Paolesse *et al.*, 2006)

MOSFET- Metal oxide semiconductor field effect transistor, MOS- Metal oxide semiconductor, SnO2- tin dioxide

Although researchers have been able to discriminate between contaminated and un-contaminated samples to a reasonable degree, a number of limitations have been identified. Most testing has been based on fungi grown on artificial media with contamination protocols which do not reflect a realistic scenario, where cereals are contaminated pre-harvest and are

subsequently harvested and dried prior to human consumption. Additionally, a majority of the studies have been focused on mycotoxins produced by *Fusarium* and *Penicillium* species in wheat, barley and oats. Studies on evaluation of electronic noses for detection of aflatoxin produced by *Aspergillus* species on maize are limited. The studies show that production of VOCs on grains by mycotoxigenic fungi is influenced by the substrate on which the fungus is grown and the environmental conditions. This could potentially have an effect on the capability of any electronic nose to detect contamination. Therefore the applicability of this technique for detection of aflatoxin across different maize genotypes artificially and naturally infected with *A. flavus* needs to be validated.

1.6 JUSTIFICATION

Maize, the main staple food in Kenya, is highly susceptible to degradation by mycotoxigenic fungi. Contamination by the fungus not only reduces the quality but also has detrimental effects on human health which can be fatal. Recommended management strategies have not been effective at a subsistence farm level. Therefore a regulatory control to prevent entry into the supply chain is of paramount importance. This is, however, limited by the nature of farming and trading systems that complicate enforcement of regulatory control, as well as limited analytical capacity non-existent at subsistence level. Available screening and analytical techniques are fraught with numerous challenges, including high cost, laborious and time consuming procedures and lack of necessary infrastructure. There is therefore an urgent need for simple, accurate, reliable, rapid and cost-effective techniques for early detection of *A. flavus* infection and aflatoxin contamination of maize that are adaptable to the regulatory and monitoring systems of developing countries. This will enable provision of real time monitoring data that would facilitate removal of contaminated lots prior to trade or consumption.

The use of VOCs produced by crops upon infection by fungi provides a novel and promising alternative to existing screening and analytical techniques for detection of mycotoxin. The volatiles are characteristic markers of fungal presence and can be used to discriminate between contaminated and un-contaminated bulk grains. GC-MS and electronic noses are the most widely used techniques for analysis of VOCs associated with mycotoxin contamination. The potential to rapidly and noninvasively monitor fungal infection and toxin presence in real time makes the techniques highly desirable. The field component of this study was conducted in Kenya because of project funding limitations and also because of the high incidences of aflatoxicosis previously reported making it easier to obtain field samples contaminated with aflatoxins.

1.7 OBJECTIVES

The main objective of this study was to evaluate the potential application of GC-MS and electronic noses for detection of aflatoxin contamination in maize flour. To achieve the objective, the study explored three specific objectives:

1. To evaluate the potential for GC-MS to detect aflatoxin contamination in Australian and Kenyan maize varieties artificially inoculated and naturally infected with *A. flavus*.
2. To compare the performance of different electronic nose sensor technologies for detection of aflatoxin contamination in maize (MOS sensors, (Fox 3000), CP sensors, (Cyranose 320) and MOS sensors with thermocycling (DiagNose)).
3. To evaluate the potential for a field portable electronic nose (DiagNose) to detect aflatoxin contamination in Kenyan maize varieties artificially inoculated and naturally infected with *A. flavus*.

1.8 OUTLINE OF THE THESIS

The first Chapter provides a background to the problem and a literature review on the topic that discusses the role of maize in food security, factors that predispose maize to aflatoxin contamination and the effect of contamination on human health and trade. The Chapter also addresses the current and emerging analytical techniques for mycotoxin analysis, the project justification and objectives. In Chapter 2, the artificial inoculation of maize samples with *A. flavus* and collection of maize samples naturally infected with *A. flavus*, that were used for evaluation of the diagnostic techniques is presented. Chapter 3 addresses the potential application of GC-MS for detection of aflatoxin contamination using Australian and Kenyan maize varieties that were artificially and naturally infected with *A. flavus*. Potential diagnostic VOCs for detection of aflatoxin contamination of maize are discussed. In Chapter 4, the capabilities of three electronic platforms, representing different sensor technologies, to detect aflatoxin contamination in maize are compared. The best performing platform is selected and evaluated with Kenyan maize varieties that were artificially and naturally infected with *A. flavus*. In Chapter 5, the potential application of a field portable electronic nose (DiagNose) to detect aflatoxin contamination in Kenyan maize varieties artificially and naturally infected with *A. flavus* is investigated. Chapter 6 summarises the key findings of the study and presents the overall conclusions and recommendations for future work.

CHAPTER 2

ARTIFICIAL INOCULATION OF MAIZE WITH *A. FLAVUS* AND COLLECTION OF FIELD SAMPLES NATURALLY INFECTED WITH *A. FLAVUS*

2.1 INTRODUCTION

The potential for GC-MS and electronic noses to detect aflatoxin contamination was evaluated with maize samples that were artificially inoculated and naturally infected with *A. flavus.* The objective was to generate samples contaminated with different strains of *A. flavus* and controls and as well samples with varying aflatoxin levels across the different sample classes. This chapter details materials and methods used for artificial inoculation of maize with *A. flavus*, the methodology for collection of field samples that were naturally infected with *A. flavus*, the ELISA procedure for determination of aflatoxin contamination levels as well as the aflatoxin contamination results and discussion for maize samples artificially inoculated and naturally infected with *A. flavus*. In the context of this thesis, aflatoxin contaminated sample refers to milled flour with aflatoxin levels ≥ 6ppb, while non-contaminated sample refers to milled flour with aflatoxin levels ≤ 5ppb. The ppb is the SI unit for quantification of aflatoxin contamination levels on a vol/vol basis.

2.2 MATERIALS AND METHODS

2.2.1 Artificial inoculation of maize with *A. flavus* under laboratory conditions

2.2.1.1 Australian and Kenyan maize varieties

The Australian maize variety (DK703w) used for laboratory inoculation with *A. flavus* was provided by Pacific Seeds (Queensland Australia). The two Kenyan maize varieties, Duma 43 and Pioneer, which are the most common cultivars in the Eastern Region where aflatoxin contamination is more prevalent, were provided by the National Irrigation Board- Mwea Irrigation Agriculture Development Centre Kenya.

2.2.1.2 Fungal isolates

The Australian variety was inoculated with the aflatoxigenic (FRR-5315) and non-aflatoxigenic (FRR4288) *A. flavus* isolates obtained from the strain collection at Commonwealth Scientific and Industrial Research Organisation (CSIRO) from the then Division of Animal, Food and Health Sciences North Ryde, NSW. The Kenyan maize varieties were inoculated with aflatoxigenic isolate 121365 and non-aflatoxigenic isolate 3VM787 obtained from the strain collection at the University of Nairobi School of Biological Sciences. They had previously been isolated from maize and soil from farms and rural households in the Makueni and Nandi counties of Kenya, as described by Okoth *et al.* (2012).

2.2.1.3 Culturing *A. flavus* isolates and preparation of fungal inoculum

A. flavus isolates were cultured on coconut agar media (270 mL coconut cream, 270 mL distilled water, 8.1 g agar, autoclaved at 121 ^{o}C for 15 minutes) and incubated for ten days at 25 ^{o}C. The aflatoxigenic status of the isolates was confirmed under UV light at 350 nm and by direct competitive ELISA (AgraQuant®total aflatoxin assay 1/20 Romer labs. Inc., Union, MO, USA) after three and ten days respectively. Spores were harvested after ten days and suspended

in sterile deionised water containing 2 % v/v Tween 20. Serial dilutions of 10^{-1}, 10^{-2} and 10^{-3} were prepared from the spore suspension and used to determine the concentration of spores in the suspension with a haemocytometer. From each dilution, 100 µl of the spore suspension was withdrawn and placed in a 1.5 mL tube, 100 µl of Trypan Blue stain (Sigma-Aldrich, Castle Hill, New South Wales, Australia) was added and the tubes were vortexed gently. A volume of 10 µl of each suspension was withdrawn and placed on a haemocytometer slide (Fisher Scientific, Australia Pty Ltd). The number of live spores (unstained by Trypan Blue) in the 5 x 5 blocks of 4 x 4 small squares was quantified under a compound microscope (Nikon Instruments Inc.) at x40 magnification. Spore density was determined by dividing the total cell count by two to adjust for the 1:2 Trypan dilution. The spore density was adjusted through serial dilution to achieve a concentration of 1×10^{6} viable spores/mL.

2.2.1.4 Inoculation of maize with *A. flavus*

Maize cobs at kernel dent stage (30 to 35 days after silking) were used for inoculation with *A. flavus.* Once received in the laboratory, the leaves and silks of the maize cobs were removed and each cob was trimmed to a uniform length of 10 cm. Treatments for the experiments included maize cobs contaminated with aflatoxigenic and non aflatoxigenic *A. flavus* at two, six and ten incisions per cob, to achieve different aflatoxin concentration levels. Two controls were used for the experiments, namely cobs inoculated with 2 % v/v Tween 20 at two, six and ten incisions and un-inoculated controls with no incisions. A set of ten cobs was used for each treatment. To inoculate the maize as per the treatments above, each cob was attached to two comb-like holders to allow for suspension of the cob in the polypropylene filter bag (Sanitex, Kingsford, Australia) (Figure 2.1a). This was to prevent the mycelia from growing out of the bag and cross-contaminating other samples and the oven. Using a sterile blade, an 8 cm incision was made on either side of the cob to wound the kernels. A volume of 100 µl of the spore suspension (or control) was spread along each incision using a pipette. The cobs were air dried for ten minutes in a Class II biological safety cabinet after which they were placed in a 10.2 x 35.6 cm, 1µm pore size polypropylene filter bag (Sanitex, Kingsford, Australia) and closed with three metal clips to contain fungal spores (Figure 2.1b). The bags were incubated at 40 ^{o}C for ten days to achieve a moisture content of 10 %.

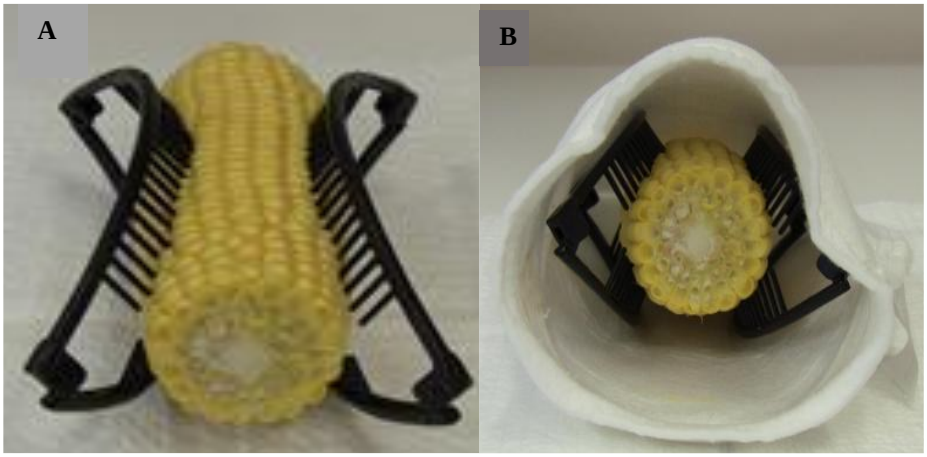


Figure 2.1: Inoculation of maize with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavu*s. **A)** Cob attached to the two comb holders; **B)** Cob placed in a one micron pore size polypropylene bag.

For each class of samples (aflatoxigenic, non aflatoxigenic and control), a separate cob, incubated under the same conditions, had its moisture content measured with a HE 60 grain moisture meter (GrainTec, Pty, Ltd) based on an electric resistance (conductive) method. Monitoring intervals were based on relative humidity levels measured with a Hobo temperature/relative humidity data logger (Hobo Temp\RH, PCE Instruments, UK) for each class of samples. The total drying time was approximately ten days. The sealed bags were then cooled and stored at room temperature in desiccators for a further ten days. The corn kernels were then harvested and ground in a laboratory mill (Perten laboratory mill 3303, Perten Instruments, Huddinge, Sweden) in a Class II Biological Safety Cabinet. The flour was stored in the original filter bags at room temperature until ready for analysis. Figure 2.2 provides a summary of the sequential stages for artificial inoculation of maize with *A. flavus.*

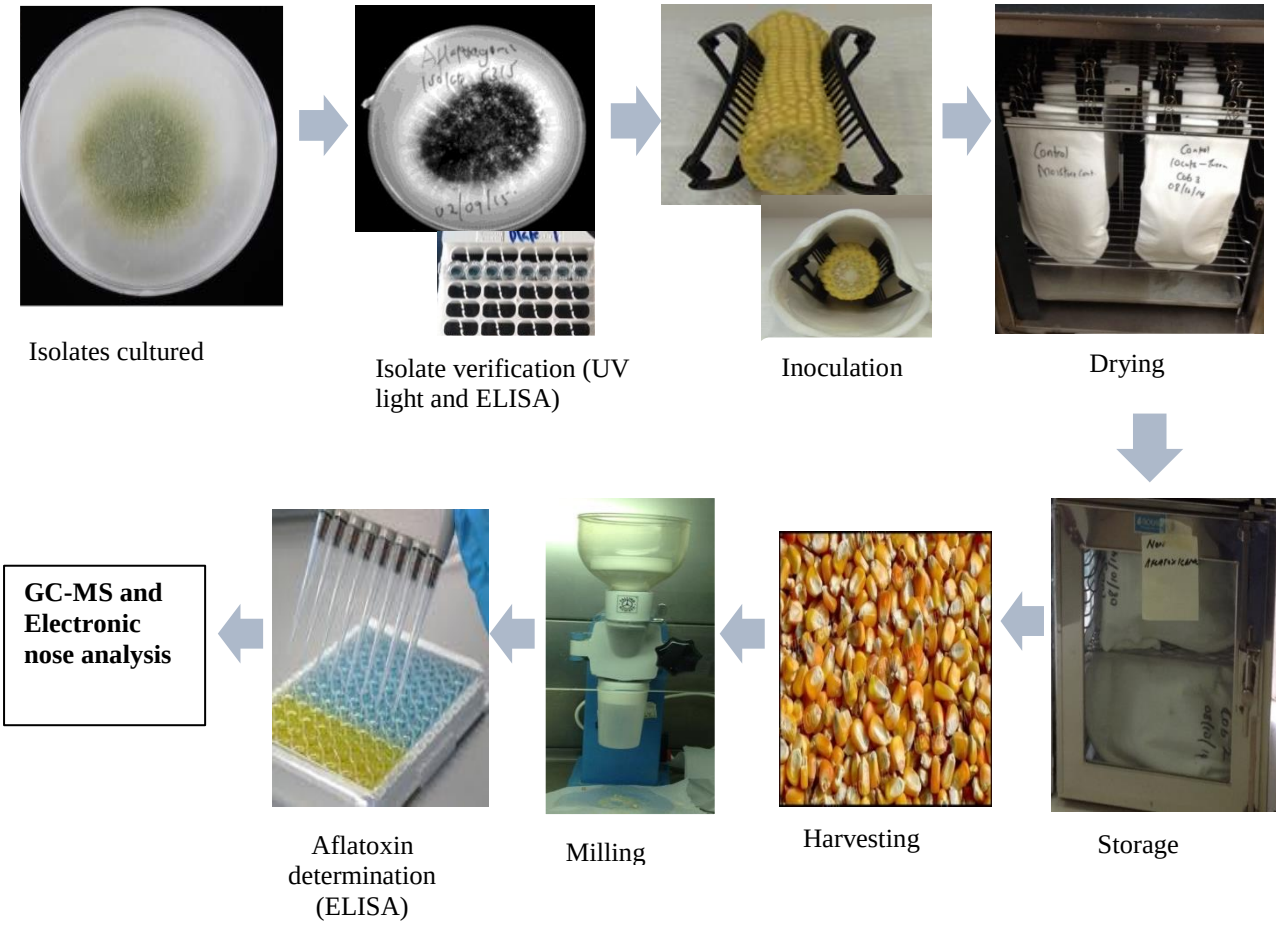


Figure 2.2: Sequential stages for artificial inoculation of maize with *A. flavus*

2.2.2 Collection of field samples naturally infected with *A. flavus*

2.2.2.1 Description of sampling sites

Maize samples potentially contaminated with aflatoxins under field conditions were collected from farms in the Bura Irrigation Scheme in Tana River County and Kaiti in Makueni County. The two sites were selected on the basis of past records that they have high prevalence of aflatoxin contamination and outbreaks of aflatoxicosis (Probst *et al.*, 2007; Nyaga, 2010; Probst *et al.*, 2011; Yarda *et al.*, 2013; Maina *et al.*, 2016). Additional samples were provided by Bioscience eastern and central Africa (BecA- ILRI Hub) (collected from market centres in Meru County). Tana River County located in the Coast Region (Figure 2.3) is hot and dry with average annual temperature of 27.5 ^{o}C. Rainfall is erratic, averaging between 220 mm to 500 mm annually. Maize production is predominantly under irrigation in settlement schemes established and managed by the government (Koech *et al.*, 2014). Makueni County, located in the Eastern Region (Figure 2.3) is classified as a semi- arid zone at an elevation of 800 to 1700 m above sea level with an annual rainfall of 300 to 600 mm, and annual average temperature of 21.5 ^{o}C. Meru County is located in the Eastern Region (Figure 2.3) and has a mean annual rainfall of 1300 mm to 2600 mm and annual average temperature of 18.4 ^{o}C (Jaetzold *et al.*, 2007).

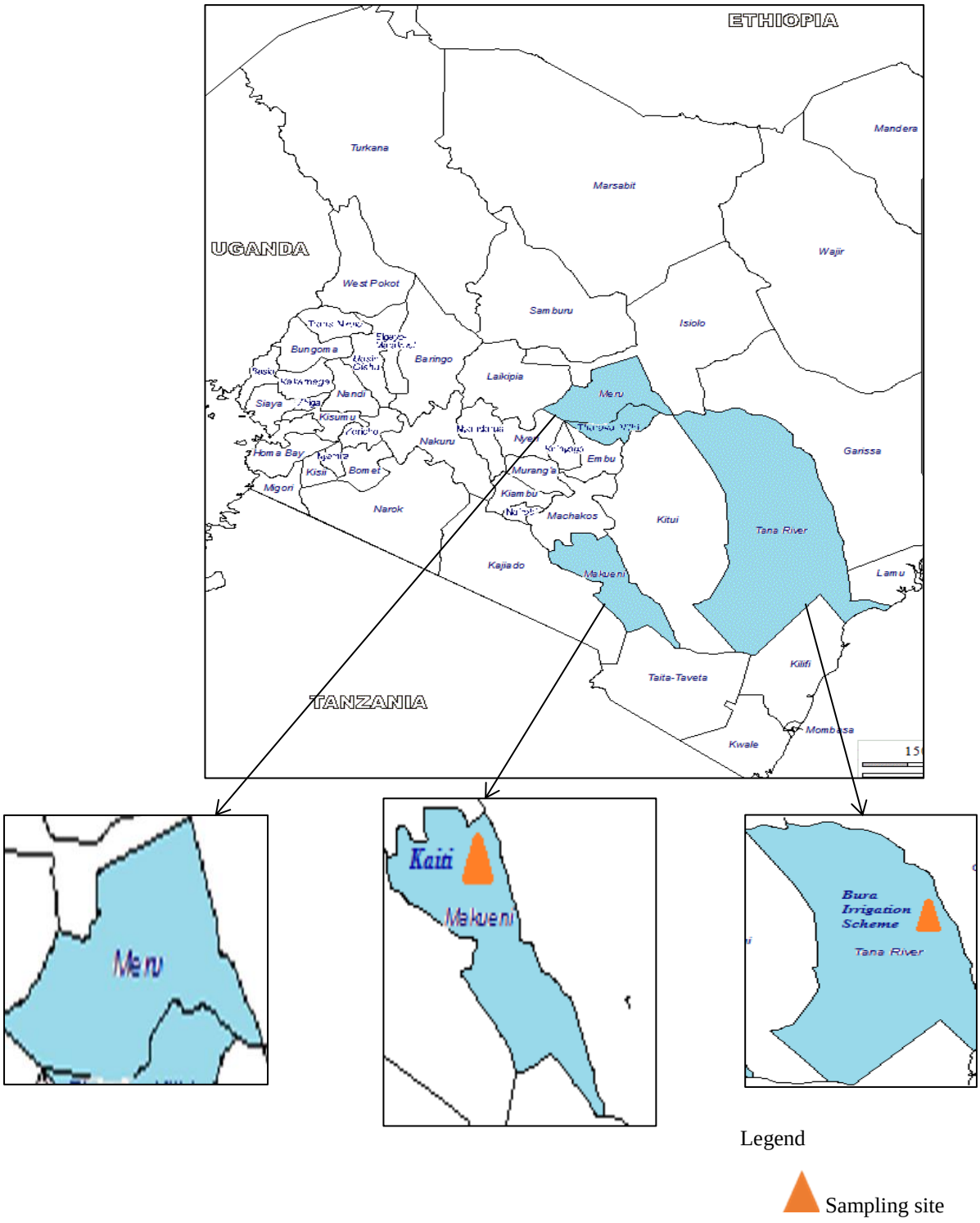


Figure 2.3: Administrative map of Kenya showing the sampling sites. Adapted from GeoCurrents maps (http://www.geocurrents.info). Samples from Meru were provided by BecA.

2.2.2.2 Sample collection

Field samples were collected in May 2016, two months after the harvest season. In the Bura Irrigation Scheme, samples were collected from farmers in three villages selected on the basis of a previous study on assessment of farmers' perceptions of and willingness to pay for aflatoxin biological control agent aflasafe by Migwi (2016). In each village, farmers were selected randomly. In Kaiti, samples were collected from seven locations that included Kola, Kee, Kivani, Ukia, Kithungu, Mukuyuni and Lumbwa. These locations were established sampling sites of the BecA Capacity and Action for Aflatoxin Reduction in Eastern Africa project. From each location, villages and farmers were selected at random. A questionnaire was administered to gather information on the farmer and the sample (Appendix 1). Questions were designed to gather information on location, geographical positioning system (GPS) co-ordinates for the location, the maize variety, the source of maize (own or purchased) and type of agronomic practices employed by the farmer.

Sampling was conducted in accordance with the ethical guidelines approved for the project where the farmer's informed consent was sought prior to sample collection. The ethics approval for the project was provided by the University of Canberra Human Research Ethics committee and endorsed by CSIRO Social Science Human Research Ethics Committee, approval number 14-251 (Appendix 2). From each selected farmer, 500 g of shelled maize kernels were collected randomly in paper bags from the household storage facilities. Maize stored in sacks was sampled from different parts using a closed spear driven through the top, middle and bottom part of the sack to obtain a 500 g sample. After collection, the samples were stored at 4 ° C in paper bags until ready for analysis. A summary of the sequential stages for collection of maize samples naturally infected with *A. flavus* is shown in Figure 2.4. A total of 200 maize samples

were collected from all locations. A summary of the number of samples collected from each location per variety is shown in Table 2.1 and 2.2.

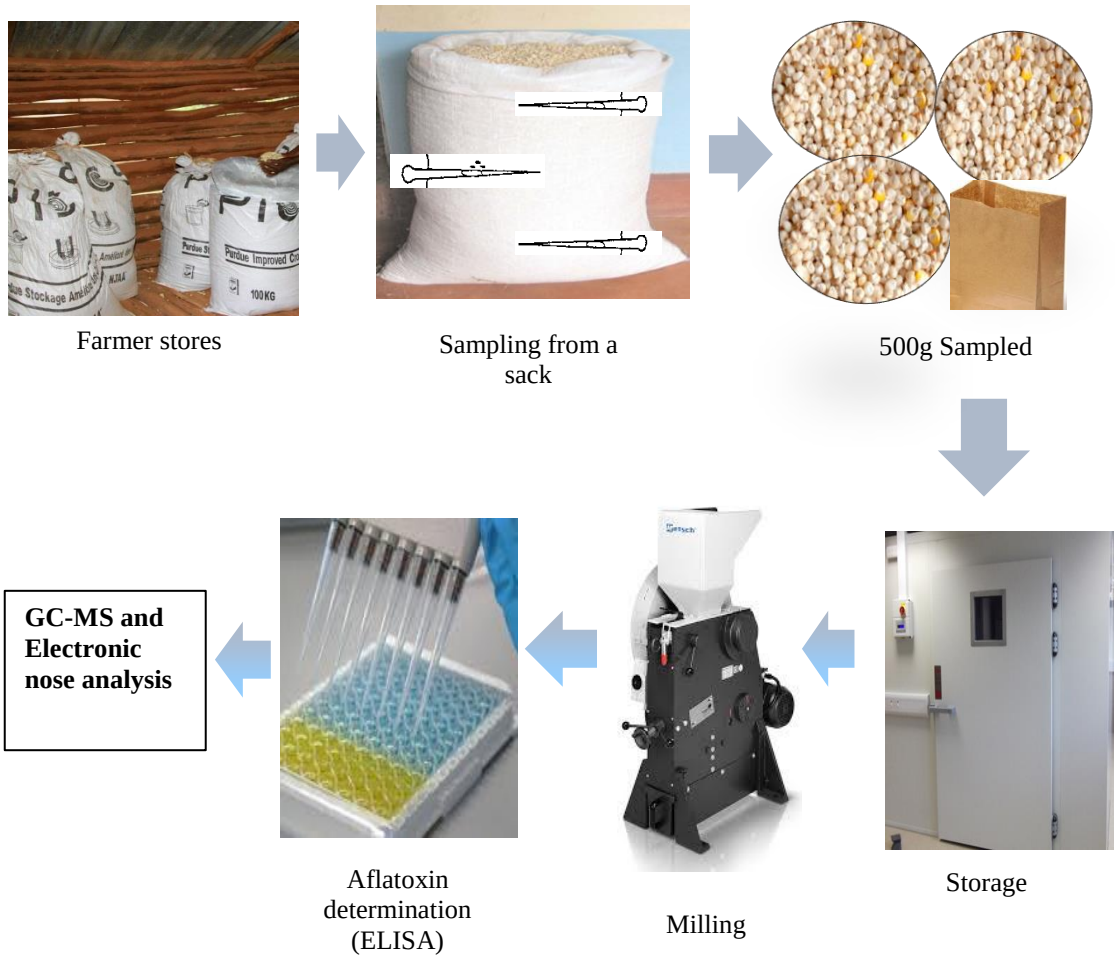


Figure 2.4: Sequential stages for collection of maize samples naturally infected with *A. flavus*

Table 2.1: Summary of maize samples collected in Bura Irrigation Scheme, Kaiti Constituency and Meru County.

Area	Locations	Number of villages	Number of samples
Bura Irrigation Scheme	-	3	52
Kaiti	Kola	9	23
	Kee	2	11
	Kivani	4	18
	Ukia	20	28
	Kithungu	1	7
	Mukuyuni	1	7
	Lumbwa	1	11
Meru *****	-		43
Total			**200**

***** Samples provided by BecA. The number of samples collected per village were dependent on availability of particular maize varities hence the inconsitency in number of samples collected from the villages.

Table 2.2: Number of maize samples collected per variety

Area	Variety	Number of Samples
Bura Irrigation Scheme	DH04	53
Kaiti	Pioneer	65
	Duma 43	40
Meru*	Mixed-Unknown	43
Total		**200**

***** Samples provided by BecA.

2.2.3 Determination of aflatoxin contamination levels

The presence and concentration of aflatoxin in inoculated and naturally contaminated samples was determined by direct competitive ELISA (AgraQuant® total aflatoxin assay 1/20 Romer labs. Inc., Union, MO, USA) as per manufacturer protocol. Briefly, 5 g of the maize flour was weighed and mixed with 50 mL of 70/30 methanol/water solution and vortexed for three minutes. The mixture was filtered through a Whatman #1 filter paper and the filtrate collected. Aflatoxin standard concentrations of 0, 1.0, 2.0, 4.0, 10.0 and 20 parts per billion (ppb) were used. A volume of 200 µl of anti-aflatoxin antibody was placed into each blue/green boarded dilution well to which 100 µl of each standard or sample was added. A volume of 100 µl of the extract (mixture of anti-aflatoxin antibody and sample or standard) was then transferred to the corresponding secondary antibody-coated microwell and incubated at room temperature for 15 minutes. The wells were washed five times with deionised water and excess water removed with absorbent paper. A volume of 100 µl of the enzyme substrate solution was added to each well and the colour allowed to develop for five minutes. The enzyme reaction was stopped by addition of 100 µl of the stop solution and the resultant colour intensity was measured using an ELISA plate reader with an absorbance window centred at 450 nm (SpectraMax, Bio-Strategy Ltd, Australia). The absorbances of the standards were used to plot a standard curve from which the aflatoxin concentration of the sample was determined by interpolation. Samples with aflatoxin levels greater than 20 ppb were serially diluted and re-analysed.

2.3 RESULTS

2.3.1 Aflatoxin contamination levels for maize varieties artificially inoculated with *A. flavus*

The Australia maize variety DK703w and two Kenyan maize varieties, Duma 43 and Pioneer, were inoculated with 2 % Tween 20 as control samples and non-aflatoxigenic and aflatoxigenic *A. flavus* isolates. To mimic the highly heterogeneous distribution of aflatoxins in contaminated matrices, the maize cobs were inoculated with 2 % Tween 20 and the *A. flavus* isolates at two, six and ten incisions per cob to achieve varied contamination levels. Higher aflatoxin levels were recorded for maize samples inoculated with the aflatoxigenic *A. flavus* isolate when compared to the control and samples inoculated with the non-aflatoxigenic isolate for the three varieties (Figure 2.5). For all three varieties, aflatoxin levels ranged from 0.1 to 1.7 parts per billion (ppb) (Log aflatoxin level 0.1 to 0.4) for the control samples, 1.2 to 5.1 ppb (Log aflatoxin level 0.01 to 0.7) for the non-aflatoxigenic *A. flavus* infected samples and 65.4 to 1064.7 ppb (Log aflatoxin level 1.8 to 3.0) for the aflatoxigenic *A. flavus* infected samples (Figure 2.5). Differences in aflatoxin contamination levels were recorded between the three varieties with higher levels for Pioneer compared to DK703w and Duma 43 (Figure 2.5). There was no consistent correlation between the number of cuts and aflatoxin contamination levels for any of the three varieties. However, contamination levels varied widely in samples inoculated with the aflatoxigenic isolate which represents the heterogeneous distribution of aflatoxin in contaminated matrix (Figure 2.5). The control and non-aflatoxigenic samples contained aflatoxins, with levels ranging between 0.1 to 5.1 pbb. This is below the maximum set regulatory limit of 10 ppb for maize and therefore these samples were considered as real negative controls for the purpose of this study. A summary of aflatoxin levels for the three varieties across the different treatments is shown in Table 2.3.

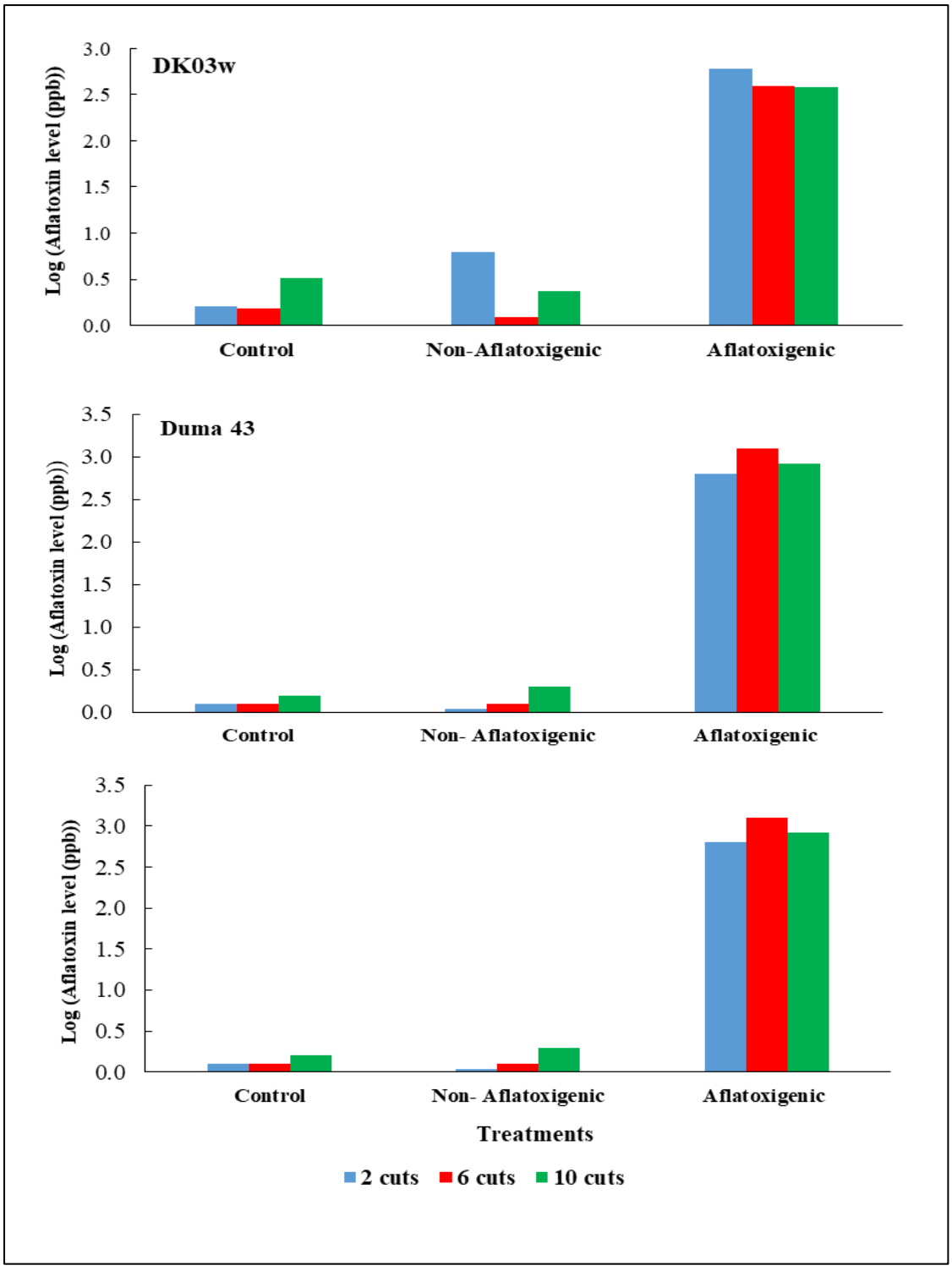


Figure 2.5: ELISA-determined log aflatoxin contamination levels in parts per billion (ppb) of maize variety DK703w, Duma 43 and Pioneer inoculated with 2 % Tween 20 as a control and non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* at 2, 6 and 10 incisions (cuts) per cob. Values on the bars represent the mean of 10 replicates per treatment.

Table 2.3: Average aflatoxin levels in parts per billion (ppb) of maize variety DK703w, Duma 43 and Pioneer inoculated with 2 % Tween 20 as a control and non-aflatoxigenic and aflatoxigenic isolates of *A. flavus*.

	Aflatoxin levels (ppb)		
Variety	**Control**	**Non-aflatoxigenic**	**Aflatoxigenic**
DK703w	0.5 ± 0.2	2.2 ± 0.8	533.8 ±275
Duma 43	0.6 ± 0.3	0.4 ± 0.2	158 ± 137
Pioneer	0.1 ± 0.05	0.7 ± 0.3	885 ± 158

Values represent the mean ± standard deviation for 30 replicates per treatment.

2.3.2 Aflatoxin levels for naturally contaminated samples.

Maize samples potentially infected with *A. flavus* under field conditions were purchased from farms in aflatoxin prone areas in Kenya and included the main varieties grown in those respective areas. Three varieties were obtained. Two of these; Duma 43 and Pioneer, were the same as those experimentally inoculated with *A. flavus.* The third variety was DH04. Market samples were also purchased, but their varieties were not known. Aflatoxin contamination levels were higher for samples purchased from the market when compared to individual varieties grown and purchased from farms (Table 2.4). Contaminaion levels ranged from 49.8 (Log aflatoxin level 1.7) ppb for Duma 43 to 12,680 (Log aflatoxin level 3.1) ppb for market samples (Table 2.4, Figure 2.6). Forty seven percent (20/43) of the market samples had contamination levels above the set regulatory limit of 5 ppb (Wu, 2004), of which 70 % (14/20) had contamination levels greater than 1000 ppb (Table 2.4). Among the farm purchased samples, the highest contamination levels were recorded for Pioneer where 48 % (31/65) of the samples had aflatoxin levels above the regulatory limit (Table 2.4). For DH04 only 21 % (11/52) of the samples had aflatoxin levels above the regulatory limit while for Duma 43 37 % (13/3) of the samples were contaminated beyond the regulatory limit (Table 2.4). For the purpose of this study samples with aflatoxin levels between 0 to 5.9 ppb were considered as

controls, whereas samples with contamination levels equal to or greater than 6 pbb were classified as contaminated. The avearge aflatoxin levels for each variety and market samples are presented in Figure 2.6.

Table 2.4: Aflatoxin contamination levels in parts per billion (ppb) of Kenyan maize varieties and market samples naturally infected with *A. flavus* under field conditions.

	Number of samples with different aflatoxin levels						
Variety	**Aflatoxin levels (ppb)**					**No. of contaminated/ Total**	**Average afIatoxin levels (ppb)**
	0-5	**6-100**	**101-500**	**501-1000**	**>1000**		
DH04	41	8	3	0	0	11/52	49.8
Pioneer	34	6	25	0	0	31/65	100
Duma 43	22	11	2	0	0	13/35	269
Market samples	23	3	3	0	14	20/43	12,680

No. – Number

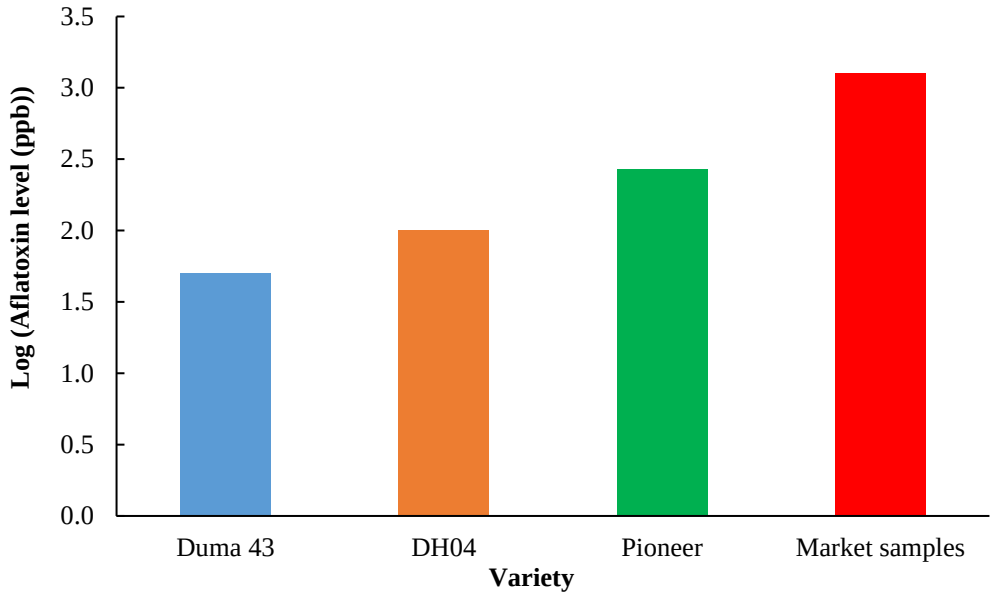


Figure 2.6: Average log aflatoxin contamination levels in parts per billion (ppb) of Kenyan maize varieties and market samples naturally infected with *A. flavus* under field conditions. Values on the bars represent the means for contaminated samples (≥ 6 ppb). n = 13 for Duma 43, n = 11 for DH04, n = 31 for Pioneer and n = 20 for market samples.

2.4 DISCUSSION

The study aimed to develop maize samples artificially contaminated with aflatoxin and collect naturally contaminated maize samples to be used to evaluate the potential for GC-MS and electronic noses to detect aflatoxin contamination. Previous evaluations of GC-MS and electronic noses for detection of mycotoxins in maize have been based on either growing the fungus on artificial media or cracked maize kernels (Falasconi *et al.*, 2005; Gobbi *et al.*, 2011). In this study, successful colonization of intact maize kernels by *A. flavus* and subsequent contamination with aflatoxins was demonstrated. Aflatoxin contamination levels were higher in samples inoculated with the aflatoxigenic *A. flavus* isolates when compared to the controls and samples inoculated with the non-aflatoxigenic isolates. This is a mimic of the natural process where maize is infected with *A. flavus* at the pre-harvest stage followed by aflatoxin production at the post-harvest stages (Hell *et al.*, 2008). Additionally, variable aflatoxin contamination levels were achieved by varying the number of cuts on the cobs which represents the highly heterogeneous distribution of aflatoxins in contaminated matrices as previously recorded by Cucullu *et al.* (1966) and Johansson *et al.* (2000) on peanuts and corn respectively. Similar results were observed by De Lucca *et al.* (2012) on sterile cracked maize kernels inoculated with aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. Contamination levels were higher in grains inoculated with the aflatoxigenic *A. flavus* when compared to grains inoculated with the non-aflatoxigenic isolates.

The maize samples naturally contaminated with aflatoxins comprised of three varieties (Pioneer, Duma 43 and DH04) purchased from farms and market samples. Aflatoxin contamination levels, as determined by ELISA, varied widely across the different varieties and market samples. Contamination levels were higher for samples purchased from the market (12, 680 ppb) when compared to individual varieties grown and purchased from farms (49.8 ppb to

269 ppb). Similar observations were reported by Mutiga *et al.* (2015) with maize samples from Western Kenya. Aflatoxin contamination levels were higher for market purchased maize when compared to homegrown maize. Hoffmann *et al.* (2013) also reported higher aflatoxin levels in maize grain meant for sale when compared to grain retained by farmers for their own consumption using data from 2000 maize samples collected from 176 small-scale hammer mills across 138 villages in Western, Rift Valley, Eastern and Nyanza provinces. In Kenya, the smallholder/individual farmer's maize is generally purchased by small-scale assemblers or brokers, who aggregate maize for sale in the market. The markets operate outside of the regulatory structure to monitor contamination prior to purchase (Grace *et al.*, 2015). Therefore the traders potentially consolidate contaminated maize from each individual farmer, hence the higher contamination levels in the market samples. Additionally, most farmers handle maize for home consumption more carefully than maize for sale through proper drying and rigorous sorting for mouldy grains (Hoffmann and Gatobu, 2014). This coupled by lack of proper drying and storage facilitates by the traders could also explain the high contamination levels recorded for the market samples. The moisture content of the grain at storage as well as storage conditions are associated with mould infestation, discolouration and occurrence of aflatoxin contamination in maize (Rashid *et al.*, 2013; Fountain *et al.*, 2014; Mutiga *et al.*, 2017).

The difference in contamination between the home purchased and market purchased maize could also be attributed to the region from which the samples were collected. Contamination level was higher for market samples collected from Meru County when compared to Pioneer, Duma 43 and DH04 collected from Makueni and Tana River Counties. Makueni and Tana River Counties lie within the semiarid agro-ecological zone. while Meru County lies within the subhumid agro- ecological zone (Jaetzold *et al.*, 2007). The region is characterized by high altitude, humid conditions and heavy rainfall that is well distributed throughout the year.

Therefore the high aflatoxin levels recorded are not likely to be attributed to pre-harvest moisture stress, but to poor drying and storage conditions as a result of the wet weather. Interestingly, the contamination levels in samples collected from Meru were higher than samples collected from Makueni and Tana River Counties where cases of aflatoxicosis fatalities have been previously reported (Nangindu *et al.*, 1982; CDC, 2004; Maina *et al.*, 2016). Aflatoxicosis outbreak has not been previously reported in Meru County, indicating that it could be a new aflatoxin hotspot. Government interventions to reduce aflatoxin contamination have been focused on areas where aflatoxicosis outbreaks have been reported (Schmidt, 2013). Therefore farmers in emerging aflatoxin hotspots lack the necessary skills and capacities to control factors that predispose maize to *A. flavus* infection and subsequent aflatoxin contamination. This could explain the high aflatoxin contamination levels in the market samples from Meru County relative to samples from Makueni and Tana River Counties. The results concur with findings from other studies. Mutiga *et al.* (2014), reported high aflatoxin levels in samples collected from three districts in Meru County compared to samples collected from Machakos, Mwala, Kitui and Makueni Counties where aflatoxicosis fatalities have been reported. Meru County had highest proportion of samples contaminated above the legal limit and the highest mean aflatoxin levels ranging from 23 ppb to 4839 ppb compared to 8 ppb to 1373 ppb reported for the other Counties.

The differences in contamination levels among the maize varieties could be attributed to differences in susceptibility of the varieties to infection by *A. flavus* and mycotoxin contamination (Hell and Mutegi, 2011; Smith *et al.*, 2016; Mutiga *et al.*, 2017). Among the three varieties, lowest contamination levels were recorded for Duma 43 while the highest was recorded for Pioneer. A similar observation was made for the maize samples artificially inoculated with *A. flavus*, where contamination levels were higher for Pioneer relative to Duma

43 (Table 2.3 and 2.4). This could be an indication that Pioneer is more susceptible to infection by *A. flavus* compared to Duma 43 and DH04. Similar variabilities in aflatoxin contamination levels for maize varieties grown in Kenya and market samples have been previously reported. Mutiga *et al.* (2015) reported higher aflatoxin levels for hybrid maize varieties (H614, H513) relative to the local landrace (Local 8) grown by farmers in Western Kenya. In contrast, Chebon *et al.* (2016), reported lower contamination levels in hybrid varieties (H613, H614, H628, H629, H6210 and H6213) compared to a local landrace (Kikamba) grown by farmers in the highland agro-ecological zone and mid-altitude agro-ecological zones in Kenya. However, based on the results from this study, it was not possible to determine which of the two factors, region from which the samples were collected and variety had a greater effect on the aflatoxin contamination levels. The factors were confounding which is a limitation in this study.

2.5 CONCLUSION

To the best of our knowledge, this is the first study to document methodology for successful artificial inoculation, colonization and contamination of kernels on intact cobs with *A. flavus* and subsequently contamination with aflatoxins. The methodology developed can be adopted to study other mycotoxigenic fungi on different cereals. The study confirms the widespread aflatoxin contamination of maize produced and consumed in Kenya and gives insights to varietal differences to *A. flavus* infection and aflatoxin contamination. The study also provides insight into new areas that are potential hotspot for aflatoxicosis outbreaks. There is however, need for long-term surveillance strategies over wider geographical areas than those covered in this study, to identify other emerging aflatoxin hotspots. The areas should be included in the national surveillance and management efforts aimed at reducing contamination. Such efforts should include improvement of drying and storage conditions and education programmes to create awareness on the effect of aflatoxins and other available management strategies.

CHAPTER 3

EVALUATION OF GC-MS POTENTIAL TO DETECT AFLATOXIN CONTAMINATION IN AUSTRALIAN AND KENYAN MAIZE VARIETIES ARTIFICIALLY INOCULATED AND NATURALLY INFECTED WITH *A. FLAVUS*

3.1 INTRODUCTION

Profiling of volatile organic compounds (VOCs) induced by infection of crops by fungi has emerged as a novel and promising alternative to existing immunochemical and chromatographic diagnostic techniques for detection of mycotoxin contamination (Sankaran *et al.*, 2010; Martinelli *et al.*, 2014). Compared to other analytical techniques such as ELISA, HPLC and LC-MS for aflatoxins, the potential to rapidly and noninvasively monitor fungal infection in real time makes GC-MS highly desirable (Sherif *et al.*, 2016). Additionally the sporadic nature of aflatoxin contamination within a matrix makes it difficult to obtain a representative sample for analysis. Therefore the use of an averaged headspace from the entirety of the sample would be very useful in overcoming representativeness problems associated with sampling maize for aflatoxin analysis (Johansson *et al.*, 2000; Cellini *et al.*, 2017).

The use of VOCs for detection of contamination is based on analysis of changes in composition of volatile compounds in the sample headspace following infection by toxigenic fungi (Falasconi *et al.*, 2012). Filamentous fungi, including *Aspergillus* species, produce a number of characteristic volatiles during their primary and secondary metabolism from a wide variety of starting compounds, e.g., acetate, amino acids, fatty acids, and keto acids (Kinderlerer, 1989;

Borjesson *et al.*, 1992; Jelen and Wasowicz, 1998). The volatiles are species and sometimes strain specific and have been used as characteristic markers of the fungal presence as well as to discriminate between contaminated and un-contaminated grains (Jeleń and Wasowicz, 1998). The use of volatiles for characterization of individual fungal species and evaluation of food quality has been demonstrated according to reviews by various authors (Jelen and Wasowicz, 1998; Schnurer *et al.*, 1999; Magan and Evans, 2000; Sahgal *et al.*, 2007). Of particular interest however, is identification of VOCs that can be used to detect mycotoxin contamination.

Using GC-MS, VOCs that can differentiate toxigenic and non-toxigenic isolates of species of mycotoxigenic fungi as well as contaminated and non-contaminated grains have been documented for *Aspergillus* (Zeringue *et al.*, 1993; Jurjevic *et al.*, 2008; De Lucca *et al.*, 2010; De Lucca *et al.*, 2012), *Fusarium* (Keshri and Magan, 2000; Jelen´ *et al.*, 1995) and *Penicillium* (Karlshoi and Larsen, 2005). *A. flavus* has been reported to produce a range of classes of VOCs including alcohols, carbonyls and hydrocarbons. However, most of the studies have been based on *in vitro* procedures with fungus cultured on artificial media or sterile grains. These conditions do not reflect the actual scenario where maize is infected with *A. flavus* at the pre-harvest stage, which generates aflatoxins mostly at the post-harvest stage (Hell *et al.*, 2008). A more diverse volatile spectrum is expected when fungus infects intact kernels or from maize naturally infected with *A. flavus*. The most important challenge is to identify a set of specific volatile biomarkers to discriminate between maize contaminated with, or free from aflatoxins.

The objective of the present study was to evaluate the potential of GC-MS analysis of VOCs to detect aflatoxin contamination in Australian and Kenyan varieties of maize artificially and naturally contaminated with *A. flavus* and identify any relevant diagnostic volatiles.

3.2 MATERIALS AND METHODS

3.2.1 Inoculation of maize with *A. flavus* and collection of naturally contaminated samples

The potential for GC-MS in combination with statistical techniques to detect aflatoxin contamination was evaluated with an Australian maize variety (DK703w) and two Kenyan maize varieties (Pioneer and Duma 43) that had been artificially inoculated with *A. flavus*. The Australian variety was inoculated with the aflatoxigenic (FRR-5315) and non-aflatoxigenic (FRR-4288) *A. flavus* isolates, while the Kenyan maize varieties were inoculated with aflatoxigenic isolate 121365 and non-aflatoxigenic isolate 3VM787 as described in Chapter 2 section 2.2.1. Treatments for the experiments included maize cobs inoculated with aflatoxigenic and non-aflatoxigenic *A. flavus* at two, six and ten incisions per cob to achieve different aflatoxin concentration levels, two controls namely; cobs inoculated with 2 % v/v Tween 20 at two, six and ten incisions and un-inoculated controls with no incisions. A set of ten cobs was used for each treatment as described in Chapter 2 section 2.2.1.4. Evaluation of GC-MS for detection of aflatoxin contamination with the Australian maize variety DK703w was conducted in two separate experiments. In the first experiment the samples were analysed 14 days after milling while in the second experiment the samples were analysed 30 days after milling. A set of five cobs was used for each treatment in the two experiments.

The potential for GC-MS in combination with statistical techniques to detect aflatoxin contamination was also investigated with maize samples that had been naturally contaminated with aflatoxin under field conditions in Kenya. A total of 200 maize samples comprising three varieties (Pioneer, Duma 43, and DH04) and market samples were collected from aflatoxin prone areas as described in Chapter 2 section 2.2.2.

3.2.2 Determination of aflatoxin contamination levels

The presence and concentration of aflatoxin in inoculated and naturally contaminated samples was determined by direct competitive ELISA (AgraQuant®total aflatoxin assay 1/20 Romer labs. Inc., Union, MO, USA) as per manufacturer protocol, as described in Chapter 2 section 2.2.3.

3.2.3 GC-MS parameters and extraction of volatiles

VOCs in the headspace of maize flour samples were extracted using a solid phase micro extraction technique (SPME). The VOCs were preconcentrated onto a SPME fibre (Aldrich, Bellefonte, PA) composed of fused silica partially cross-linked with 65 μm polydimethylsiloxane/divinylbenzene prior to analysis with the GC-MS. A mass of 1.65 g of the maize flour sample was weighed and transferred to a 10 mL glass vial (Supelco, Bellefonte, PA), which was sealed with silicon/teflon magnetic autosampler vial caps (Agilent Technologies, Australia). The vials were incubated at 35 ^{o}C for ten minutes to increase the concentration of the volatiles in the headspace. The SPME fibre was inserted by the autosampler into the vial head space for 20 minutes. After absorption, head space volatiles were transferred to the GC injection port equipped with a 0.8 mm i.d splitless glass liner at 250 ^{o}C. Desorbed volatile compounds were separated in a GC (Varian 3800 GC for Australian variety DK703w, Agilent 7890A GC for Kenyan variety Duma 43 and Bruker 451 GC for Pioneer and samples naturally infected with *A. flavus*) equipped with a 30 m x 0.25 mm, 0.25 μm film thickness ZB-5MS fused capillary column (Zebron, NSW, Australia). The oven temperature was programmed to hold the temperature of 35 ^{o}C for four minutes then rise to 65 ^{o}C at 2.5 ^{o}C min^{-1} and rise again to 225 ^{o}C at 5 ^{o}C min^{-1} with a final hold of two minutes. The GC column

output was fed into a mass selective detector /mass spectrometer (Varian 1200 MS for Australian variety DK703w, Agilent 240 MS for Kenya variety Duma 43 and Bruker Scion Single Quad MS for Pioneer and samples naturally infected with *A. flavus*). The GC-MS transfer line was heated at 250 ^{o}C with the flow rate of the helium carrier gas set at 1 mL min^{-1}. Mass spectrometry was performed in electron impact mode at 70eV over a mass range of 35 to 350 *m/z* in a 1-s cycle.

The samples were analysed with three different GC-MS equipment based on availability. The Australian variety DK703w artificially inoculated with *A. flavus* was analysed with a Varian 3800 GC and Varian 1200 MS which was available at CSIRO where the experiments were conducted. The Kenyan variety Duma 43 artificially inoculated with *A. flavus* was analysed with an Agilent 7890A GC and Agilent 240 MS available at BecA where the experiments were conducted. However, the Agilent GC-MS brokedown, hence the remaining Kenyan samples (Pioneer artificially inoculated with *A. flavus* and all samples naturally infected with *A. flavus*) were imported into CSIRO and analysed with the Bruker 451 GC and Bruker Scion Single Quad MS available at that time. During analysis, the different classes of samples, control, non-aflatoxigenic and aflatoxigenic were alternated to minimize carry-over or memory effects from previous samples. It is noted there could have been differences in responses across the three GC-MS instruments used.

3.2.4 Data processing and statistical analysis

The objective of data analysis was to identify features of the VOC profiles that can distinguish the different classes of samples, namely control, non-aflatoxigenic *A. flavus* infected maize and aflatoxigenic *A. flavus* infected maize and potentially identify VOCs associated with aflatoxin presence.

3.2.4.1 Data processing

Compounds in each chromatogram were identified by comparison of their MS fragmentation patterns with the mass spectrum data base of the National Institute of Standards and Technology (NIST 08) library via spectrum matching. The compounds were identified from the list of most probable compounds based on a probability-based matching system and the value of the relative match factor (RMF). The RMF is a similarity score that represents the goodness of fit between a measured spectrum and a library reference spectrum. The RMF was used to determine the quality of the mass spectral match as per the NIST library guidelines where, RMF of 900 and above represents an excellent match, 800-900 a good match, 601- 700 a fair match and < 600 a poor match (Gujar *et al.*, 2018). The chromatograms and mass spectra were evaluated using the MS Workstation software for each of the respective GC instruments used for sample analysis as described earlier (Varian MS Workstation version 6.6, Agilent MS Workstation version 7.00 and Bruker MS Workstation version 8.2). Chromatograms representing the different classes of samples (control, non-aflatoxigenic and aflatoxigenic) were randomly selected and the compounds present identified as described above. The reports of compounds present in each chromatogram were used to generate a compound table which was used as a template to produce compound reports for the other samples. The relative amounts of individual compounds were expressed as relative peak areas (relative to total peak area) and used for

statistical analysis. These were calculated by automatically integrating the peaks using the respective MS workstation software described above for each individual sample. Background peaks corresponding to compounds from the SPME fibre and the blank vial were considered contaminants and were excluded from the analysis.

3.2.4.2 Statistical analysis

Principal component analysis (PCA) was performed using of The Unscrambler software version 10.3 (CAMO ASA, Oslo, Norway) to determine the utility of VOCs profiles to discriminate between the different classes of samples, in a number of pairwise comparisons namely; control versus (vs) all *A. flavus* infected maize, control vs aflatoxigenic *A. flavus* infected maize, control vs non-aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize. PCA provides an efficient approach for reducing the dimensionality of data, while retaining much of the information present in the original data. The approach generates eigenvectors for the data matrix referred to as principal components and the magnitude of each eigenvector expressed by its eigenvalue indicates the variance assigned to each principal component. Principal components (PC) are ranked so that PC1 accounts for the highest variability in the data and other PCs follow in the order of decreasing variance (Zhang and Wang, 2007). The results of a PCA are presented as score plots used to visualize the separation of different classes of samples and loading plots that show the relative significance of the various VOCs to each PC and correlation between VOCs and PCs.

Loading plots were used to select VOCs contributing to the differences between the classes for each of the pairwise comparisons mentioned above. From the loading plot, VOCs that were oppositely correlated with the *A. flavus* infected samples in the score plots were selected as potential markers for aflatoxin contamination. An unpaired t-test was performed on VOCs

selected from the loading plots using GraphPad Prism version 7.04 (GraphPad Software, La Jolla, California, USA) to assess if they significantly separated the different classes of samples at the confidence level represented by p = 0.05. SVM classification, described below, was performed on VOCs that significantly separated the different classes of samples. Classification accuracies achieved by each of the VOCs were compared to accuracies achieved with the entire profile. VOCs that achieved classification accuracies comparable to what was achieved with the entire profile were selected as markers for *A. flavus* infection and aflatoxin contamination. The VOCs were identified based on their fragmentation patterns using authentic standards, when available specifically for ethyl acetate, 4-ethyl-2- methoxy phenol, 2,3 butanedione, 2,3-hyrodxy butanone (acetoin), p-xylene, naphthalene, toluene, benzaldehyde, or comparison with NIST mass spectral library. To confirm these identifications, the retention indices were determined as the average of three replicates of the authentic standard where available and by comparasion with those reported in the literature (Zeringue, 2000; De Lucca *et al.*, 2010; De Lucca *et al.*, 2012; Sun *et al.*, 2014; Sun *et al.*, 2016).

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The effectiveness of the VOCs in discriminating between the different classes of samples was evaluated with SVM classification. SVM constructs a hyperplane to separate training data in different classes. It classifies any new datum according to which side of the plane it lies (Cortes and Vapnik, 1995) The leave-one-out cross-validation method was used to train the classifier where the dataset was partitioned into *N* pairs of a training set (*N*-1 data samples) and a test/validation set (the remaining 1 data sample), where N is the size of the data set. The process was repeated *N* times to cover all samples in the data set. The accuracy of SVM is therefore scored as the average correct classification rate obtained over the *N* training-test pairs. Based

on the best classification rate obtained, the number and type of misclassified samples were identified (false positives and false negatives).

3.3 RESULTS

3.3.1 Evaluation of potential for GC-MS to detect aflatoxin contamination in maize varieties artificially inoculated with *A. flavus*

3.3.1.1 Aflatoxin contamination levels

The potential of GC-MS in combination with statistical techniques to detect experimentally introduced aflatoxin contamination in maize was evaluated using the Australia maize variety DK703w and two Kenyan maize varieties, Duma 43 and Pioneer, inoculated with 2% Tween 20 as control samples and non-aflatoxigenic and aflatoxigenic *A. flavus* isolates. Aflatoxin contamination levels for the different classes of samples are presented and discussed in Chapter 2 section 2.3.1 and 2.4 respectively.

3.3.1.2 Classification accuracy for experimentally inoculated varieties

The number of VOCs identified from the headsapce of controls and maize infected with aflatoxigenic and non-aflatoxigenic *A. flavus* varied across the varieties. A total of 61, 58 and 77 VOCs were detected from headspace of maize varieties DK703w, Duma 43 and Pioneer respectively (Appendix 3, 4 and 5). The effectiveness of VOCs to discriminate between the different classes of samples was evaluated by SVM using the leave-one out cross-validation method through a number of class comparisions that consisted of control vs all *A. flavus* infected maize, control vs. aflatoxigenic *A. flavus* infected maize, control vs non-aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize. The classification accuracies presented are the average correct classification rates over the *N* fold cross-validation partitions. The potential of statistical techniques (PCA and SVM) applied to the volatile profile measured by GC-MS to detect aflatoxin contamination in the Australian maize variety DK703w was conducted in two experiments. In the first experiment the samples

were analysed 14 days after milling while in the second experiment the samples were analysed 30 days after milling. PCA analysis of the combined data showed variations between the two experiments rather than separation between the different classes of samples (Figure 3.1), hence experiments were analysed separately.

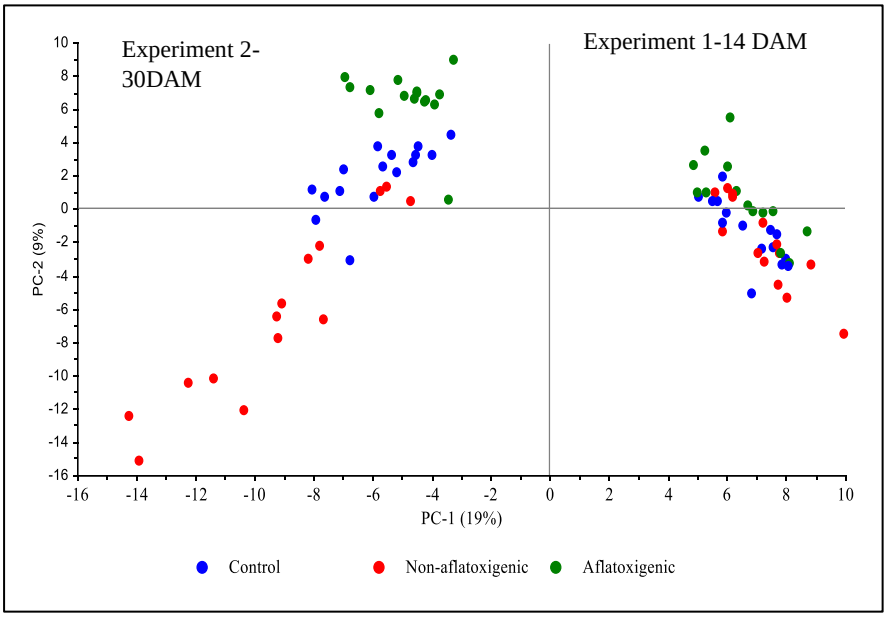


Figure 3.1: PCA score plot for GC-MS analysis of maize flour for variety DK703w inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* for experiment 1 and 2. Each marker on the plot represents a single sample; the markers are filled with different colours representing different classes of samples as shown in the legend.

30 DAM- Samples analysed at 30 days after milling; 14 DAM- Samples analysed at 14 days after milling.

The differences between the two experiments may be due to differences in storage time of the samples prior to analysis. As earlier mentioned in the first experiment the maize samples were analysed 14 days after milling while in the second experiment the samples were analysed 30 days after milling. These could have potentially lead to a decline in concentration of VOCs in the headspace of the second experiment samples compared to the first experiment samples The level of VOCs (Mcounts) decreased with 65 % for the control samples, 61 % for the non-aflatoxigenic *A. flavus* infected maize and 64 % for the aflatoxigenic *A. flavus* infected maize with increase in time from milling to analysis from 14 days in the first experiment to 30 days in the second experiment (Figure 3.2). The volatile profile and classification accuracies were however, relatively similar between the two experiments across the different pairwise comparisons.

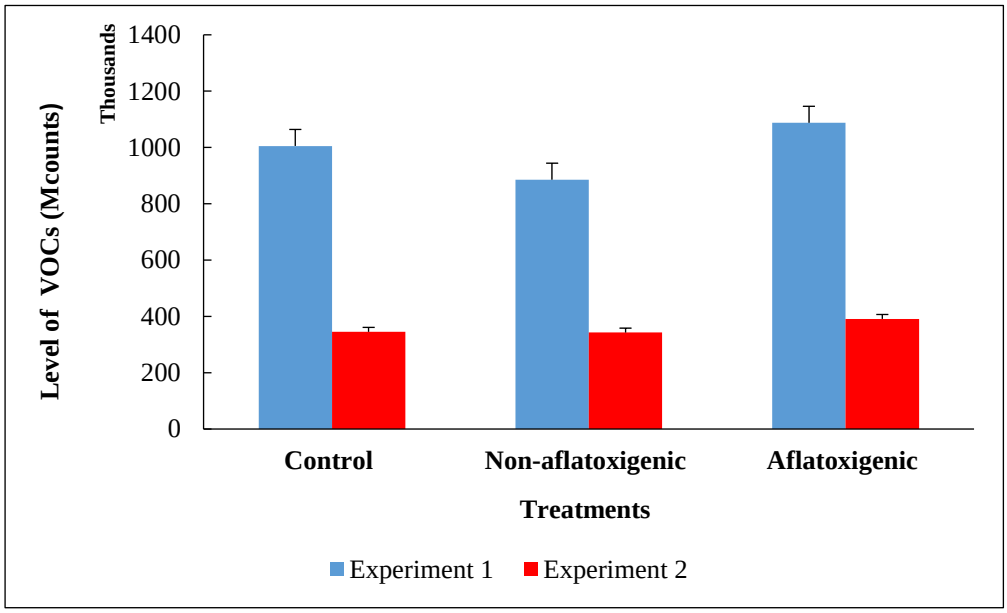


Figure 3.2: Entire VOCs levels in the head space of maize flour samples for variety DK703w inoculated with 2 % Tween 20 as control, non-aflatoxigenic and aflatoxigenic isolates of *A .flavus* for experiment 1 and 2. The bars represent mean ± standard deviation of 30 samples per treatment.

Classification accuracy for the control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize, which represent the actual contaminated samples ranged between 81 % and 98 % (Table 3.1). In practice the results imply ability of GC-MS in combination with statistical techniques to discriminate between aflatoxin contaminated as well as samples infected with *A. flavus* from un-contaminated/uninfected samples with 81 % to 98 % accuracy. Therefore based on the current results, the GC-MS using internal validation would correctly detect contamination in 98 out of 100 samples in the best case scenario and 81 out of 100 samples in the worst case scenario. The classification accuracies were actually relatively higher for the control vs aflatoxigenic *A. flavus* infected maize for the three varieties which may be more representative of the actual field situation when compared to the control vs all *A. flavus* infected maize. Recorded classification accuracy for the control vs aflatoxigenic *A. flavus* infected maize for DK703w was 97 % in both experiments, 86 % for Duma 43 and 98 % for Pioneer while for the control vs all *A. flavus* infected maize it was 91 % and 96 % for DK703w in experiment 1 and 2, 85 % for Duma 43 and 81 % for Pioneer (Table 3.1).

The potential for GC-MS in combination with statistical techniques to discriminate between the different classes of samples differed among the three varieties. Classification accuracy was higher for the Australian variety DK703w when compared to the Kenyan varieties Duma 43 and Pioneer. Classification accuracy for DK703w ranged from 91 % to 100 % correct classification across the different pairwise comparison in the two experiments while for Pioneer and Duma 43 it ranged from 81 % to 96 % correct classification and 85 % to 92 % correct classification respectively (Table 3.1).

Table 3.1: Cross-validated classification accuracy for GC-MS analysis of maize flour for varieties DK703w, Duma 43 and Pioneer inoculated with 2 % Tween 20 as a control, non-aflatoxigenic or aflatoxigenic isolates of *A. flavus*.

Variety	**Pairwise Comparison**	**Best correct classification (%)**
DK703w (Experiment 1)	Control vs all *A. flavus*	92
	Control vs aflatoxigenic	97
	Control vs non-aflatoxigenic	93
	Non-aflatoxigenic vs aflatoxigenic	100
DK703w (Experiment 2)	Control vs all *A. flavus*	96
	Control vs aflatoxigenic	97
	Control vs non-aflatoxigenic	100
	Non-aflatoxigenic vs aflatoxigenic	97
Duma 43	Control vs *A. flavus*	85
	Control vs aflatoxigenic	86
	Control vs .non-aflatoxigenic	82
	Non-aflatoxigenic vs aflatoxigenic	92
Pioneer	Control vs all *A. flavus*	81
	Control vs aflatoxigenic	98
	Control vs non-aflatoxigenic	87
	Non-aflatoxigenic vs aflatoxigenic	96

The ability of GC-MS in combination with statistical techniques to discriminate between different classes of samples was high as evidenced by high classification accuracies achieved, with some minor variations among the pairwise comparisons. The GC-MS was most effective in discriminating maize samples infected with the non-aflatoxigenic *A. flavus* from controls and samples infected with aflatoxigenic *A. flavus* isolates. It was least effective in discriminating between control and combined aflatoxigenic and non-aflatoxigenic samples. Among the four pairwise comparisons, the highest classification accuracy for DK703w was for the non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize (100 % correct classification) in the first

experiment and control vs non-aflatoxigenic *A. flavus* infected maize at 100 % correct classification in the second experiment. Similar results were recorded for Duma 43 where the highest classification accuracy was non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize (92 % correct classification) (Table 3.1). For Pioneer the highest classification accuracy was for the control vs aflatoxigenic *A. flavus* infected maize (98 % correct classification) (Table 3.1). Lower classification accuracies were recorded for the control vs all *A. flavus* infected maize for DK703w in experiments 1 and 2 (92 % and 96 % correct classification) and Pioneer (81 % correct classification). For Duma 43 lowest classification accuracy was recorded for the control vs non-aflatoxigenic *A. flavus* infected maize (82 % correct classification) (Table 3.1).

3.3.1.3 Misclassified samples in experimentally inoculated varieties

The desired outcome of a diagnostic technique is to discriminate between different samples with 100 % accuracy which, in practice, is rarely achieved. Therefore, in addition to classification accuracy the type of misclassified samples are important in determining the applicability of the technique. The sum of false positives and false negatives as a fraction of the total number of classifications represents the percentage of predictions that are misclassified. A false positive represents uncontaminated samples classified as contaminated. A false negative is a contaminated sample classified as uncontaminated. Misclassification rates for DK703w ranged from 0 to 8 % across the different pairwise comparisons. For Duma 43 and Pioneer they ranged from 8 % to 18 % and 2 % to 19 % respectively (Table 3.2). Most notable is the ability of the GC-MS to classify correctly all non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize and control vs non-aflatoxigenic *A. flavus* maize for DK703 in experiment 1 and 2 respectively (Table 3.2). The highest misclassification rates were recorded for the control vs all *A. flavus* infected for all varieties apart from Duma 43 (Table 3.2). The number of false

positives was higher compared to the number of false negatives across the different pairwise comparisons for all three varieties (Table 3.2).

Table 3.2: Misclassification errors for GC-MS analysis of maize flour for varieties DK703w, Duma 43 and Pioneer inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

			No. of misclassified samples/total	
Variety	**Class comparison**	**Misclassification (%)**	**False +ves**	**False –ves**
DK703w	Control vs all *A. flavus*	8	3/48	1/48
(Exp1)	Control vs. aflatoxigenic	3	1/30	0/30
	Control vs non-afla	7	1/29	1/29
	Non-afla vs aflatoxigenic	0	0/28	0/28
Total			**5/135**	**2/135**
DK703w	Control vs all *A. flavus*	4	1/44	0/44
(Exp 2)	Control vs aflatoxigenic	3	1/29	0/29
	Control vs non-afla	0	0/29	0/29
	Non-afla vs aflatoxigenic	3	1/29	1/29
Total			**3/131**	**1/131**
Duma 43	Control vs all *A. flavus*	15	7/59	1/59
	Control vs aflatoxigenic	14	3/44	3/44
	Control vs non-afla	18	7/38	0/38
	Non-afla vs aflatoxigenic	8	1/38	2/38
Total			**18/179**	**6/179**
Pioneer	Control vs *A. flavus*	19	11/68	1/68
	Control vs aflatoxigenic	2	1/45	0/45
	Control vs non-afla	13	3/46	3/46
	Non-afla vs aflatoxigenic	3	1/45	1/45
Total			**17/204**	**5/204**

Exp1- Experiment 1; Exp 2- Experiment 2; No. – Number; False +ve - Number of false positive samples (control samples classified as either *A. flavus* infected, Aflatoxigenic or Non-aflatoxigenic); False –ve- Number of false negative samples (*A .flavus* infected, Aflatoxigenic and Non-aflatoxigenic samples classified as control); Non-afla- non-aflatoxigenic.

3.3.1.4 Identification of diagnostic volatiles for detection of aflatoxin contamination in experimentally inoculated varieties

A wide spectrum of VOCs was produced in the headspace of maize flour that had been artificially inoculated with *A. flavus.* Using the PCA loading plots, the VOCs contributing to the differences between the different classes of samples were identified. The type and number of VOCs differed across varieties and sample types and included quantitative variation in the peak levels of some VOCs.

3.3.1.4.1 Australian variety DK703W artificially inoculated with *A. flavus*

Tetramethyl pyrazine was significantly ($p < 0.0001$) effective in discriminating controls from all *A. flavus* infected maize; maize inoculated with aflatoxigenic *A. flavus* and between maize inoculated with the aflatoxigenic and non-aflatoxigenic *A. flavus.* In all cases the peak levels for tetramethyl pyrazine was higher in *A. flavus* infected samples when compared to the control (Table 3.3). Classification accuracies achieved with tetramethyl pyrazine for the three pairwise comparisons above were relatively similar to accuracies achieved with the entire volatile profile (61 compounds - Appendix 3). Classification accuracy for control vs all *A. flavus* infected maize, control vs aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize were 96 %, 96 % and 100 % respectively, while accuracies achieved with tetramethyl pyrazine were 80 % for the control vs all *A. flavus* infected maize and 97 % for the control vs aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize (Table 3.3). Ethyl acetate was also significantly ($p < 0.0001$) effective in discriminating the control vs all *A. flavus* infected maize and control vs non-aflatoxigenic *A. flavus* maize with 80 % accuracy which was relatively lower than accuracies achieved with the entire VOCs profile. The peak levels for ethyl acetate was significantly higher ($p < 0.0001$) in the control samples compared to the sample infected with the non-aflatoxigenic *A. flavus* in

both cases (Table 3.3). The compound 2, 6, 7 trimethyl decane significantly differentiated ($p < 0.001$) between maize samples infected with aflatoxigenic *A. flavus* from controls with an accuracy of 93 % which was relatively close to accuracy achieved with the entire VOCs profile at 97 % (Table 3.3). Three compounds, 4-ethyl-2-methoxy phenol, 2, 3 butanedione and ethyl acetate, were identified as effective for discrimination of control from maize infected with non-aflatoxigenic *A. flavus.* Among these, 4-ethyl-2-methoxy phenol was the most effective achieving classification accuracy of 90 % which was relatively close to the 100 % achieved with the entire VOC profile. The peak leves for these compounds was higher in the control than maize infected with non-aflatoxigenic *A. flavus* (Table 3.3). In addition to tetramethyl pyrazine, acetoin (2, 3 butanone -3-hydroxy) and 2, 3 butanediol, were also significantly ($p < 0001$ and $p = 0.022$) effective in discriminating between maize samples infected with non-aflatoxigenic and aflatoxigenic *A. flavus* (Table 3.3).

Table 3.3: Identified diagnostic VOCs above the headspace of maize flour for the Australian maize variety DK703w inoculated in the laboratory with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Class comparison	RT	CI	Compound name	Classification accuracy (%)- whole VOC profile (61 compounds)	Classification accuracy (%)- individual VOC	Levels
Control vs all *A. flavus*	21.1 ± 0.2	54	Tetramethyl pyrazine	96	80	all *A. flavus* > control
	2.7 ± 0.1	43	Ethyl acetate	96	80	Control > *Aspergillus*
Control vs. aflatoxigenic	21.1 ± 0.2	54	Tetramethyl pyrazine	97	97	Aflatoxigenic > control
	21.9 ± 0.1	57	2, 6,7 trimethyl decane	97	93	Aflatoxigenic > control
Control vs non-aflatoxigenic	27.9 ± 0.1	137	4-ethyl-2- methoxy phenol	100	90	Control > non-afla
	2.5 ± 0.1	43	2,3 butanedione	100	80	Control > non-afla
	2.7 ± 0.1	43	Ethyl acetate	100	80	Control > non-afla
Non-aflatoxigenic vs aflatoxigenic	21.1 ± 0.2	54	Tetramethyl pyrazine,	100	97	Aflatoxigenic > non-afla
	4.2 ± 0.2	45	Acetoin (2,3 butanone -3-hydroxy)	100	90	Aflatoxigenic > non-afla
	5.8 ± 0.2	45	2,3 butanediol	100	86	Aflatoxigenic > non-afla

Non-afla – Non-aflatoxigenic; RT- mean retention time (minutes) ± the standard deviation; CI- Characteristic ion; VOC- Volatile organic compounds; >- Denotes the class where the level / concentration of VOC is higher. Whole VOCs profile for DK703w = 61 compounds- Appendix 4; Mass spectra for each identified compound shown in Appendix 6.

3.3.1.4.2 Kenyan maize varieties artificially inoculated with *A. flavus*

For the two artficially inoculated varieties, a more diverse volatile profile was recorded for Pioneer when compared to Duma 43. The type of compounds discriminating between the different classes of samples also varied between the two varities (Table 3.4). For Duma 43, 3-methyl butanal was significantly ($p < 0.001$) effective in discrimating between controls and all *A. flavus* infected maize, with an accuracy of 84 % compared to 85 % achieved with the entire VOC profile (58 compounds - Appendix 4). 1-Octadecyne significantly ($p < 0.001$) discriminated between controls and maize infected with the aflatoxigenic *A. flavus* with an accuracy of 81 %, while 3-aminopyrrolidine significantly ($p = 0.005$) discriminated between controls and maize infected with the non- aflatoxigenic *A. flavus* with an accuracy of 74 % which was relatively lower than the accuracy achieved with the entire VOC profile at 82 % (Table 3.4). 6-Ethyl-2-methyl octane was effective ($p < 0.001$) in discriminating between the non-aflatoxigenic and aflatoxigenic *A. flavus* infected maize with a similar accuracy to one achieved with the entire volatile profile (Table 3.4). For Pioneer, p-xylene was effective ($p < 0.0001$) in discriminating controls from all *A. flavus* infected maize and maize infected with the aflatoxigenic *A. flavus* with 81 % and 98 % accuracies respectively, which is close to accuracies achieved with the entire volatile profile (77 compounds - Appendix 5) (Table 3.4). Other compounds; 2, 3 butanediol and naphthalene were also significantly ($p < 0.0001$) effective in discriminating the controls from all *A. flavus* infected maize and aflatoxigenic *A .flavus* infected maize, while 4-choloro-2, 4-dimethyl hexane effectively discriminated controls from maize infected with both aflatoxigenic and non-aflatoxigenic *A. flavus* (Table 3.4). 1-Octadecyne achieved a classification accuracy of 93 % for discrimination of maize infected with non-aflatoxigenic and aflatoxigenic *A. flavus*, which was statistically significant ($p < 0.001$) and close to classification accuracy achieved with entire VOC profile (Table 3.4). The peak levels

for compounds discriminating the different classes of samples were higher in the *A. flavus* infected samples compared to the control for all the pairwise comparisons (Table 3.4).

Table 3.4: Identified diagnostic VOCs above the headspace of maize flour for varieties Duma 43 and Pioneer inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Class comparison	RT	CI	Compound name	Classification accuracy (%) whole VOC profile	Classification accuracy (%) individual VOC	Levels
Duma 43				**(58 compounds)**		
Control vs all *A. flavus*	3.3 ± 0.4	44	3-methyl butanal	85	84	all *A. flavus* > control
Control vs aflatoxigenic	20.4 ± 0.2	81	1-octadecyne	86	81	Control > afla
Control vs non-afla	3.2 ± 0.1	43	3-aminopyrrolidine	82	74	Non-afla > control
Non-afla vs aflatoxigenic	20.5 ± 0.1	43	6-ethyl-2-methyl octane,	92	92	Non-afla > afla
Pioneer				**(77 compounds)**		
Control vs *A. flavus*	9.5 ± 0.6	91	p-xylene	81	88	Infected > control
	5.8 ± 0.1	45	2,3 Butanediol	81	80	Infected > control
Control vs aflatoxigenic	9.5 ± 0.6	91	p-xylene	98	98	Afla > control
	12.6 ± 0.1	55	4-choloro-2,4- dimethyl hexane	98	89	Afla > control
	24.7 ± 0.1	128	Naphthalene	98	84	Afla > control
Control vs non-afla	32.9 ± 0.1	41	Dichloroacetic acid, tetradecyl ester	87	89	Non-afla > control
	12.6 ± 0.1	55	4-chloro-2,4-dimethyl hexane	87	87	Non-afla > control
Non-afla vs aflatoxigenic	20.4 ± 0.2	81	1-octadecyne	96	93	Afla > non-afla
	9.5± 0.6	91	p-xylene	96	82	Afla > non-afla

Afla- Aflatoxigenic; Non-afla – Non-aflatoxigenic; RT- mean retention time (minutes) ± the standard deviation ; CI- Characteristic ion; VOC- Volatile organic compounds; >- Denotes the class where concentration of VOC is higher; Whole VOCs profile for Duma 43 = 58 compounds- Appendix 4, Whole VOCs profile for Pioneer= 77 compounds- Appendix 5. Mass spectra for each identified compound shown in Appendix 6.

3.3.2 Evaluation of GC-MS potential to detect aflatoxin contamination in Kenyan maize naturally infected with *A. flavus*

3.3.2.1 Aflatoxin levels for naturally contaminated samples

The potential for GC-MS in combination with statistical techniques to detect aflatoxin contamination was investigated with maize flour samples naturally contaminated with aflatoxins. The samples were purchased from farms in aflatoxin prone areas in Kenya and included the main varieties grown in those respective areas. Three varieties were obtained. Two of these; Duma 43 and Pioneer, were the same as those experimentally inoculated. The third variety was DH04. Market samples were also purchased and used for validation of the technique, but their varieties were not known. Aflatoxin contamination levels as determined with ELISA are described and discussed in Chapter 2 section 2.3.2 and 2.4 respectively.

3.3.2.2 Classification accuracy for maize naturally infected with *A. flavus*

The capability of GC-MS in combination with statistical techniques to differentiate between controls and aflatoxin contaminated samples was evaluated in Kenyan maize samples naturally infected with *A. flavus.* A total of 77 VOCs were identified from headspace of maize varieties Duma 43, Pioneer, DHO4 and market samples (mixture of unknown varieties) (Appendix 5). The effectiveness of identified VOCs to discriminate between the two classes of samples was evaluated with SVM, as previously described in section 3.3.1.2. Classification accuracies ranged from 48 % to 80 % across the different sample types, and was 70 % for the market samples (Table 3.5). The highest classification accuracy was for DH04 at 80 % while the lowest accuracy was for Pioneer (48 %) (Table 3.5).

Table 3.5: Cross-validated classification accuracy for GC-MS analysis of maize flour samples for Kenyan varieties infected with *A. flavus* under field conditions.

Variety	Pairwise comparison	Best correct classification (%)
Duma 43	Control vs contaminated	69
Pioneer	Control vs contaminated	48
DH04	Control vs contaminated	80
Market samples	Control vs contaminated	70

3.3.2.3 Number of misclassified samples for maize naturally infected with *A. flavus*

The number and type (false positive and false negative) of misclassified samples were identified based on the percent correct classification rate achieved as previously described in section 3.3.1.3. Misclassification rates ranged from 20 % to 52 % for the different varieties and market samples (Table 3.6). The lowest misclassification rate was recorded for DH04 at 20 % while the highest was recorded for Pioneer at 52 % (Table 3.6). The numbers of false positives were high compared to false negatives for Pioneer and the market samples, while for Duma 43 and DH04 the false negatives were more than the false positives (Table 3.6).

Table 3.6: Misclassification errors for GC-MS analysis maize flour for Kenyan varieties naturally infected with *A. flavus*.

			No. of misclassified samples /total	
Variety	**Pairwise comparison**	**Misclassification (%)**	**False +ves**	**False –ves**
Duma 43	Control vs contaminated	31	4/32	6/32
Pioneer	Control vs contaminated	52	18/61	14/61
DH04	Control vs contaminated	20	7/46	9/46
Market samples	Control vs contaminated	30	7/43	6/43
Total			**36/182**	**35/182**

No. – Number; False +ve - Number of false positive samples (control samples classified as contaminated); False –ve- Number of false negative samples (contaminated samples classified as control).

3.3.2.4 Identification of diagnostic volatiles for detection of aflatoxin contamination in Kenyan maize naturally infected with *A. flavus*

The compounds effective in discriminating between contaminated and uncontaminated samples for Kenyan maize varieties contaminated with *A. flavus* under field conditions were different from compounds discriminating similar types of artificially inoculated samples. P-xylene was effective in discriminating uncontaminated and contaminated samples for both artificially and naturally infected varieties. For DH04 which had the highest classification accuracy among the naturally infected samples, p-xylene supported a classification accuracy of 80 %, which was statistically significant ($p = 0.0017$) and similar to the accuracy achieved with the entire VOC profile of 77 compounds (Table 3.7, Appendix 5). Benzaldehyde was also effective ($p = 0.0004$) in discriminating DH04 uncontaminated and contaminated samples (Table 3.7). For Duma 43, 2, 3 butanediol significantly differentiated between the contaminated and uncontaminated samples with accuracy higher than that achieved with the entire VOC profile (Table 3.7). P-xylene was also effective in discriminating uncontaminated and contaminated market samples ($p = 0.023$) with relatively similar accuracy to what was achieved

with entire VOC profile of 77 compounds (Table 3.7, Appendix 5). Apart from 2, 3 butanediol whose peak level was higher in contaminated samples compared to control, the peak levels for all the other compounds was higher in the controls compared to the contaminated samples (Table 3.7).

Table 3.7: Identified diagnostic VOCs above the headspace of flour from Kenyan maize varieties infected with *A. flavus* under field conditions.

Variety	Pairwise comparison	RT	CI	Compound name	Classification accuracy (%) whole VOC profile (77 compounds)	Classification accuracy (%) individual VOC	Levels
Duma 43	Control vs. contaminated	5.8 ± 01	45.0	2,3 Butanediol	69	72	Conta > con
		5.8 ± 0.1	91	Toluene	69	63	Con > conta
DHO4	Control vs. contaminated	9.5± 0.6	91	p-xylene	80	80	Con > conta
		13.8± 0.2	105	Benzaldehyde	80	78	Con > conta
Market samples	Control vs. contaminated	9.5± 0.6	91	p-xylene	70	67	Con > conta

Conta- contaminated; Con- control RT- mean retention time (minutes) ± the standard deviation ; CI- Characteristic ion; VOC –Volatile organic compound; >-Denotes the class where concentration of VOC is higher; Whole VOCs profile for Appendix 5, Mass spectra for each identified compound shown in Appendix 6.

3.4 DISCUSSION

In this study the potential for GC-MS in combination with statistical techniques to detect aflatoxin contamination was evaluated with Australian and Kenyan maize varieties artificially inoculated and naturally infected with *A. flavus*. The results demonstrate the potential of GC-MS in combination with statistical techniques to discriminate controls from all maize artificially infected with aflatoxigenic and non-aflatoxigenic *A. flavus* as well between samples infected with non-aflatoxigenic and aflatoxigenic *A. flavus*, but only within the same variety and under the same conditions. GC-MS in combination with statistical techniques was able to discriminate between the different classes of samples with accuracies that ranged from 81 % to 100 % for the three maize varieties (Table 3.1). Arguably the most important class comparisons are control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize which represents the actual situation in the field where maize is either uncontaminated or contaminated with aflatoxins. Classification accuracy for these comparisons ranged from 81 % to 98 % (Table 3.1). This implies that, if deployed as a diagnostic technique for initial screening of maize for aflatoxin contamination, GC-MS in combination with statistical techniques could discriminate uncontaminated samples from contaminated samples with an accuracy of 81 % to 98 %. These could potentially reduce the number of samples to undergo more expensive and time-consuming quantitative analyses.

Among the class comparisons the GC-MS in combination with statistical techniques was most effective in discriminating maize samples infected with the non-aflatoxigenic *A. flavus* from controls and samples infected with aflatoxigenic *A. flavus* isolates. It was least effective in discriminating between controls and maize samples inoculated with both aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. This indicates that the GC-MS could be less effective in discriminating maize samples infected with both aflatoxigenic and non-aflatoxigenic *A. flavus*

compared to maize infected with either of the strains. This result could possibly be attributed to the fact that aflatoxigenic and non-aflatoxigenic *A. flavus* have been documented to have different volatile profiles (De Lucca *et al.*, 2010). The higher classification accuracy achieved for the non-aflatoxigenic *A. flavus* pairwise comparisons could be attributed to differences in abundance of VOCs from aflatoxin producing isolates. Non-aflatoxigenic *A. flavus* has been documented to produce significantly higher VOCs compared to the aflatoxigenic *A. flavus*. This could be due to increased metabolism directed towards aflatoxin production in aflatoxigenic *A. flavus* and less towards VOCs production (Sun *et al.*, 2016). Though the non- aflatoxigenic *A. flavus* does not produce aflatoxin, it has been tried as a biological control for managing aflatoxin contamination (Abbas *et al.*, 2006; Probst *et al.*, 2011; Ehrlich, 2014; Alaniz *et al.*, 2016). Identification of VOCs that can discriminate between the aflatoxigenic and non-aflatoxigenic strains might be a valuable for identifying non-aflatoxigenic *A. flavus* isolates for biological control of aflatoxins.

The potential for GC-MS in combination with statistical techniques to discriminate between the different classes of samples differed among the three varieties. Classification accuracy was higher for the Australian variety DK703w when compared to the Kenyan varieties Duma 43 and Pioneer. These could be attributed to inherent differences between the varieties where DK703w is a yellow corn variety whereas Duma 43 and Pioneer are white maize varieties. Concentration of VOCs was higher in the yellow maize variety compared to the white maize. Additionally the varieties were grown under different environmental conditions which could have an influence on the type and quantity of VOCs produced and in turn the potential to detect aflatoxin contamination or *A. flavus* infection using GC-MS. Production of VOCs by plants induced by biotic or abiotic factors is strongly regulated by both genetics and the environment and has been demonstrated to be species specific (Niederbacher *et al.*, 2015). The type of GC-

MS instrument used, Varian 3800 GC and 1200 MS for Australian variety DK703w, Agilent 7890A GC and 240 MS for Duma 43 and Bruker 451 GC and Scion Single Quad MS for Pioneer, did not appear to have an effect on the type of VOCs identified and classification accuracies achieved. Classification accuracy ranged from 92 % to 100 % for DK703w, 82 % to 92 % for Duma 43 and 81 % to 98 % for Pioneer across the different class comparisons. Additionally, comparision of chromatograms for similar sample types and standards that included a maize flour sample and a blank 10ml glass vial (Supelco, Bellefonte, PA- sealed with silicon/teflon magnetic autosampler vial caps (Agilent Technologies, Australia)) across the different GC-MS instruments did not show differences in responses (Appendix 7 and 8).

In addition to the classification accuracies achieved, the misclassification rates ranged from 0 to 19 % across the different pairwise comparison for the three varieties, with a higher number of false positives compared to false negatives (Table 3.2). In practice the higher proportion of false positives implies that if the GC-MS were used as an initial screening technique for detection of aflatoxin contamination, it would classify more of the uncontaminated samples as contaminated, which although undesirable, is less concerning than classification of contaminated samples as uncontaminated. A bias towards a false positive result is preferable because few genuinely contaminated samples could be released for consumption. A high proportion of false negatives would be less safe because more contaminated samples would be released for consumption.

Previous studies of evaluation of GC-MS based on VOC profiles for detection of aflatoxin contamination in maize have been more focused on identification of volatile profiles that discrimination between the aflatoxigenic and non-aflatoxigenic strains of *A. flavus* in culture and sterile kernels (Jurjevic *et al.*, 2008; De Lucca *et al.*, 2010; De Lucca *et al.*, 2012; Sun *et*

al., 2016). To the best of our knowledge, this is the first study reporting the use of VOC profiles produced by *A. flavus* for discrimination of aflatoxin uncontaminated and contaminated maize. The results are however, in line with previous studies on other cereals infected with different mycotoxigenic fungi. Presicce *et al.* (2006) reported discrimination of controls from contaminated samples for wheat flour inoculated with *Fusarium poae* using VOCs identified from the headspace of samples inoculated with the toxigenic strain and PCA as a multivariate technique. The accuracy to which the VOCs were able to discriminate between the different classes of samples was however not reported. Olsson *et al.* (2002) classified barley samples naturally contaminated with *Aspergillus, Penicillium* and *Fusarium* species as either below or above the 5 μg/ kg regulatory limit for ochratoxin A and deoxynivalenol using VOCs detected by GC-MS in combination with PCA and partial least squares multivariate data analysis techniques. The GC-MS misclassified three out of 37 samples, with a higher proportion of false positives than false negatives.

Differences in the peak levels for VOCs were reported between the first and second experiment for the Australian variety DK703w. Peak levels for VOCs was higher in samples that were stored for a shorter period prior to analysis when compared to samples that were stored for longer. This could be an indication that the length of sample storage prior to analysis could have an effect on the concentration of VOCs on the head space of samples and hence on the ability of GC-MS to detect contamination. Previous studies on the accumulation of VOCs in stored cereals during fungal colonization show a sequential increase in the quantities of VOCs produced with storage time up to a certain peak time after which the concentration begins to decline (Abramson *et al.*, 1980). Richard-Molard *et al.* (1976) reported a sequential increase in quantities of fungal VOCs produced in maize with storage time at room temperature. Abramson *et al.* (1980) found 10 to 15 fold increases in the quantities of VOCs produced by *Penicillium*

verrucosum on wheat, oats and barley samples by the seventh week of storage at room temperature, with levels decreasing by the sixteenth week. In this study however, the concentration of VOCs was relatively higher in samples stored for two weeks after milling in experiment one when compared to maize samples stored for four weeks after milling in experiment two. Though the classification accuracies were relatively similar across the two experiments, the difference in peak levels for VOCs between the two experiments suggest the potential effect of storage time on concentration of VOCs in the headspace of maize infected with *A. flavus*.

Besides storage time, the temperature at which samples are stored can influence the composition and quality of volatiles produced in grains infected with mycotoxigenic fungi (Magan and Evans, 2000; Garcia-Cela *et al.*, 2018). Garcia-Cela *et al.* (2018) reported an increase in the total number and concentrations of secondary metabolites including VOCs produced in stored wheat naturally contaminated and that inoculated with *Fusarium graminearum* with increase in temperature from 10 ^{o}C to 25 ^{o}C. Maximum production occurred at 15 to 25^{o} C where up to 50 out of the total 121 metabolites were produced. There is therefore a need to conduct a detailed study of changes in the concentration of VOCs in the head space of maize infected with *A. flavus* for storage periods of longer than four weeks at varied temperatures. These would provide information on the duration of storage and temperature at which maize samples remain viable for GC-MS analysis to detect aflatoxin contamination.

The potential for GC-MS in combination with statistical techniques to detect aflatoxin contamination of maize was further evaluated using maize samples that were naturally infected with *A. flavus* under field conditions in Kenya. The samples were collected from aflatoxin prone areas and comprised of different maize varieties and market samples with varying aflatoxin

contamination levels. Classification accuracy ranged from 48 % to 80 % (Table 3.5) indicating that the potential for GC-MS in combination with statistical techniques to discriminate between samples contaminated and uncontaminated with aflatoxin could be variety dependent. Among the three varieties (Duma 43, Pioneer and DH04) and the market samples, the highest classification accuracy was for DH04 at 80 % while the lowest was for Pioneer at 48 %. These could be attributed to inherent genetic differences between the varieties which could have an influence on the type and quantity of VOCs produced and hence on the capability of GC-MS to detect contamination. Pioneer is a hybrid variety with a stay green trait, also referred to as improved late season plant health, therefore it is normally left in the field for extended periods of time after physiological maturity (Arriola, 2006). Infection of maize by *A. flavus* and probably production of VOCs as a result of the infection occurs at the pre-harvest stage. The extended period between infection and harvest could have resulted in decline of VOCs and hence a decline in the capability of the GC-MS in combination with statistical techniques to discriminate between uncontaminated and contaminated samples.

Results indicated potential for GC-MS in combination with statistical techniques to correctly detect contamination in 80 out of 100 samples in the best case scenario but only 48 out of 100 in the worst case scenario, if deployed as a screening technique for detection of aflatoxin contamiation in practice. The classification accuracies are lower than for similar types of samples inoculated in the laboratory where control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize ranged from 81 % to 98 % (Table 3.1). There is therefore need to further investigate the applicability of GC-MS in combination with statistical techniques for detecting aflatoxin with naturally contaminated maize samples. Production of VOCs in maize and other cereals as a result of infection by mycotoxigenic fungi has been demonstrated to differ considerably among varieties. Busko *et al.* (2016) reported significant

differences in volatile compounds present in the grains of bread wheat, durum wheat and triticale wheat contaminated with various species of mycotoxigenic fungi. Sherif *et al.* (2016) reported differences in volatiles produced by hybrid and dwarf maize varieties infected with different strains of *Fusarium graminearum* and *F. verticillioides* at flowering stage. Fauguel *et al.* (2017) reported differences in amount and profiles of VOCs produced by six maize genotypes in response to infection by *F. verticillioides*. These could indicate that VOC profile from fungi is highly dependent on the substrate on which they grow.

In addition to inherent genetic differences the variability in potential for GC-MS to detect aflatoxin contamination in maize samples naturally infected with *A. flavus* could be attributed to the climatic conditions and crop management practices under which the maize was grown. Production of VOCs by maize in response to *A. flavus* infection has been shown to be correlated to initiation and decline of aflatoxin synthesis (Zeringue *et al.*, 1993). Therefore factors that impact on the degree of fungal infection and mycotoxin contamination level are more likely to have an impact on the VOC production patterns and hence on the potential for GC-MS to detect contamination. Among these factors weather conditions, virulence of the *A. flavus* strain, agronomic practices such as nutrition, relative humidity, moisture content of the grain during storage as well as storage conditions play an important role (Rashid *et al.*, 2013; Fountain *et al.*, 2014; Mutiga *et al.*, 2017). Gouinguene´ and Turlings (2002) reported variations in VOCs produced by maize produced under different soil humidity, air humidity, temperature, light, and fertilization rate. Changes in these abiotic factors resulted in significant changes in intensity and variability of VOCs produced.

The maize samples naturally infected with *A. flavus* used in this study were collected from farmers in different agro-ecological zones in Kenya and were therefore presumably produced

under different ecological, agronomic and climatic conditions which could account for the variability in VOCs produced hence the classification accuracies. DH04 was grown under irrigated conditions compared to all the other varieties and market samples that were grown under rain fed conditions. The market samples were collected from Meru County which due to the high altitude is relatively humid compared to the other areas. The crop management practices adapted during production could have differed based on the agro-ecological zone and the socio-economic circumstances of the farmer. Differences in maize production practices in terms of planting date, fertilizer application, time of harvesting and post-harvest handling have been shown to differ with the agro-ecological zone and socio-economic status of the farmer (Hassan, 1996). Additionally, varieties could also have been infected with different strains of *A. flavus* which have been documented to vary across the different agro ecological zones in Kenya (Okoth *et al.*, 2012; Odhiambo *et al.*, 2013; Salano *et al.*, 2016; Okoth *et al.*, 2018). The type and quantity of VOCs produced by aflatoxigenic *A. flavus* on maize have been shown to differ across different strains (De Lucca *et al.*, 2012). The variability in potential for GC-MS in combination with statistical techniques to detect aflatoxin contamination in maize naturally infected samples across the different varieties could be attributed to above discussed factors. Therefore, in order to improve the specificity and potential for GC-MS in combination with statistical techniques to detect aflatoxin contamination, it would be useful to evaluate the potential with a wide range of varieties grown in field trials under similar and climatic and environmental conditions. These would compensate for the variabilities and facilitate identification of specific VOCs produced by maize as a result of natural infection by *A. flavus* hence selection of diagnostic VOCs for detection of aflatoxin contamination.

The second objective of this study was to identify diagnostic VOCs for detection of aflatoxin contamination in maize flour. A wide variety of VOCs were identified from the headspace of

maize varieties artificially inoculated and naturally infected with *A. flavus.* The varieties were characterized by different profiles of VOCs. Using PCA, unpaired t-test and SVM classification it was possible to identify VOCs that can discriminate between the different classes of samples for each variety. The results demonstrated the capability of individual VOCs to discriminate between the different classes of samples, within a variety, with accuracy relatively similar to what was achieved with the entire VOCs profile. A number of VOCs that included tetramethyl pyrazine, ethyl acetate, 2, 6, 7 trimethyl decane, 4-ethyl-2-methoxy phenol, 2, 3 butanedione, acetoin and 2, 3 butanediol were identified from the Australian maize variety DK703w as capable of discriminating the different classes of samples (Table 3.3). These compounds have previously been identified from maize infected with *A. flavus* and other cereals infected with different mycotoxigenic fungi (Jelen and Wasowicz, 1998; Magan and Evans, 2000; Jurjevic *et al.*, 2008; De Lucca *et al.*, 2010; De Lucca *et al.*, 2012; Laddomada *et al.*, 2014; Lippolis *et al.*, 2014). Among these compounds tetramethyl pyrazine appeared to be the most important in discriminating between the controls and maize inoculated with *A. flavus.* Tetramethyl pyrazine was effective in discriminating between controls vs all *A. flavus* infected maize, control vs. aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize. The concentration of this compound was significantly higher in all *A. flavus* infected and aflatoxigenic *A. flavus* infected maize when compared to the control. The peak level was also significantly higher in the maize infected with the aflatoxigenic *A. flavus* compared to maize infected with the non-aflatoxigenic *A. flavus.* The compound was not identified as important for discrimination of the control vs maize infected with the non-aflatoxigenic *A. flavus.* Therefore the compound may be unique to the aflatoxigenic *A. flavus* isolate used in this study and a potential marker for *A. flavus* infection and aflatoxin contamination. Tetramethyl pyrazine has been reported previously as a fermentation product of microorganisms including *Aspergillus* species on food. The actual mechanism of formation in

microorganisms has yet to be elucidated, but it is hypothesized to be synthesised by the condensation reaction between acetoin and ammonia (Kempler, 1983; Jelen and Wasowicz, 1998).

The VOCs identified as significant in discriminating the different classes of samples differed among the Kenyan maize varieties artificially inoculated and naturally infected with *A. flavus.* A total of four compounds 3-methyl butanal, 1-octadecyne, 3-aminopyrrolidine and 6-ethyl-2-methyl octane, were identified from the headspace of Duma 43 as capable of discriminating between the different classes of samples (Table 3.4). However, there was no VOCs that showed consistency in discriminating between similar classes of samples across the different pairwise comparisons. For example 3-methyl butanol was only effective in discriminating between control vs all *A. flavus* infected maize and was not identified as a potential marker for other pairwise comparison with similar samples. A similar pattern was observed for the other identified VOCs and therefore no potential markers for *A. flavus* infection or aflatoxin contamination were identified for Duma 43. A different set of VOCs were identified in the headspace of Pioneer artificially inoculated with *A. flavus* and Duma 43, DH04 and market samples naturally infected with *A. flavus*. p-xylene, 2, 3 butanediol, naphthalene, 4-choloro-2, 4-dimethyl hexane, dichloroacetic acid, tetradecyl ester, 1-octadeycene, toluene, and benzaldehyde were identified as effective for discriminating between the different classes of samples across the varieties and market samples. The compounds have been previously reported from head space of maize infected with *A. flavus* (Jurjevic *et al.*, 2008; De Lucca *et al.*, 2010; De Lucca *et al.*, 2012) and in wheat that was artificially and naturally contaminated with *Fusarium* species (Presicce *et al.*, 2006; Laddomada *et al.*, 2014; Maciej *et al.*, 2016).

P-xylene was effective in discriminating between the control and various samples infected with the aflatoxigenic *A. flavus* (control vs all *A. flavus* infected maize, control vs aflatoxigenic *A. flavus* infected maize, non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize and control vs contaminated samples) for variety DH04 and market samples that were naturally infected with *A. flavus*. Apart from the naturally contaminated DH04 and market samples, the peak levels of p-xylene was significantly higher in *A. flavus* contaminated samples compared to the control. Similar to tetramethyl pyrazine for the Australian variety DK703w, p-xylene was not identified as significant for discrimination of the control vs non-aflatoxigenic *A. flavus* infected maize which could be an indication that the compound is specific to the aflatoxigenic *A. flavus* and thus a potential marker for *A. flavus* infection in maize and subsequently aflatoxin contamination. P-xylene is an aromatic hydrocarbon produced as a secondary metabolite during the conversion of glucose to pyruvate in the glycolytic pathway (Magan and Evans, 2000; Li *et al.*, 2013; Show *et al.*, 2015). Production of p-xylene by *Aspergillus* species fungi has been previously reported by various authors. Jelen and Grabarkiewicz-szczeüsna (2005) reported production of p-xylene by *Aspergillus* strains with different abilities to produce ochratoxin A on culture media. Jurjevic *et al.* (2008), De Lucca *et al.* (2010) and De Lucca *et al.* (2012), reported production of p-xylene by *A. flavus* on culture media and cracked maize kernels. Production of p-xylene has also been reported with other mycotoxigenic fungi. Busko *et al.* (2016) identified p-xylene as one of the compounds effective in discriminating between contaminated and uncontaminated wheat grains naturally infected with *F. poae*. Perkowski *et al.* (2012) also reported p-xylene as an important compound in development of a model for discriminating between contaminated and uncontaminated grains of barley, rye and oats naturally infected with *F. poae*. Laddomada *et al.* (2014) reported higher concentrations of aromatic VOCs including p-xylene in wheat grains artificially inoculated with *F. poae* when compared to un-inoculated controls.

3.5 CONCLUSION

The results from this study include novel insights into the potential application of GC-MS in combination with statistical techniques for detection of aflatoxin contamination in maize. Previous studies have mainly focused on the ability of GC-MS to discriminate between aflatoxigenic and non-aflatoxigenic *A. flavus* strains, with fungus cultured on artificial media or sterile grain. The current study provides initial investigation on the capability of GC-MS in combination with statistical techniques to detect aflatoxin contamination in maize that was artificially and naturally infected with *A. flavus.* Results show potential for GC-MS in combination with statistical techniques to discriminate between uncontaminated and contaminated maize samples that were artificially inoculated with *A. flavus* with accuracy of 81 % to 100 %, but only within the same variety. This indicates potential for GC-MS in combination with statistical techniques as a rapid and cost-effective diagnostic technique for initial screening of maize to detect aflatoxin contamination to allow for separation of contaminated and uncontaminated lots prior to trade or human consumption. These results were however not consistent for maize samples naturally infected with *A. flavus*. Unlike the maize samples artificially inoculated with *A. flavus,* classification accuracies for naturally infected samples were highly variable across the varieties and ranged from 48 % to 80 %. These could be attributed to inherent genetic differences between the varieties and environmental conditions under which the varieties were grown, which presumably had an effect on the type and quantity of VOCs produced and hence on the capability of GC-MS to detect contamination. There is therefore need for further studies on the potential for GC-MS to detect contamination in maize naturally infected with *A. flavus* with a wide range of varieties grown in field trails under similar and different agronomic and environmental conditions to eliminate the above variabilities.

The study further investigated the type of VOCs produced on maize upon infection by *A. flavus* and identification of potential diagnostic volatiles for discrimination of aflatoxin contaminated and uncontaminated maize. Though the VOC profiles varied widely across the different varieties evaluated, a number of VOCs capable of discriminating between maize contaminated and not contaminated with aflatoxin were identified. Among these compounds, tetramethyl pyrazine and p-xylene exhibited consistent discriminatory ability for maize samples artificially inoculated and naturally infected with *A. flavus* with classification accuracies similar to accuracies achieved with the whole VOC profile. The compounds were effective in discriminating maize infected with aflatoxigenic *A. flavus* isolates from the controls and maize infected with the non-aflatoxigenc *A. flavus* isolates indicating they could be characteristic of the aflatoxigenic *A. flavus* and could hence be potential markers for aflatoxin contamination. However, there is need for further studies to confirm the production of these compounds with a wide range of maize varieties and *A. flavus* isolates evaluated under similar environmental and agronomic conditions. These will aid identification of unique discriminatory diagnostic VOCs that are uniform across different varieties infected with different strains of *A. flavus*. Additionally it will be useful to evaluate the VOC profiles of *A. flavus* infected maize over time on live plants to establish a reliable set of VOCs markers that are correlated to aflatoxin production.

CHAPTER 4

COMPARISON OF THE PERFORMANCE OF METAL OXIDE AND CONDUCTING POLYMERS ELECTRONIC NOSES FOR DETECTION OF AFLATOXIN USING ARTIFICIALLY CONTAMINATED MAIZE

4.1 INTRODUCTION

The electronic nose offers potential as a portable, rapid, cost-effective, non-invasive, high throughput field diagnostic technique, for initial screening of commodities to detect mycotoxin contamination (Falasconi *et al.*, 2012; Alam and Saeed, 2013). Electronic nose detection is based on changes in the spectrum of VOCs in the sample headspace due to growth of mycotoxigenic fungi (Cheli *et al.*, 2009). Electronic noses consist of an array of non-specific chemical sensors, which interact with different volatile compounds and provide an electronic signal that is characteristic of the mixture of volatiles present in the sample (Di Natale *et al.*, 2001; Sliwinska *et al.*, 2014). The signal is analysed with pattern recognition and multivariate statistical techniques to differentiate and classify samples according to their volatile composition (Loutfi *et al.*, 2015). Advances in sensory technology have resulted in the development of a range of sensor formats that include metal oxide semiconductors, conducting organic polymers, piezoelectric crystals and most recently mass detection-based electronic noses. Of these, the metal oxide semiconductors and conducting polymer sensors are the most widely used in commercially available electronic nose devices/instruments (Schaller *et al.*, 1998; Ampuero and Bosset, 2003; Rock *et al.*, 2008; Berna, 2010; Loutfi *et al.*, 2015; Peris and Escuder-Gilabert, 2016).

The potential of electronic noses to determine the mycological quality of grains has been investigated by various researchers. Capability to discriminate between different strains of toxigenic fungal species as well as grain samples infected with mycotoxigenic and non-mycotoxigenic strains has been evaluated (Borjesson *et al.*, 1996; Jonsson *et al.*, 1997; Olsson *et al.*, 2002; Paolesse *et al.*, 2006; Cheli *et al.*, 2009; Campagnoli *et al.*, 2011; Gobbi *et al.*, 2011; Lippolis *et al.*, 2014). However, the majority of these studies have been focused on mycotoxins produced by *Fusarium* and *Penicillium* species in wheat, barley and oats. Evaluations of electronic noses for detecting aflatoxins produced by *Aspergillus* species on maize are very limited (Campagnoli *et al.*, 2009; Cheli *et al.*, 2009). No known previous study has comprehensively compared the performance of different sensor technologies for detecting aflatoxins.

The objective of the current study was to compare the performance of three electronic nose instruments for detecting aflatoxin contamination in maize. The three instruments are based on different sensor technologies namely metal oxide semiconductor sensors (Fox 3000), conducting polymer sensors (Cyranose 320) and metal oxide semiconductor sensors with thermocycling (DiagNose).

4.2 MATERIALS AND METHODS

4.2.1 Inoculation of maize with *A. flavus* and collection of naturally contaminated samples

The potential for the three electronic nose instruments, Fox 3000, Cyranose 320 and DiagNose to detect aflatoxin contamination was evaluated with an Australian maize variety (DK703w) that had been artificially inoculated with *A. flavus*. The maize was inoculated with the aflatoxigenic (FRR-5315) and non-aflatoxigenic (FRR-4288) *A. flavus* isolates. Treatments for the experiments included maize cobs contaminated with aflatoxigenic and non-aflatoxigenic *A. flavus* at two, six and ten incisions per cob to achieve different aflatoxin concentration levels, two controls namely; cobs inoculated with 2 % v/v Tween 20 at two, six and ten incisions and un-inoculated controls with no incisions. A set of five cobs was used for each treatment as described in Chapter 2 section 2.2.1.

4.2.2 Electronic nose analysis

The differences in headspace volatile profiles between maize flour contaminated with aflatoxigenic or non-aflatoxigenic *A. flavus* as well as the control treatments were evaluated with electronic noses. The performances of different electronic nose instruments for detection of aflatoxins namely, the Fox 3000, Cyranose 320 and DiagNose (Figure 4.1), were compared. Experiments were performed in duplicate under similar conditions, with five samples per treatment. However, for DiagNose the instrument pump failed during the second experiment hence only the result for one experiment is presented (Appendix 7).

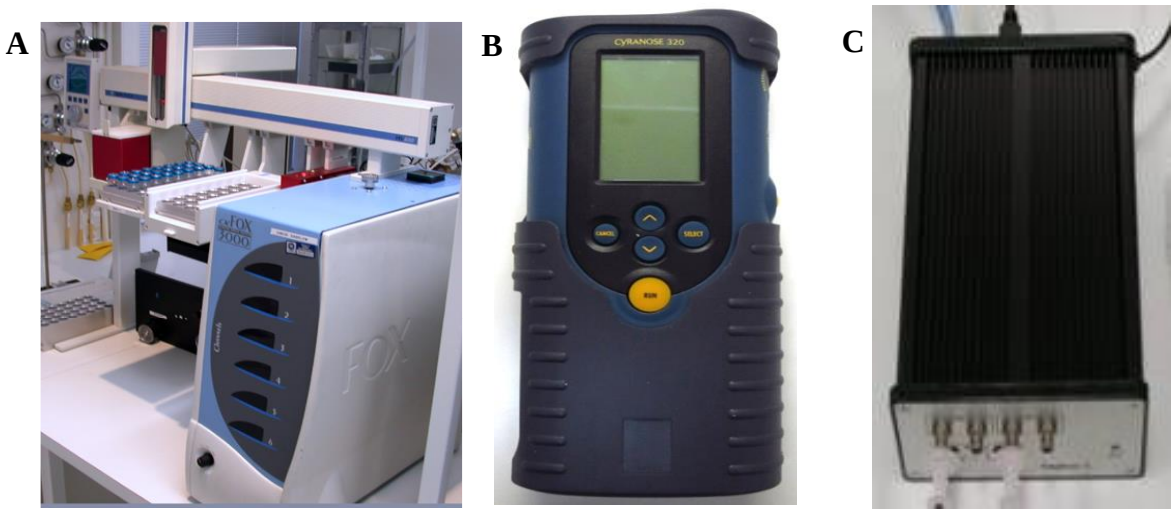


Figure 4.1: Electronic nose instruments evaluated for detecting aflatoxin associated volatiles in maize. **(A)** Fox 3000, **(B)** Cyranose 320 and **(C)** DiagNose.

4.2.2.1 FOX 3000 (Alpha-M.O.S., Toulouse, France)

The Fox 3000 (Figure 4.1A) is equipped with an array of twelve semiconducting sensors comprising of six standard doped tin oxide sensors (SnO_2) and six chromium titanium oxide (CTO) sensors. For the six CTO sensors, five are coated with zeolite and one is uncoated. To extract the volatiles, 1.65 g of maize flour sample was weighed into a 10 mL glass vial (Supelco, Bellefonte, PA) fitted with a silicon/teflon magnetic autosampler vial cap (Agilent Technologies, Australia). Prior to head space analysis, the glass vials were transferred to a heating block and agitated at 35 ^{o}C for five minutes at 500 rpm, to facilitate volatile production. The headspace was sampled using an auto injector (CombiPAL, Switzerland) set at 45 ^{o}C, which transferred 2.5 mL of the headspace using a 2.5 mL syringe into the carrier gas (zero air-20.9 % oxygen, 78.1 % nitrogen and 0.9 % argon (BOC Australia, North Ryde, NSW)) flowing at 150 mL/min. The carrier gas carries the sample over the array of 12 metal oxide sensors. The headspace was sampled for ten minutes. After each analysis, the instrument was purged with zero air at a flow rate of 150 mL/min for 10 min to allow the sensors to return to baseline for subsequent samples. During analysis the different classes of samples, control,

non-aflatoxigenic *A. flavus* infected maize and aflatoxigenic *A. flavus* infected maize were alternated to minimize the memory effect from previous sample. The instrument generates one data points for each sensor for every second of analysis hence a total of 600 data points for each sensor.

4.2.2.2 Cyranose 320 (Sensigent, Inc. Balfwin Park, CA, USA)

Cyranose 320 (Figure 4.1B) is a portable hand held system consisting of 32 individual conducting polymer sensors blended with carbon black composite, configured as an array. Volatiles were extracted by weighing 1.65 g of maize flour into a 10 mL glass vial (Supelco, Bellefonte, PA) fitted with a silicon/teflon magnetic autosampler vial cap (Agilent Technologies, Australia). The vials were incubated in the block heater for nine and a half minutes at 35 ^{o}C to allow for the release of volatiles into the sample headspace. The instrument was first flushed with zero air (20.9 % oxygen, 78.1 % nitrogen and 0.9 % argon (BOC Australia, North Ryde, NSW)) from an air bag for 50 seconds using its internal pump at 120 mL/min. Headspace was drawn from the sample for 30 seconds at the same flow rate. A bag containing zero air was connected to the vial to replace air removed during sampling. After sampling the sensors and the sample line were purged with zero air at 180 mL/min for 20 seconds to allow the instrument to return to baseline for subsequent samples. During analysis the different classes of samples, control, non-aflatoxigenic *A. flavus* infected maize and aflatoxigenic *A. flavus* infected maize were alternated to minimize the memory effect from previous sample. The instrument generates 18 data points for each sensor for every second of analysis hence a total of 540 data points for each sensor.

4.2.2.3 DiagNose (C-it, The Netherlands)

The DiagNose (Figure 4.1C) is field portable and consists of twelve *n* type metal oxide sensors. The sensor array comprises: three SnO_2 sensors doped with palladium (SnO_2-Pd), three SnO_2 sensors doped with platinum (SnO2-Pt), one SnO_2 sensor doped with silver (SnO_2-Ag), one SnO_2 sensor doped with copper (SnO_2-Cu), three undoped WO_3 sensors and an experimental sensor (Extype 1) with composition undisclosed by the manufacturer. In this instrument, sensor array temperature is modulated by driving sensor heater elements with a triangular voltage waveform. The temperature waveforms are generated with a period of 20 seconds during which the sample is measured. This represents a "one time index". Within each 20 second period (time index), the sensor surface temperature is varied over 32 steps following a quasi-sinusoidal signal, between temperatures of 260 ^{o}C and 340 ^{o}C. Each sensor therefore generates 32 data points every 20 seconds (Figure 4.2). The instrument was operated with zero air (20.9 % oxygen, 78.1 % nitrogen and 0.9 % argon (BOC Australia, North Ryde, NSW) at a flow rate of 400mL/min carrier gas. A mass of 1.65 g of maize flour was weighed and transferred to a 10 mL glass vial (Supelco, Bellefonte, PA) fitted with a silicon/teflon magnetic autosampler vial cap (Agilent Technologies, Australia). The vials were incubated in a block heater for 30 min at 35 ^{o}C to allow for the release of volatiles into the sample headspace. After incubation, the headspace was carried to and from the electronic nose chamber via Teflon tubing at a flow rate of 40mL/ min. Headspace was sampled for five minutes. After each analysis, the instrument was purged with zero air at a flow rate of 300 mL/min for 35 min to allow the instrument to return to baseline for subsequent samples. To prevent contamination of the electronic nose chamber with flour or fungal spores, PTFE filters 25 mm x 0.2 μm (Supelco, Bellefonte, PA) were placed at the inlet and outlet sampling ports. During analysis the different classes of samples, control, non-aflatoxigenic *A. flavus* infected maize and aflatoxigenic *A. flavus* infected maize were alternated to minimize the memory effect from previous sample.

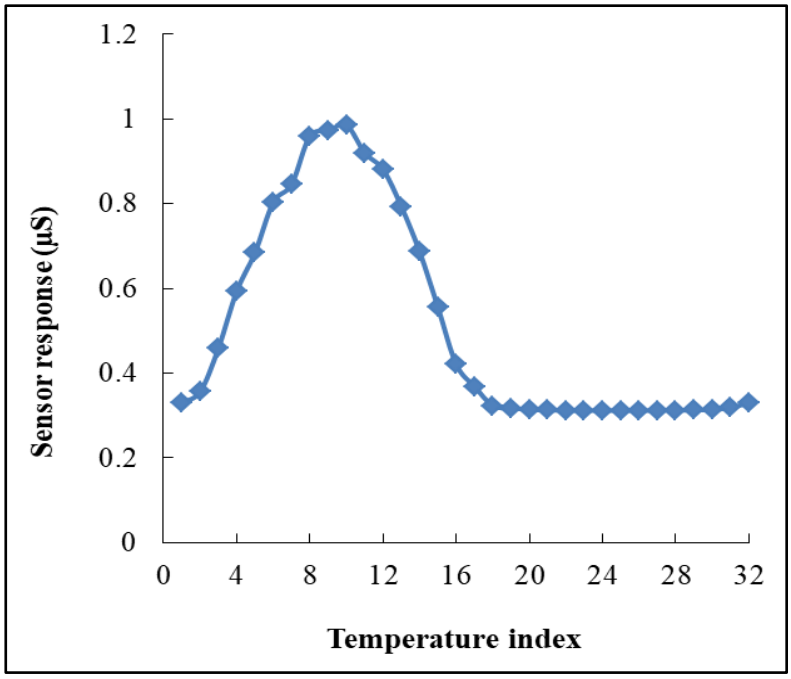


Figure 4.2: Sample raw DiagNose data at a single time index (20 seconds) for one sensor. Each data point represents a certain temperature between 260 ^{o}C and 340 ^{o}C varied over 32 steps following a quasi-sinusoidal signal.

4.2.3 Data analysis

4.2.3.1 Data pre-processing

The Fox 3000 data was pre-processed using *eNose explorer* software written by Dr.Thomas Nowotny (Sussex University, UK). The raw data had its baseline subtracted and was normalised across sensors to correct for day of analysis effect. The sample data and standards (blank vial and maize flour) analysed on the same day was first normalised by their mean and standard deviation (i.e. z-scored): $(x-\mu)/\sigma$, where x is a data point, μ is the mean of all data for that sensor that day, and σ is the standard deviation of all data for that sensor. The Cyranose 320 data was also pre-processed in *eNose explorer*. Processing involved detrending to remove linear drift in the data, baseline subtraction and division to normalize data across the sensors

and normalization within the day of analysis to remove any day effect as described above under Fox 3000.

The DiagNose data was extracted and pre-processed using *"e-nose explorer"* algorithms coded in MatLab (Mathworks, USA) by Dr. Rosalind Wang (CSIRO) and Dr. Thomas Nowotny (Sussex University, UK). During the extraction, the interval before and after the peak was considered. Depending on the time of the peak, 300 seconds of the data leading to and including the peak (actual analysis time), plus another 100 seconds after the peak (baseline recovery time) were processed. Since the instrument was found not to make sensor measurements at exactly the prescribed 20 seconds intervals, to compare data across different samples, linear interpolation was applied to the raw data to derive virtual sensor readings at exactly 20 second intervals. The data was extracted for 25 time indices per sample (500 seconds equivalent). The data was normalized to correct for day of analysis effects as described above under Fox 3000.

4.2.3.2 Comparison of electronic nose instrument data reduction methods and feature selection with relative mutual information (MI)

Data reduction/feature selection is a critical step in analysis of sensor response data. Its aim is to reduce the high dimensionality of data and identify a small set of parameters that are most informative. Removal of redundant, irrelevant and noisy sensors through data reduction/ feature selection can enhance the accuracy of classification algorithms. Data reduction for Fox 3000 and Cyranose 320 was performed with *eNose explorer* software as described below in section 4.2.3.2.1 The methodologies were applied to select data points contributing to the differences between the different sample comparisons that included: control maize vs all *A. flavus* infected maize, control maize vs aflatoxigenic *A. flavus* infected maize, control maize vs non-

aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize. SVM classification with leave-one-out cross-validation was used to determine the capability of the data points selected with the instrument associated data reduction methods to discriminate between the different sample classes. The classification accuracies across the different sample comparisons achieved with instrument associated data reduction methods was compared with accuracies achieved with MI as a feature selection method.

For DiagNose feature selection was done by calculating the Euclidean distance between the classes (Q-value) as described in section 4.2.3.2.1. The classification accuracies across the different sample comparisons achieved with features selected with Q-values were compared to classification accuracies achieved with MI as a feature selection method.

4.2.3.2.1 Electronic nose associated data reduction methods

A) Fox 3000 data reduction method

In order to reduce dimensionality for the Fox 3000 data, representative features were extracted from the most responsive sensors (Figure 4.3 A). Analysis of the maize samples showed that the sensor response curves stabilized after 20 to 40 seconds and therefore for each selected sensor, data was extracted from the first 40 seconds out of the total 600 seconds. These included the first 10 seconds of data leading to and including the peak and an additional 30 seconds after the peak (Figure 4.3 B). Data was extracted from the seven most responsive sensors which included six SnO_2 and one CTO sensor; hence a total of 287 data points for each class comparisons.

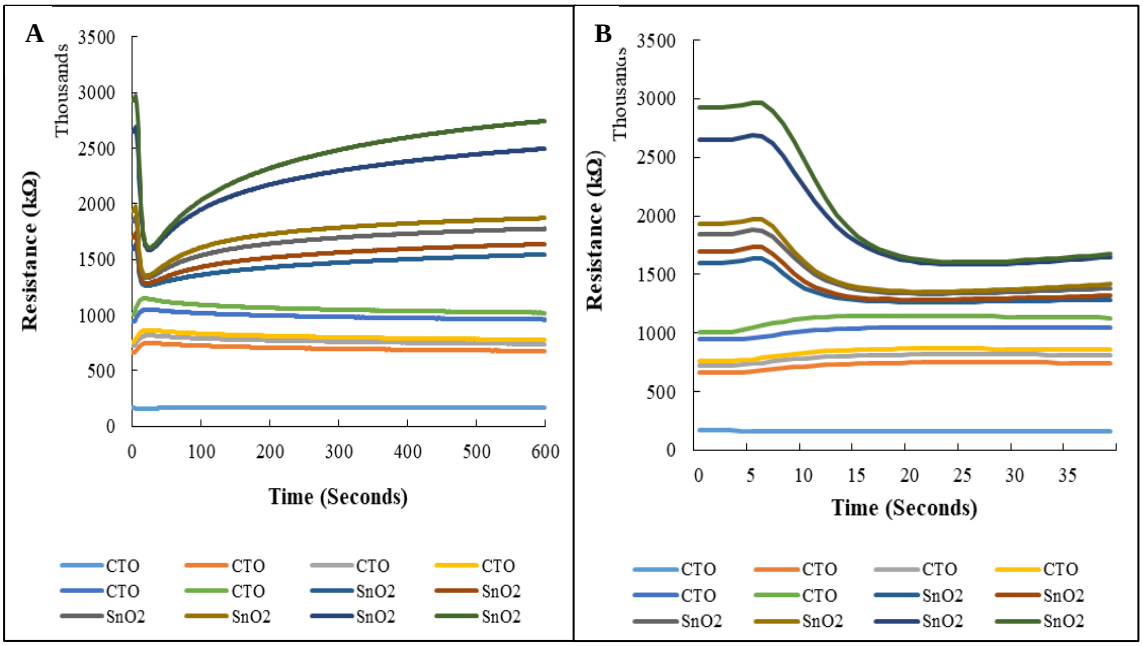


Figure 4.3: Representative responses of Fox 3000 tin oxide sensors (SnO_2) and chromium titanium oxide (CTO) sensors to maize flour samples inoculated aflatoxigenic *A. flavus* isolates. **(A)** Responses over the full 600 seconds. **(B)** Extracted sampling time over the first 40 seconds.

B) Cyranose 320 data reduction method

Representative data points for the Cyranose data were extracted from all the 32 sensors using the *eNose explorer* software. Data was extracted from the 30 seconds of analysis time referred to as the sample draw and an additional ten seconds after the peak (Figure 4.4). A total of 74 data out of 540 points were selected for each sensor, resulting in 2368 points for each class comparison.

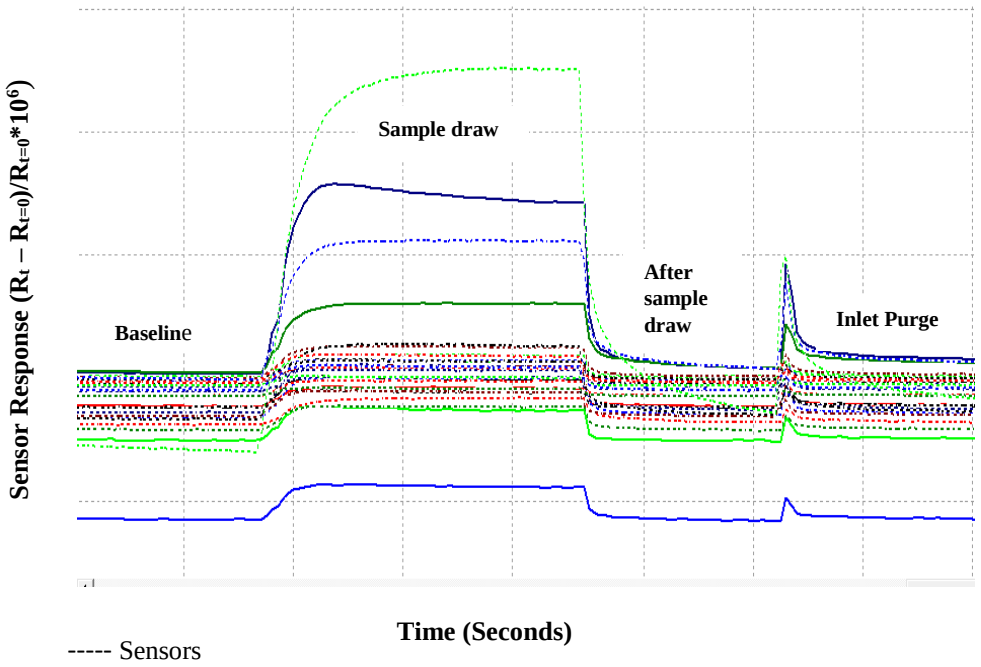


Figure 4.4: Representative responses for Cyranose 320 sensors to maize flour samples inoculated with aflatoxigenic *A. flavus* isolate. The vertical axis represents the sensor responses, ($R_t - R_{t=0}$)/$R_{t=0}$*10^6) where, R_t- sample resistance and $R_{t=}$0 second is the baseline.

C) DiagNose feature selection (Q-values)

Feature selection for DiagNose data was performed by calculating the Euclidean distance between the different sample class comparisons. For each class, the mean (centroid) was calculated over all samples in the class. The Euclidean distance between samples *i* and the class *k* centroid was calculated as:

$$d_{ik} = \sqrt{(x_i - \bar{x}_k).(x_i - \bar{x}_k)^T}$$

where x_i is the *i*th sample described by a row vector with *n* variables (*n* denotes the number of sensors), $\bar{x}_k$ is the centroid of class *k*, and T denotes the transpose of a vector (Zhang *et al.*, 2012). The number of features selected was based on a cut off value Q, which is a quotient of

Euclidian distance between the two classes under evaluation divided by sum of the co-efficient of variation for each class.

$$Q = d_{ik} / (\sum(cv\{i\} + \sum cv\{k\})$$

Where, is d_{ik} $\{i,k\}$ is the Euclidian distance between class *i* and *k*, $\sum cv\{i\}$ is the sum of the co-efficient of variation for class *i* and $\sum cv\{k\}$ is the sum of the co-efficient of variation for class *k*.

4.2.3.2.2 Feature selection with relative mutual information

MI, an information theoretic approach was used to select features for classification of Fox 3000, Cyranose 320 and DiagNose data (Wang *et al.*, 2014). The objective was to select a subset of features, $\upsilon \subseteq \{1,2,\ldots,m\}$, where m is the total number of features, such that the resulting subset of features gives the best classification performance for the given size constraint n on the number of features in U. The subsets of features were selected by maximizing the *mutual information* $I(\mathbf{Z}^u, C)$ between the selected features $\mathbf{Z}^\upsilon = \{Z^{i_1},\ldots,Z^{i_n}\}$ and class C: This approach is equivalent to selecting the class which maximises the probability *p* ($\mathbf{Z}^v$,*C*) of the observation given the class, and is also equivalent to maximising the posterior *p* (*C*|$\mathbf{Z}^v$) under the assumption of the marginal distribution for each class p(c) being equiprobable.

$$I(\mathbf{Z}^u, C) = \sum_{\mathbf{Z}^u, C} p(\mathbf{Z}^u, C) \log_2 \frac{p(C \mid \mathbf{Z}^u)}{p(C)}$$

The upper band of size constraint was set to 100, that is, the first 100 features with the most information about the differences between classes were selected. The Kraskov–Grassberger technique (estimator 2) (Kraskov *et al.*, 2004) was employed to estimate the mutual

information. The code used for the estimation is publicly available as a Java Information Dynamics Toolkit (Lizier, 2014).

4.2.3.3 Cross-validation and classification

The leave-one-out cross-validation method was used to train the classifier by selecting appropriate features and testing the classifier with new data. The datasets were partitioned into *N* pairs of a training set (*N*-1 data samples) and a test validation set (the remaining 1 data sample), where *N* is the size of the data set. The process was repeated *N* times to cover all data samples in the data set. Feature selection was performed on each training set to select the features to train a given classifier, and then the classifier was applied to the test set. The performance of a given classifier is therefore scored as the average correct classification rate it obtained over the *N* training-test pairs. The effectiveness of selected features to discriminate between the different classes of samples was evaluated by two common classifiers namely SVM and KNN. SVM constructs a hyperplane to separate training data in different classes, i.e. it 'divides' the data into separate clusters. It then classifies a new unseen data according to which side of the plane the new data lays (Cortes and Vapnik, 1995). KNN classifies each new unseen data point based on the *k* nearest (Euclidean distance) training data from the new data point. The new data point's class is determined to be that with the most training data points in its neighborhood (Gou *et al.*, 2012). Based on the best classification rates obtained by the classifiers the number and type of misclassified samples were identified. This included identification of false positive (uncontaminated samples classified as contaminated) and false negative (contaminated samples classified as uncontaminated).

4.3 RESULTS

4.3.1 Aflatoxin contamination levels

The potential of the three electronic nose instruments to detect aflatoxin contamination in maize was evaluated using maize variety DK703w inoculated with 2% tween 20 as control samples, non-aflatoxigenic and aflatoxigenic *A. flavus* isolates. Aflatoxin contamination levels for the different classes of samples are presented and discussed in Chapter 2 section 2.3.1 and 2.4 respectively.

4.3.2 Comparison of electronic nose instrument data reduction methods and feature selection with relative mutual information

4.3.2.1 Fox 3000 data reduction method vs MI

For the Fox 3000 data reduction method, the best correct classification rates with 287 selected data points/features were; 64 % and 54 % for the control vs aflatoxigenic *A. flavus* infected maize and 75 % and 70 % for the control vs non-aflatoxigenic *A. flavus* infected maize in the first and second experiment respectively (Table 4.1). Classification rates were higher with MI compared to the Fox 3000 data reduction method for the control vs aflatoxigenic *A. flavus* infected maize (79 % and 70 %) and control vs non-aflatoxigenic *A. flavus* infected maize (76 % and 91 %) in the first and second experiment respectively. The number of features used for classification was also lower with MI and ranged from 8 to 64 across the different class comparisons (Table 4.1).

Table 4.1: Classification accuracies for Fox 3000 analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control and non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons using features selected by instrument associated data reduction method and mutual information.

		Fox 3000 data reduction method		**Mutual Information**	
Experiment	**Class comparison**	**Best correct classification (SVM) (%)**	**No. of data points/features**	**Best correct classification(SVM) (%)**	**No. of features**
Exp 1	Control vs aflatoxigenic *A. flavus*	64	287	79	8
	Control vs non-afla *A. flavus*	75	287	76	64
Exp 2	Control vs aflatoxigenic *A. flavus*	54	287	70	19
	Control vs non-afla *A. flavus*	70	287	91	23

Exp- Experiment; No.-Number; SVM- Support vector machines classification; Non-afla- Non-aflatoxigenic.

4.3.2.2 Cyranose 320 data reduction method vs MI

A similar pattern to Fox 3000 was observed when the Cyranose 320 data reduction method was compared to MI. Using the 2368 selected data points/features, the best correct classification rates for the Cyranose 320 data reduction method were 32 % and 39 % for the control vs aflatoxigenic *A. flavus* infected maize and 48 % and 34 % for the control vs non-aflatoxigenic *A. flavus* infected maize in the first and second experiment respectively (Table 4.2). The classification rates were higher with MI for different class comparisons and ranged from 66 % and 69 % for the control vs aflatoxigenic *A. flavus* infected maize and 76 % and 58 % for the control vs non-aflatoxigenic *A. flavus* infected maize in the first and second experiment respectively. The number of features selected with MI was lower and ranged from 10 to 55 across the different class comparisons (Table 4.2).

Table 4.2: Classification accuracies for Cyranose 320 analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons using features selected by instrument data reduction method and mutual information.

		Cyranose 320 data reduction method		Mutual Information	
Experiment	**Class**	**Best correct classification (SVM) (%)**	**No. of data points/ features**	**Best correct classification (SVM) (%)**	**No. of features**
Exp1	Control vs Aflatoxigenic *A. flavus*	32	2368	66	32
	Control vs Non-afla *A. flavus*	48	2368	76	55
Exp 2	Control vs Aflatoxigenic *A. flavus*	39	2368	69	18
	Control vs Non-afla *A. flavus*	34	2368	58	10

Exp-Experiment; No. - Number; SVM- Support vector machines classification; Non-afla- Non-aflatoxigenic.

4.3.2.3 DiagNose Q-values vs MI

Though the percent best classification rates were relatively similar for the DiagNose associated method (Q-values) and MI, more features were used with the Q-values when compared to MI (Table 4.3). Classification rates with the Q-values were 84 % with 1831 features for the control vs aflatoxigenic samples and 83 % with 402 features for the control vs non-aflatoxigenic samples in the first experiment (Table 4.3). With MI the classification rates for the control vs aflatoxigenic and control vs non-aflatoxigenic samples were 80 % and 91 % with 13 and 4 features respectively (Table 4.3).

Table 4.3: Classification accuracies for DiagNose analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons using features selected by Q-values and mutual information.

		DiagNose Q-values		Mutual Information	
Experiment	**Class**	**Best correct classification (SVM) (%)**	**No. of features**	**Best correct classification (SVM) (%)**	**No. of features**
Exp1	Control vs Aflatoxigenic *A. flavus*	84	1831	80	13
	Control vs Non-afla *A. flavus*	83	402	91	4

N/B For, DiagNose the instrument pump failed during the second experiment hence only results for one experiment are presented (Appendix 9); Exp- Experiment, No. - Number, SVM- Support vector machines classification; Non-afla- Non-aflatoxigenic.

4.3.3 Relative performance of different electronic nose technologies

MI as a feature selection method gave relatively higher percent best classification rates when compared to the Fox 3000 and Cyranose 320 instrument associated data reduction methods and Q-values for DiagNose across the different class comparisons. Additionally relatively fewer features were used with MI compared to the instrument associated data reduction methods and Q-values, resulting in reduced computation time and effort. The results demonstrate that MI could be a superior method for selecting useful features for discrimination of control and aflatoxin contaminated samples. As a result MI was adopted as the feature selection method for this study.

The potential for Fox 3000, Cyranose 320 and DiagNose to detect aflatoxin contamination in maize was evaluated by the ability of the systems to discriminate between the different sample classes. MI was calculated between individual features and the respective classes to determine the sensor and time indices that contained discriminating information related to the classes. Results for MI between the classes are presented as a heat map showing the sensor responses to the different samples. Cells with high MI are represented by many colour gradations from dark red to cyan indicating those features that contain higher information about the different classes. MI close to or equal to zero indicate features that contain no information about the classes, represented by cells compressed into a few shades of blue.

Based on the MI values, up to 100 of the most informative features were selected. The effectiveness of features selected by leave-one out cross-validation to discriminate between the different classes of samples was evaluated two classifiers SVM and KNN. For each class comparison a significance level at 95 % confidence interval was determined based on the number of samples. Classification accuracy rates below the significance level value are

considered non-significant. The naïve classification level, which represents classification rates expected by chance, was also determined. The performance of a given classifier is the average correct classification rate obtained over the *N* fold cross-validation partitions which was plotted against the number of features for each class comparison. The number and type of misclassified samples (false positive and false negative) were investigated from the classifier that achieved the best correct classification rate.

4.3.3.1 Fox 3000

4.3.3.1.1 Fox 3000 sensor responses

Using MI the sensors contaning the most discriminating information on differences between the sample classes were identified across a number of class comparsions namely; control vs all *A. flavus* infected maize, control vs aflatoxigenic *A. flavus* infected maize, control vs non-aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize. Sensor response was higher for the control vs aflatoxigenic *A. flavus* infected maize and non- aflatoxigenic *A. flavus* infected maize in both experiments when compared to the other class comprasions (Figure 4.5). Lowest reponse was recored for the control vs all *A. flavus* infected maize in both experiments (Figure 4.5). The response of the different sensor types varied across the sample classes in the two experiments. The SnO_2 sensors contained highest information on the difference between control vs all *A. flavus* infected maize in the first and second experiment, while the CTO sensors contained highest information on difference between the non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize in both experiments (Figure 4.5). Both sensor types contained discriminating information for the control vs non-aflatoxigenic *A. flavus* infected maize in the two experiments (Figure 4.5). Variations in sensor response to the control vs aflatoxigenic *A. flavus* infected maize were recorded in the two experiments. In the first experiment the SnO_2 contained most information on the difference

between the sample classes, while in the second experiment information was contained in the CTO sensors (Figure 4.5).

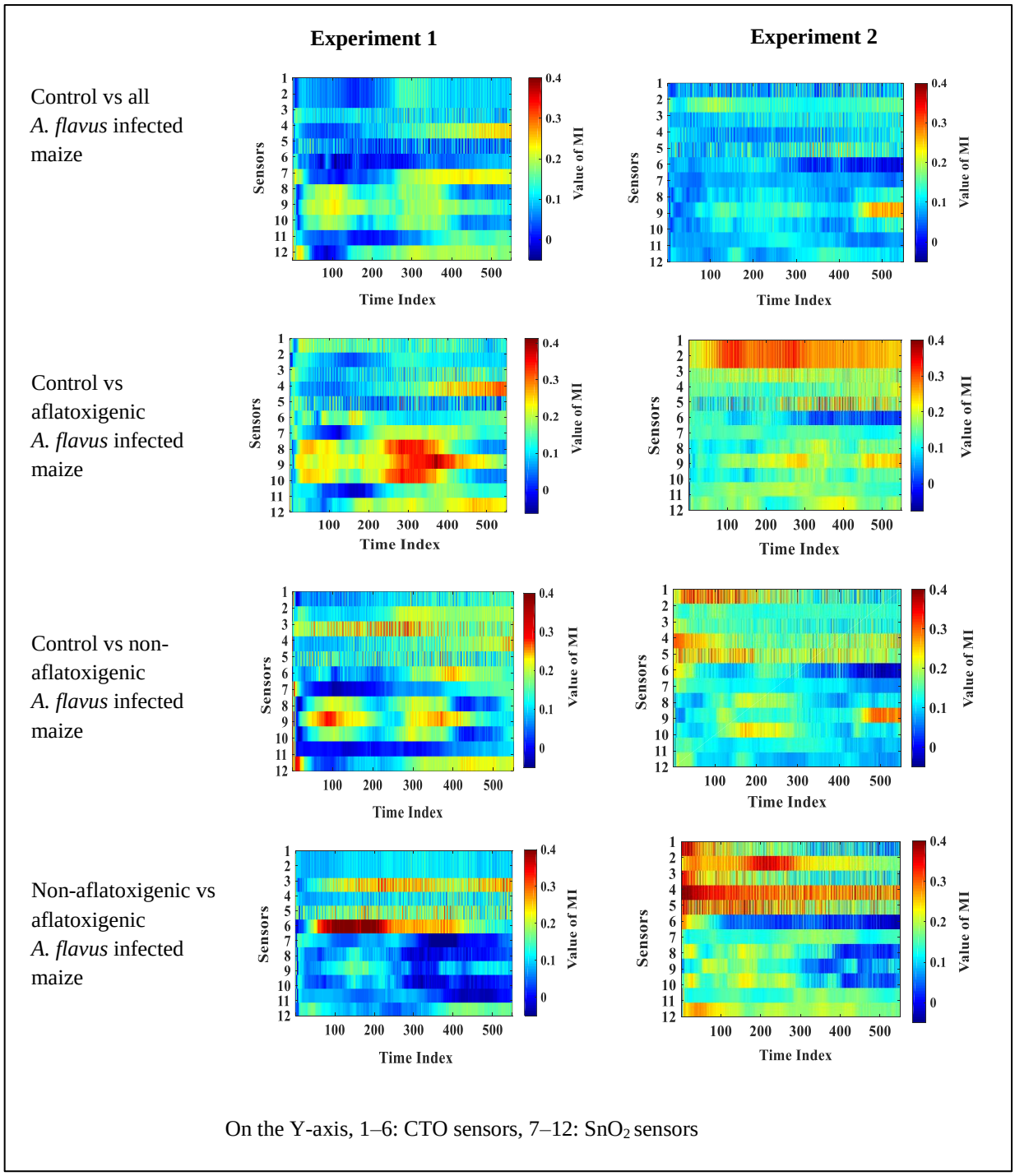


Figure 4.5: Fox 3000 relative mutual information between classes for Australian maize variety DK703w; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour.

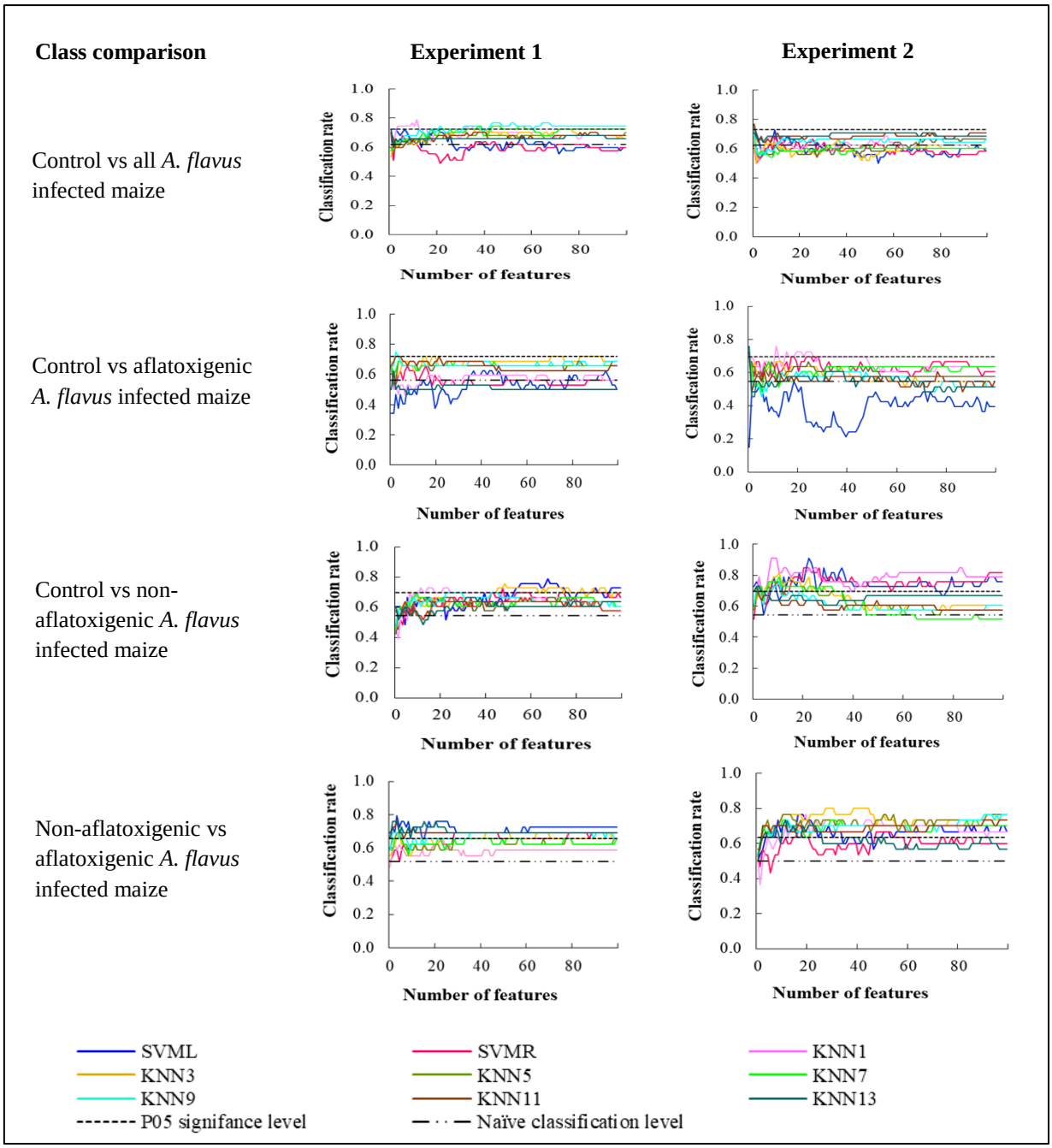
4.3.3.1.2 Fox 3000 classification accuracy

The capability of Fox 3000 to detect aflatoxin contamination was demonstrated by the ability to correctly classify different classes of samples through a number of class comparasions. Classification accuracies across the different class comparasions were higher in the second experiment (76 % to 91 %) compared to the first experiment (75 % to 79 %) (Figure 4.6, Table 4.4). Fox 3000 was able to correctly classify 75 % and 77 % of all *A. flavus* infected maize from the controls in the first and second experiment respectively (Figure 4.6, Table 4.4). Out of the nine classifiers, six achieved classification rates equal to or above the P05 significance level (72 %) in the first experiment and five out of nine classifiers achieved classification rates equal to or above the P05 significance level (73 %) in the second experiment (Figure 4.6, Table 4.4). However, the classification rates were not sustained across the different number of features, indicating a non-robust classification performance (Figure 4.6). Significant classification accuracies were also recorded for the control vs aflatoxigenic *A. flavus* infected maize in both experiments. Classification accuracies of 75 % and 76 % were recorded in the first and second experiment respectively (Figure 4.6, Table 4.4). Five out of the nine classifiers achieved classification rates equal to or above the P05 significance level (70 %) in the first experiment, while eight out of nine classifiers achieved classification rates above the P05 significance level (70 %) in the second experiment (Figure 4.6, Table 4.4). Similar to the control vs all *A. flavus* infected maize the classification accuracies were not sustained across the different number of features indicating a weak classification performance (Figure 4.6).

Fox 3000 was most effective in discriminating controls from maize infected with the non-aflatoxigenic *A. flavus* in both experiments (Figure 4.6, Table 4.4). Fox 3000 was able to classify 76 % and 91 % of the samples correctly in the first and second experiment respectively. In both cases the classification rate was sustained across the the different number of features

which indicates a robust classification performance (Figure 4.6, Table 4.4). In the first experiment, six out of the nine classifiers evaluated achieved classification rates equal to or greater than the P05 significance level (70 %), while in the second experiments all classifiers achieved classification rates above the P05 significance level (70 %) (Figure 4.6, Table 4.4). A similar pattern was recorded for non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize. Fox was able to correctly classify 79 % and 80 % of the samples in the first and second experiment respectively. In the first experiment eight out of the nine classifiers achieved classification rates above the P05 significance level (66 %), while in the second experiment all classifiers achieved classification rates above the P05 significance level (63 %) (Figure 4.6, Table 4.4).

KNN exhibited the best classification rates for most of the class comparisons in both experiments except for control vs non-aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize in the first experiment which were achieved by SVM (Table 4.4). The number of feature sets used to achieve the best classification rates ranged from 1 to 64 across the different class comparisons (Table 4.4).



SVML- Support vector machines - linear function; SVMR- Support vector machines - radial function; KNN - *k*-nearest neighbour with different values of n; n= 1, 3,5,7 9,11,13; Classification rate above or equal to the P05 significance level is significant; Naïve classification level represents classification by random chance.

Figure 4.6: Fox 3000 cross-validated classification accuracy versus number of features using up to 100 of the most informative features selected by relative mutual information for Australian maize variety DK703w; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour.

Table 4.4: Summary of results for Fox 3000 analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Exp	Class comparisons	Best correct classification (%)	P05 significance level (%)	Naïve classification (%)	No. classifiers ≥ P05	Best classifier	Most responsive sensors
Exp 1	Control vs all *A. flavus*	79	72	62	6/9	KNN1	SnO_2
	Control vs aflatoxigenic	75	70	56	3/9	KNN9	SnO_2
	Control vs non-afla	76	70	55	6/9	SVML	SnO_2 & CTO
	Non-afla vs aflatoxigenic	79	66	52	8/9	SVML	CTO
Exp 2	Control vs all *A. flavus*	77	73	63	5/9	KNN7	SnO_2
	Control vs aflatoxigenic	76	70	55	8/9	KNN1 KNN13	CTO
	Control vs non-afla	91	70	55	9/9	KNN1 SVML	SnO_2 & CTO
	Non-afla vs aflatoxigenic	80	63	50	9/9	KNN3	CTO

Exp – Experiment; Non-afla- Non-aflatoxigenic; No. Number; Best correct classification (%)-Highest classification rate recorded; P05 significance level (%) - significance level at 95% confidence level; Naïve classification- classification by random chance based on number of samples in each class; Number classifiers ≥ P05 significance- Number of classifiers out of the total nine that achieved classification rates equal to or greater than the P05 significance level; Best classifier- Classifier that achieved the highest classification rate, SVML- Support vector machines linear function; KNN - *k*-nearest neighbour with different values of n; n= 1, 3, 9, 11, 13; Number of features- Number of features used to achieve the highest classification rate; Most responsive sensors- Sensors from which features that achieved best classification rates were selected; SnO_2- tin oxide sensors; CTO- chromium titanium oxide sensors.

4.3.3.1.3 Fox 3000 number and type of misclassified samples

Apart from the classification accuracy the number and type of misclassified samples are important in determining the applicability of the technique. This represents the percentage of predictions that are correctly classified out of the total presented to the system. The Fox 3000 was able to classify the majority of samples across the different class comparisons correctly. Misclassification rates were relatively similar between the two experiments and ranged from 9 % to 25 % across the different class comparisons. Lowest misclassification was recorded for control vs non-aflatoxigenic *A. flavus* infected maize in the second experiment where only three samples out of 33 were misclassified. The control vs aflatoxigenic *A. flavus* infected samples were the most difficult to classify and recorded the highest misclassification rates in the two experiments. The number of false positives was higher compared to the false negatives across the different comparisons in the two experiments (Table 4.5). In practice, the high proportion of false positives relative to false negatives indicate that, Fox 3000 when used as a screening technique for detection of aflatoxin contamination of maize would classify more of the uncontaminated samples as contaminated versus contaminated samples as non-contaminated. Therefore few genuinely contaminated samples could be released for consumption which is less detrimental, as opposed to a high proportion of false negatives which is less safe as more contaminated samples would be released for consumption.

Table 4.5: Misclassification errors for Fox 3000 analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Experiment	Class comparison	Misclassification (%)	Misclassified / Total samples	Misclassification type	
				False +ve	False -ve
Experiment 1	Control vs all *A. flavus*	21	10/47	6/47	4/47
	Control vs aflatoxigenic	25	8 /32	4/32	4/32
	Control vs non-aflatoxigenic	24	8/33	4/33	4/33
	Non-aflatoxigenic vs aflatoxigenic	21	6/29	4/29	2/29
Total				**18/141**	**14/141**
Experiment 2	Control vs all *A. flavus*	23	11/48	8/48	3/48
	Control vs aflatoxigenic	24	8/33	5/33	3/33
	Control vs non-aflatoxigenic	9	3/33	2/33	1/33
	Non-aflatoxigenic vs aflatoxigenic	20	6/30	3/30	3/30
Total				**18/144**	**10/144**

False +ve - Number of false positive samples (control samples classified as either *A. flavus* infected, aflatoxigenic or non-aflatoxigenic); False –ve- Number of false negative samples (*A. flavus* infected, aflatoxigenic and non-aflatoxigenic samples classified as control); In case of non-aflatoxigenic vs. aflatoxigenic: False +ve – Non-aflatoxigenic sample classified as aflatoxigenic; False -ve-aflatoxigenic sample classified as non-aflatoxigenic.

4.3.3.2 Cyranose 320

4.3.3.2.1 Sensor responses

The Cyranose 320 is portable system consisting of 32 individual polymer conducting sensors blended with carbon black composite, configured as an array. Sensor response was higher in the first experiment compared to the second experiment across all the pairwise comparisons (Figure 4.7). The value of MI was higher for the most responsive sensors in the first experiment compared to the second experiment and ranged from 0.2 to 0.3 as shown by cells with colour gradations from dark red to cyan (Figure 4.7). In the second experiment the value of MI for most responsive sensors ranged from 0.1 to 0.2 as shown by cells compressed into a few shades of blue (Figure 4.7). The cause of difference in sensor response between the two experiments could not be established, but can presumably be attributed to lower concentration of VOCs in the second experiment samples relative to the first experiment samples. Cyranose sensors were most responsive to the control vs non-aflatoxigenic *A. flavus* infected maize and the non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize in the first experiment (Figure 4.7). Information on the differences between the control and maize infected with non-aflatoxigenic *A. flavus* was contained mainly in sensors 1, 4, 12, 13, 15, 23 and 30. Information on the differences between the non-aflatoxigenic and aflatoxigenic *A. flavus* infected maize was contained in sensors 1, 6 15 and 23 (Figure 4.7). Lowest sensor response in the first experiment was recorded for the control vs all *A. flavus* infected maize (Figure 4.7).

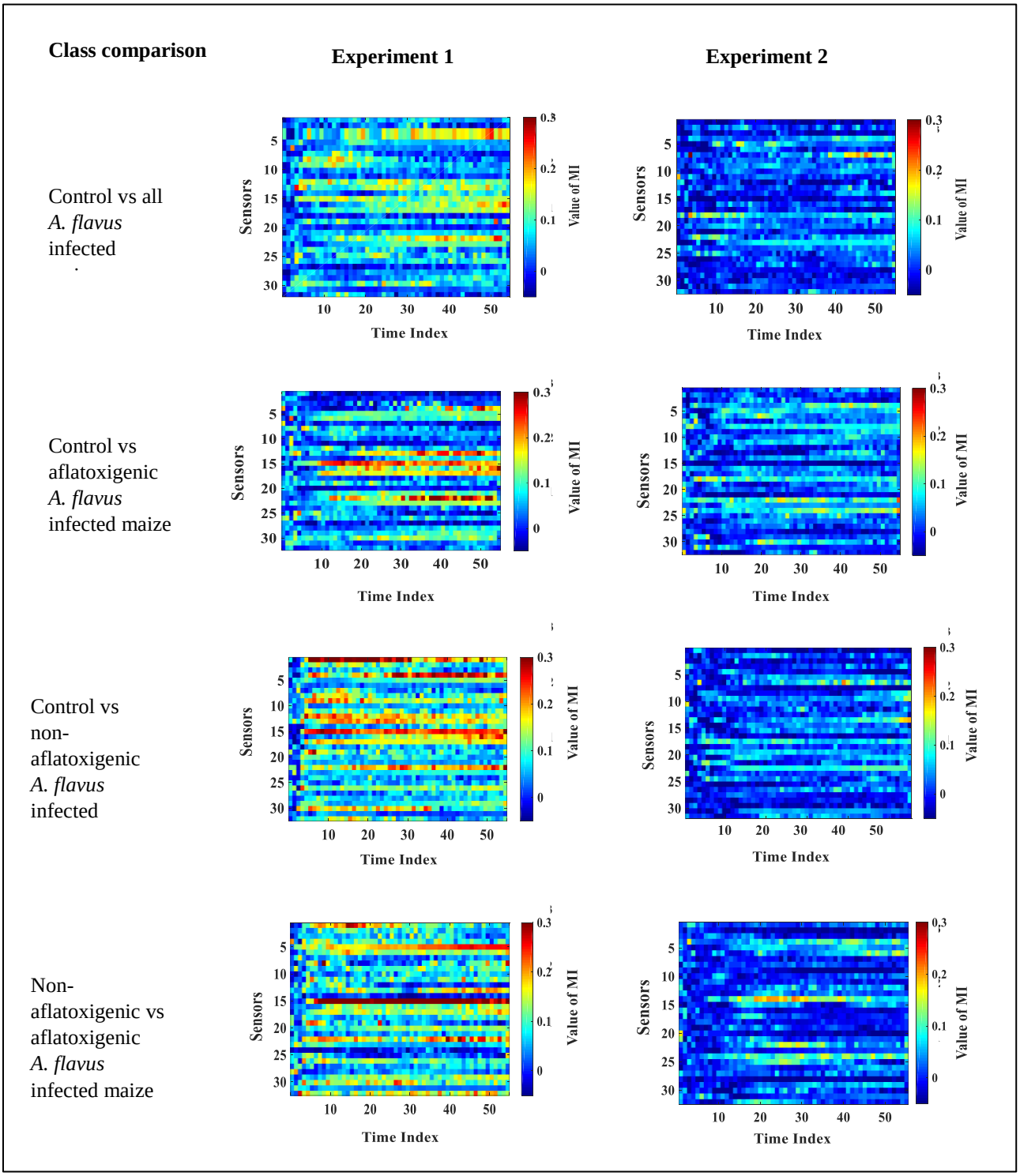


Figure 4.7: Cyranose 320 relative mutual information between classes for Australian maize variety DK703w; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour.

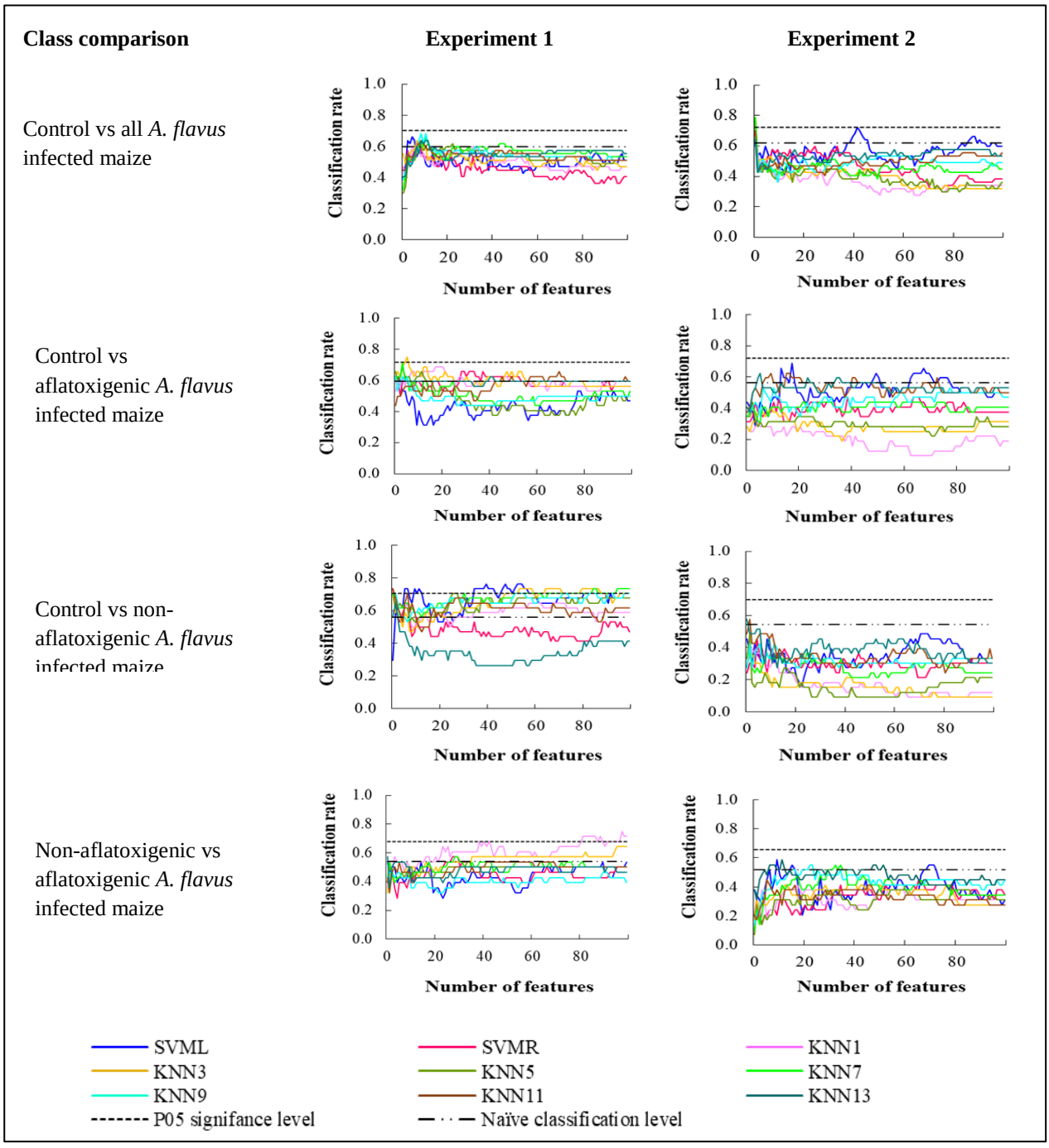
4.3.3.2.2 Classification accuracy

The capability of Cyranose 320 to detect aflatoxin contamination varied widely between the two experiments across the different class comparisons as shown by the variations in percent correct classification rates (Figure 4.8, Table 4.6). In the first experiment percent correct classification rates ranged from 68 % to 76 % for the different class comparisons, where three out of the four class comparison had classification rates above the P05 significant level. In the second experiment classification rates ranged from 58 % to 79 % with only one class out of four with classification rate above the P05 significance level (Figure 4.8, Table 4.6). The difference in classification accuracies could be attributed to the lower sensor response in the second experiment as previously mentioned above in section 4.3.3.2.1. Classification accuracy for the control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize which represent the actual contaminated samples ranged from 68 % to 79 % in the two experiments (Figure 4.8, Table 4.6). In practice the results indicate that Cyranose 320 as a diagnostic technique for aflatoxins would correctly detect contamination in 79 out of 100 samples in the best case scenario and 68 out of 100 samples in the worst case scenario.

In the first experiment, a non-significant classification accuracy of 68 % was recorded for the control vs all *A. flavus* infected maize. All classifiers tested achieved classification rates below the P05 significance level (70 %) (Figure 4.8, Table 4.6). Cyranose was able to correctly classify 75 % of maize samples infected with aflatoxigenic *A. flavus* from the controls. The classification accuracy was significant with two out of the nine classifiers achieving classification rates equal to or above the P05 significance level (72 %). The classification rates were however, not sustained across the different number of features, indicating a non-robust classification performance (Figure 4.8, Table 4.6).

Significant classification accuracies were also recorded for the control vs non-aflatoxigenic *A. flavus* infected maize at 76 % and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize at 75 % in the first experiment (Figure 4.8, Table 4.6,). For the control vs non-aflatoxigenic *A. flavus* infected maize seven out of nine classifiers achieved classification rates equal to or greater than the P05 significance level (71 %), while for non-aflatoxigenic vs aflatoxigenic *A. flavus* only one classifier achieved classification rate above the P05 significance level (68 %). For both comparisons the classification rates were not sustained across the different number of features, indicating a weak classification performance (Figure 4.8, Table 4.6).

In the second experiment, Cyranose 320 was most effective in discriminating controls from all *A. flavus* infected maize with a significant classification accuracy of 79 %. Five out of nine classifiers achieved classification rates above or equal to the P05 significance level (72 %). The classification rate was however not sustained across the different number of features, indicating a non-robust performance (Figure 4.8, Table 4.6). Classification accuracies for control vs aflatoxigenic *A. flavus* infected maize (69 %), control vs non-aflatoxigenic *A. flavus* infected maize (58 %), and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize (59 %) were below the P05 significance level (72 %, 70 % and 68 % respectively) therefore non-significant (Figure 4.8, Table 4.6). A higher number of correct classifications for the different pairwise comparisons were achieved with KNN compared to SVM (Table 4.6). The number of feature sets used to achieve the best classification rates varied across the different pairwise comparisons and ranged from 1 to 98 (Table 4.6).



SVML- Support vector machines- linear function; SVMR- Support vector machines- radial function , KNN - *k*-nearest neighbour with different values of n; n= 1, 3,5,7, 9,11,13; Classification rate above or equal to the P05 significance level is significant; Naïve classification level represents classification by random chance.

Figure 4.8: Cyranose 320 cross-validated classification accuracy versus number of features using up to 100 of the most informative features selected by relative mutual information for Australian maize variety DK703w; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour.

Table 4.6: Summary of results for Cyranose 320 analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Exp	Class comparisons	Best correct classification (%)	P05 significance level (%)	Naïve classification (%)	No. of classifiers ≥P05	Best classifier	No. of features
Exp1	Control vs all *A. flavus*	68	70	60	0/9	KNN9	11
	Control vs aflatoxigenic	75	72	59	2/9	KNN3	61
	Control vs non-afla	76	71	56	7/9	SVML	55
	Non-afla vs aflatoxigenic	75	68	54	1/9	KNN1	98
Exp 2	Control vs all *A. flavus*	79	72	62	5/9	KNN3	1
	Control vs. aflatoxigenic	69	72	56	0/9	SVML	18
	Control vs. Non-afla	58	70	55	0/9	KNN11	2
	Non-afla vs. aflatoxigenic	59	68	52	0/9	SVML	10

Exp – Experiment; Non-afla- Non-aflatoxigenic; No. Number; Best correct classification (%)-Highest classification rate recorded; P05 significance level (%) - significance level at 95 % confidence level; Naïve classification- classification by random chance based on number of samples in each class; Number classifiers ≥ P05 significance- Number of classifiers out of the total nine that achieved classification rates equal to or greater than the P05 significance level; Best classifier- Classifier that achieved the highest classification rate, SVML- Support vector machines linear function; KNN - *k*-nearest neighbour with different values of n; n= 1, 3, 9, 11, 13; Number of features- Number of features used to achieve the highest classification rate; Most responsive sensors- Sensors from which features that achieved best classification rates were selected.

4.3.3.2.3 Cyranose 320 number and type of misclassified samples

As previously mentioned the number and type of misclassified samples, in addition to the classification accuracy are important in determining the applicability of a diagnostic technique. The number of samples misclassified by Cyranose 320 varied among the different class comparison and between the two experiments. In the first experiment misclassification rates ranged from 24 % to 32 % across the different class comparisons, while in the second experiment they ranged from 21 % to 42 % (Table 4.7). The highest number of misclassified samples was recorded for the control vs aflatoxigenic *A. flavus* infected maize and the non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize in the second experiment where more than 40 % of the total samples were incorrectly classified. The lowest misclassification rate was recorded for the control vs all *A. flavus* infected maize in the second experiment where ten out of 47 samples were misclassified (Table 4.7). The number of false positives was higher than the false negative across the different class comparisons in the two experiments, except for the control vs aflatoxigenic *A. flavus* infected maize in the first experiment and control vs non-aflatoxigenic *A. flavus* infected maize in the second experiment where the number of false negatives was higher than false positives (Table 4.7). The preference for a diagnostic technique is to be able to classify 100 % of the samples correctly. However, since this is rarely the case, to minimize the risk of releasing contaminated samples for consumption, the preference as mentioned earlier is to have a higher proportion of false positives relative to the false negatives as was the case with Cyranose 320.

Table 4.7: Misclassification errors for Cyranose 320 analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Experiment	Class comparison	Misclassification (%)	Misclassified/Total samples	Misclassification type	
				False +ve	**False –ve**
Experiment 1	Control vs all *A. flavus*	32	15/47	11/47	4/47
	Control vs aflatoxigenic	25	8/32	3/32	5/32
	Control vs non-aflatoxigenic	24	8/34	4/34	4/34
	Non-aflatoxigenic vs aflatoxigenic	25	7/28	4/28	3/28
Total				**22/141**	**16/141**
Experiment 2	Control vs all *A. flavus* infected	21	10/47	6/47	4
	Control vs aflatoxigenic	31	10/32	6/32	4
	Control vs non-aflatoxigenic	42	14/33	6/33	8
	Non-aflatoxigenic vs aflatoxigenic	41	12/29	6/29	6
Total				**24/141**	**22/141**

False +ve - Number of false positive samples (Control samples classified as either *A. flavus* infected, aflatoxigenic or non-aflatoxigenic); False –ve- Number of false negative samples (*A. flavus* infected, aflatoxigenic and non-aflatoxigenic samples classified as control). In case of non-aflatoxigenic vs. aflatoxigenic: False +ve – Non-aflatoxigenic sample classified as aflatoxigenic; False -ve-aflatoxigenic sample classified as non-aflatoxigenic.

4.3.3.3 DiagNose

Potential for DiagNose to detect aflatoxin contamination in maize variety DK703W artificially inoculated with *A. flavus* was evaluated in two experiments as mentioned earlier in section 4.2.2. However, the instrument pump failed during the second experiment hence only results for one experiment are presented (Appendix 9).

4.3.3.3.1 DiagNose sensor response

The DiagNose instrument deploys of an array of 12 *n* type metal oxide sensors that comprise tin dioxide sensors doped with palladium (SnO_2-Pd), platinum (SnO_2-Pt), silver (SnO_2-Ag), copper (SnO_2-Cu), undoped tungsten trioxide (WO_3) sensors and an experimental sensor (Extype 1) with composition undisclosed by the manufacturer. The sensor response varied across the different sample class comaprasions (Figure 4.9). Highest response was observed for the control vs non-aflatoxigenic *A. flavus* infected maize with MI value of 0.3 to 0.4 for the most responsive sensors as shown by cells with colour gradations from dark red to cyan (Figure 4.9). Information on the difference between the two class of samples was mainly contained in SnO_2-Pd (sensor 1 and 3) and SnO_2-Pt (sensor 8) sensors (Figure 4.9). The DiagNose sensors were also resposive to the control vs aflatoxigenic *A. flavus* infected maize with an MI value of 0.2 to 0.3 for the responsive sensors as shown by cells with different colour gradations of light to dark red (Figure 4.9). Information on the difference between the control and maize infected with aflatoxigenc *A. flavus* was contained in both SnO_2 (SnO_2- Pd- sensor 1 and 12, SnO_2- Pt- sensor 8, SnO_2- Ag- sensor 11) and WO_3 sensors (sensor 6 and 9) (Figure 4.9). Lowest sensor response was recorded for the non-aflatoxigenic vs aflatoxigenic infected maize with an MI value of 0.1 to 0.3 for the responsive sensors as shown by cells with different colour gradations of yellow to light red (Figure 4.9).

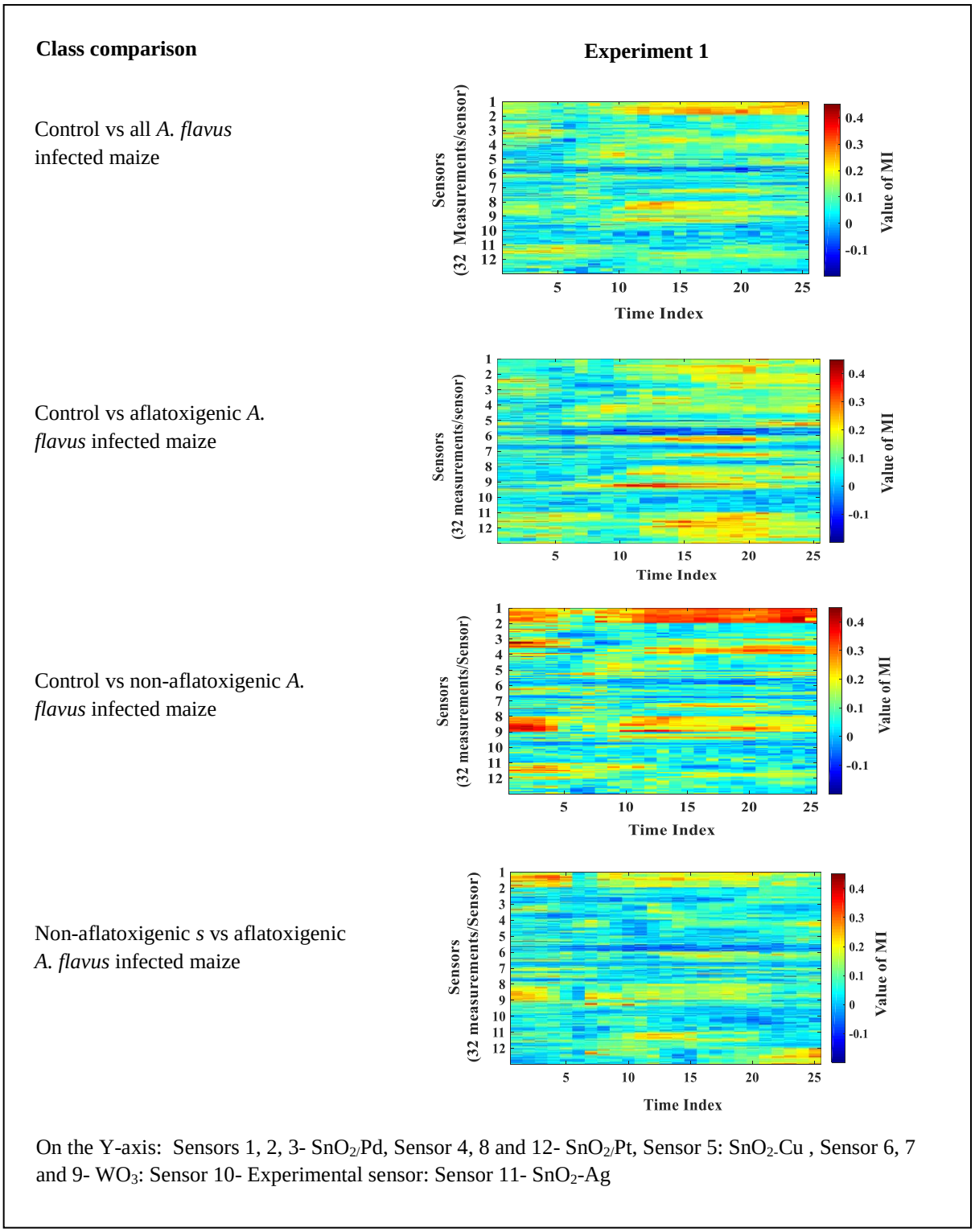
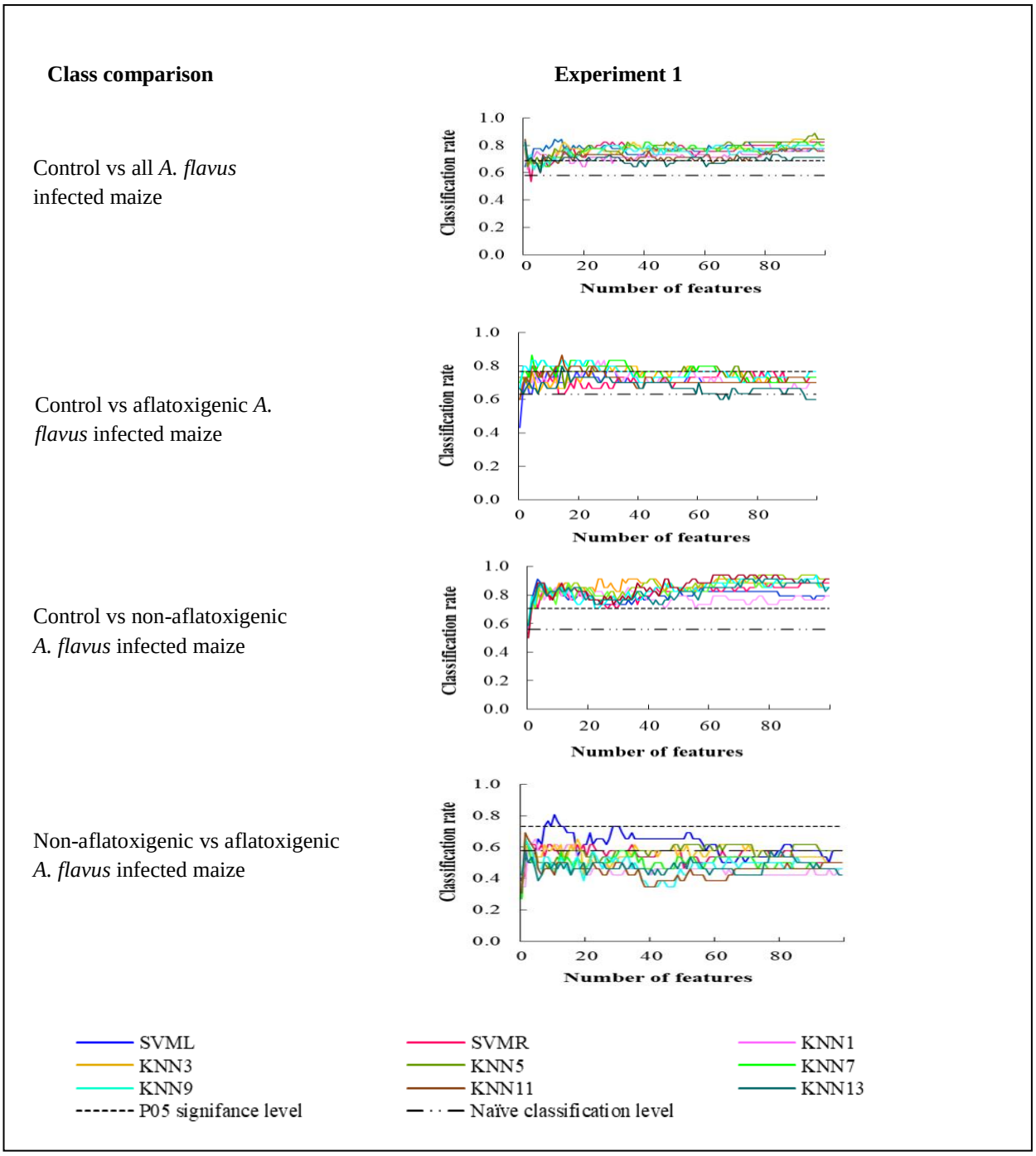


Figure 4.9: DiagNose relative mutual information between classes for Australian maize variety DK703w; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour.

4.3.3.3.2 DiagNose classification accuracy

The results indicate the potential for DiagNose to detect aflatoxin contamination in maize samples artificially inoculated with *A. flavus*. Classification accuracies ranged from 81 % to 94 % across the different class comparisons. Three out of the four class comparisons namely control vs all *A. flavus* infected maize, control vs aflatoxigenic *A. flavus* infected maize and control vs non-aflatoxigenic *A. flavus* infected maize had classifications accuracies above the P05 significance level for all the classifiers tested (Figure 4.10, Table 4.8). The classification rates for these comparisons were sustained across the different number of features indicating robust classification performances (Figure 4.10). Significant classification accuracy was achieved for the control vs all *A. flavus* infected maize (89 %) and the control vs aflatoxigenic *A. flavus* infected maize (87 %), which represents the actual situation practice. All classifiers achieved classification rates equal to or greater than the P05 significance level (77 %). In practice, results indicate potential for DiagNose, if deployed as diagnostic technique, to correctly detect aflatoxin contamination in 89 out of 100 samples at best performance and 87 out of 100 samples at the worst performance. DiagNose was most effective in discriminating between the controls and maize samples infected with non-aflatoxigenic *A. flavus* (91 %) and least effective in discriminating the non-aflatoxigenic vs aflatoxigenic *A. flavus* infected samples. For the non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize a significant classification accuracy of 81 % was achieved. However, only one out of the nine classifiers achieved classification accuracies equal to or greater than the P05 significance level (73 %). The classification rates were also not sustained across the different number of features indicating a weak classification performance (Figure 4.10, Table 4.8). KNN achieved the best classification rates for three out of the four class comparisons. The number of features used to achieve the best classification rates ranged from 11 to 97 across the different class comparisons (Table 4.8).



SVML- Support vector machines- linear function; SVMR- Support vector machines- radial function; KNN - *k*-nearest neighbour with different values of n; n= 1, 3,5,7 9,11,13; Classification rate above or equal to the P05 significance level is significant; Naïve classification level represents classification by random chance.

Figure 4.10: DiagNose cross-validated classification accuracy versus number of features using up to 100 of the most informative features selected by relative mutual information for Australian maize variety DK703w; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour.

Table 4.8: Summary of results for DiagNose analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Exp	Class comparisons	Best correct classification (%)	P05 significance level (%)	Naïve classification (%)	No. of classifiers ≥P05	Best classifier	No. of feature sets	Most responsive sensors
Exp 1	Control vs all *A. flavus*	89	69	58	9/9	KNN5	97	SnO_2
	Control vs aflatoxigenic	87	77	63	9/9	KNN7 &11	15	SnO_2 & WO_3
	Control vs non-afla	94	71	56	9/9	KNN5,9 & 11	96	SnO_2
	Non-afla vs aflatoxigenic	81	73	58	1/9	SVML	11	SnO_2

Exp – Experiment; Non-afla- Non-aflatoxigenic; No. Number; Best correct classification (%)-Highest classification rate recorded**;** P05 significance level (%) - significance level at 95 % confidence level. Naïve classification- classification by random chance based on number of samples in each class ; Number classifiers ≥ P05 significance- Number of classifiers out of the total nine that achieved classification rates equal to or greater than the P05 significance level; Best classifier- Classifier that achieved the highest classification rate, SVML- Support vector machines-linear function, KNN - *k*-nearest neighbour with different values of n; n= 1, 3, 9, 11, 13; Number of features- Number of features used to achieve the highest classification rate; Most responsive sensors- Sensors from which features that achieved best classification rates were selected; SnO_2- tin dioxide sensors; WO_3- Tungsten trioxide sensors.

4.3.3.3.3 DiagNose number and type of misclassified samples

Misclassification rates for DiagNose analysis of maize variety DK703w ranged from 6 % to 19 % (Table 4.9). Lowest misclassification was recorded for the control vs non-aflatoxigenic samples where only two out 34 samples were misclassified. Highest misclassification was recorded for the non-aflatoxigenic vs aflatoxigenic samples (Table 4.9). The number of false positives was higher relative to the false negatives across the different class comparisons indicating that, the DiagNose when used as an initial screening technique for aflatoxin detection would classify more of the non-contaminated samples as contaminated versus contaminated samples as non-contaminated with the latter being more detrimental (Table 4.9).

Table 4.9: Misclassification errors for DiagNose analysis of maize flour for variety DK703w samples inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Experiment	Class comparison	Misclassification (%)	Misclassified/Total samples	Misclassification type	
				False +ve	False –ve
Experiment 1	Control vs all *A. flavus*	11	5/45	3/45	2/45
	Control vs aflatoxigenic	13	4/30	2/30	2/30
	Control vs non-afla	6	2/34	2/34	0/34
	Non-afla vs. aflatoxigenic	19	5/26	2/26	3/26
Total				**9/135**	**7/135**

Non-afla- Non-aflatoxigenic; False +ve - Number of false positive samples (Control samples classified as either *A. flavus* infected, aflatoxigenic or non-aflatoxigenic); False –ve- Number of false negative samples (*A. flavus* infected, aflatoxigenic and non-Aflatoxigenic samples classified as control). In case of non-aflatoxigenic vs aflatoxigenic: False +ve – Non-aflatoxigenic sample classified as aflatoxigenic; False -ve-aflatoxigenic sample classified as non-aflatoxigenic.

4.3.3.4 Summary of the relative performance of the three electronic nose technologies

The summary presents a comparison of performance of the three electronic nose technologies based on the best classification performance achieved in the two experiments for Fox 3000 and Cyranose 320 and one experiment for DiagNose across the different sample class comparisons. The capability to detect aflatoxin contamination varied across the different electronic nose technologies. Classification accuracies ranged from 75 % to 91 % for Fox 3000, 58 % to 79 % for Cyranose 320 and 81 % to 94 % for DiagNose across the different pairwise comparisons (Table 4.10). For the control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize which represents the contaminated samples in practice, best classification accuracies ranged from 75 % to 79 % for Fox 3000, 68 % to 79 % for Cyranose 320 and 87 % to 89 % for DiagNose (Table 4.10). In practice the results imply that in the best case scenario, Fox 3000 and Cyranose 320 would correctly detect aflatoxin contamination in 79 out of 100 samples, while DiagNose would correctly detect contamination in 89 out of 100 samples. In the worst case scenario the Fox 3000 would correctly detect contamination in 75 out of 100 samples, Cyranose 320, 68 out of 100 samples and DiagNose 87 out of 100 samples. Therefore based on the current results, DiagNose was the most effective in discriminating aflatoxin contaminated samples from uncontaminated samples, followed by Fox 3000, while Cyranose 320 was the least effective. In addition to the classification accuracies, the classification performance for DiagNose was more robust compared to Fox 3000 and Cyranose 320. For DiagNose three out of the four class comparisons had all classifier tested achieve classification accuracies equal to or above the P05 significance level (Table 4.10). The classification rates for these three comparisons were also sustained across the different number of features (Figure 4.9). Though the classification accuracies for Fox 3000 were lower than DiagNose, they were all statistically significant and consistent across the two experiments (Table 4.10). Classification accuracies for Cyranose 320 were inconsistent across the two

experiments. In the first experiment, significant classification accuracies were recorded for three out the four class comparisons, while in the second experiment only one out of four class comparisons achieved significant classification accuracy (Table 4.10). For all the three technologies the number of false positives was higher than the false negatives indicating that, the electronic noses when used as an initial screening technique for aflatoxin detection would classify more of the uncontaminated samples as contaminated which is less detrimental as compared to classification of contaminated samples as uncontaminated. The latter would result in release of contaminated samples for consumption (Table 4.5, 4.7 and 4.9).

Table 4.10: Summary of best classification performance for Fox 3000, Cyranose 320 and DiagNose analysis of maize flour for variety DK703w samples inoculated with 2% Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

E-nose technology	Class comparison	Experiment 1			Experiment 2		
		Best correct classification (%)	P05 significance level (%)	No. of classifiers ≥P05	Best correct classification (%)	P05 significance level (%)	No. of classifiers ≥P05
Fox 3000	Control vs all *A. flavus*	79	72	6/9	77	73	5/9
	Control vs aflatoxigenic	75	70	3/9	76	70	8/9
	Control vs non-afla	76	70	6/9	91	70	9/9
	Non-afla vs aflatoxigenic	79	66	8/9	80	63	9/9
Cyranose	Control vs all *A. flavus*	68	70	0/9	79	72	5/9
	Control vs aflatoxigenic	75	72	2/9	69	72	0/9
	Control vs non-afla	76	71	7/9	58	70	0/9
	Non-afla vs aflatoxigenic	75	68	1/9	59	68	0/9
Diagnose	Control vs all *A. flavus*	89	69	9/9	**	**	**
	Control vs aflatoxigenic	87	77	9/9	**	**	**
	Control vs non-afla	94	71	9/9	**	**	**
	Non-afla vs aflatoxigenic	81	73	1/9	**	**	**

**-Represents missing results for DiagNose experiment 2 (For DiagNose the instrument pump failed during the second experiment hence only results for one experiment are presented-Appendix 9); Non-afla- Non-aflatoxigenic; No. Number; Best correct classification (%)-Highest classification rate recorded for the class comparison across the two experiments; P05 significance level (%) - significance level at 95 % confidence level; Number classifiers ≥ P05 significance- Number of classifiers out of the total nine that achieved classification rates equal to or greater than the P05 significance level.

4.4 DISCUSSION

The capabilities of three electronic nose technologies namely, metal-oxide semiconductor sensors (Fox 3000), conducting polymer sensors (Cyranose 320) and metal-oxide semiconductor sensors with thermocycling (DiagNose) in detecting aflatoxin contamination in maize using artificially inoculated samples have been compared. The objective was to determine the most appropriate sensor technology for discriminating between controls and maize artificially contaminated with aflatoxin, to be used for further investigations with maize samples naturally infected with *A. flavus* under field conditions.

To compare performance of the different electronic nose technologies, the choice of an appropriate feature selection method for identification of useful features for discriminating controls from aflatoxin contaminated samples was crucial. The capability of the electronic nose instrument associated data reduction methods and MI to select features that discriminate between control and aflatoxin contaminated samples was compared. Classification success improved from 54 % to 75 % without MI to 76 % to 91 % for Fox 3000 across the different class comparisons. For Cyranose 320 classification performance improved from 32 % to 48 % without MI to 58 % to 76 % with MI across the different class comparisons, while for DiagNose it improved from 83 % to 84 % without MI to 80 % to 91 % with MI (Tables 4.1, 4.2 and 4.3). Additionally relatively fewer numbers of features were used with MI when compared to the instrument associated data reduction methods. Similar observations were recorded by Wang *et al.* (2014) while evaluating the performance of MI as an efficient filter approach in feature selection for classifying chemicals using an array of metal oxide sensors in comparison to a wrapper approach. Features selected with MI gave consistently better classification performances than randomly selected features across the classifiers tested and with up to three times less computational effort.

Due to the high dimensionality of electronic nose data, use of both supervised and unsupervised classification methods is not only time consuming but also results in high classification errors as observed in the instrument associated data reduction methods. The high classification errors could be attributed to contributions of redundant and irrelevant sensors/features that may contain incomplete and inconsistent information which degrades the classification accuracy. MI on other hand ranks features according to their predictive power based on the feature's relevance and non-redundancy (Bennasar *et al.*, 2015). MI based feature selection aims at selecting few features with high relevance but low redundancy which could explain the higher classification rates achieved. MI selects features on the basis of unique and synergistic information they contain with regard to the target classes. A new feature is selected only if contains information about the classes that has not been captured by the preceding features.

The three electronic nose technologies evaluated were able to discriminate between the controls and samples inoculated with aflatoxigenic and non-aflatoxigenic *A. flavus* isolates with varying accuracies. The metal oxide based instruments (Fox 3000 and DiagNose) were more effective in discriminating the control and aflatoxin contaminated samples when compared to the conducting polymer based instrument (Cyranose 320). Classification accuracies ranged from 75 % to 91 % for Fox 3000, 81 % to 94 % for DiagNose and 58 % to 79 % for Cyranose 320 across the different class comparisons (Table 4.10). Among the three platforms the higher classification accuracies were recorded for DiagNose, followed by Fox 3000, then Cyranose 320 across the different class comparisons. The ability of the electronic nose instruments to classify the different sample types as represented by the class comparisons differed. Highest classification accuracy for Fox 3000 and DiagNose was recorded for the control vs non-aflatoxigenic *A. flavus* infected maize at 91 % and 94 % respectively, while for Cyranose 320 it was recorded for the control vs all *A. flavus* infected maize at 79 %. Most notable was the

ability of the three instruments to discriminate between samples inoculated with the aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. The higher classification accuracy achieved for the control vs non-aflatoxigenic *A. flavus* infected maize could be attributed to prolific growth of the non-aflatoxigenic *A. flavus* isolates compared to the aflatoxigenic isolates, hence higher concentration of VOCs in the sample head space as previously documented by Sun *et al.* (2014).

In a practical sense the most important samples to discriminate would be the control vs all *A. flavus* infected maize and the control vs aflatoxigenic *A. flavus* infected maize. The class comparisons are a representation of the actual situation, where maize is either contaminated or uncontaminated with aflatoxins. In this study successful discrimination of the control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize was demonstrated with all three instruments. Classification accuracies ranged from 75 % to 79 % for Fox 3000, 68 % to 79 % for Cyranose and 87 % to 89 % for DiagNose (Table 4.10). The results are consistent with those reported by other authors, who used maize and other cereals contaminated with different mycotoxins. Falasconi *et al.* (2005) reported 94 % accuracy for classification of contaminated and non-contaminated cracked maize grains inoculated with fumonisin producing and non-producing strains of *F. verticillioides* using metal oxide based electronic nose. Gobbi *et al.* (2011), demonstrated the capability of an electronic nose equipped with six metal oxide sensors to discriminate between samples of maize inoculated with different strains of *Fusarium* based on their fumonisin content. Balasubramanian *et al.* (2007) recorded up to 86 % accuracy for classification of barley samples that were clean, naturally or artificially infected with *Fusarium* using a conducting polymer-based electronic nose system.

Apart from the classification accuracies, the type of misclassified samples plays an important role in determining the applicability of a diagnostic technique. The desired outcome of a diagnostic technique is the capability to discriminate the different classes of samples with 100 % accuracy. However since this is rarely the case, the preference is to have a higher proportion of false positive samples than false negatives samples. This minimizes the chances for contaminated samples being released for consumption. In this study the number of false positives was higher compared to the false negatives for all three electronic nose instruments. This implies that if electronic noses are deployed as diagnostic techniques for initial screening of maize for aflatoxin, the likelihood of uncontaminated samples being classified as contaminated would be less than contaminated samples classified as uncontaminated, with the latter being more detrimental.

Detection of mycotoxin contamination by electronic nose is based on changes in the composition of VOCs produced by mycotoxigenic fungi during their growth and biochemical processes. The volatiles are potential taxonomic markers used to differentiate fungal species as well as contaminated and un-contaminated produce (Keshri and Magan, 2000; Magan and Evans, 2000). VOCs occurring as mixtures of hydrocarbons, heterocyclics, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives have been associated with mycotoxigenic fungi (Korpi *et al.*, 2009). The ability of an electronic system to detect aflatoxin contamination is therefore dependent on the sensitivity of the sensors to the various VOCs. Conducting polymer sensors are more sensitive and selective to polar organic compounds and less sensitive to non-polar compounds such as the VOCs. The sensors are also operated at room temperature which makes it difficult to detect the VOCs most of which are non-reactive at room temperature. As a result of the low operating temperature the sensors are extremely sensitive to moisture and their selectivity is reduced at high humidity levels (Bai *et al.*, 2007). The metal

oxide semiconductor sensors on the other hand respond to a wide range of volatile compounds including aldehydes, alcohols and ketones. They operate at high temperature (200-600 ^{o}C) and are therefore less sensitive to changes in relative humidity (Wilson and Baietto, 2009). The above difference could explain the better performance of the metal oxide based electronic nose instruments in detecting aflatoxin contamination when compared to the conducting polymer based instruments.

The performance of two electronic nose platforms based on metal oxide semiconductor sensors to discriminate between control and aflatoxin contaminated samples was compared. The capability to discriminate between control and aflatoxin contaminated samples was relatively higher for DiagNose when compared to the Fox 3000. The Fox is equipped with an array of 12 semiconducting sensors comprising of six standard doped SnO_2 and six CTO sensors. The DiagNose consist of an array of 12 *n* type oxide sensors, doped and undoped with SnO_2 and WO_3 with thermo modulation of the sensors. In Fox the SnO_2 sensors were more responsive to the different classes of maize samples inoculated with *A. flavus* when compared to the CTO sensors and contained the most information used for discriminating between the different sample comparisons. A similar observation was recorded for DiagNose where the SnO_2 sensors were more responsive compared to the WO_3 sensors. Similar observations were made by Nowotny *et al.* (2013) while using the Fox 3000 to classify a set of 20 chemicals that represented different chemical groups. Higher performance of SnO_2 sensors compared to the CTO sensors was recorded. Since SnO_2 sensors were the most responsive in both the Fox 3000 and DiagNose, the difference performance between the two instruments could be attributed to the doping of the DiagNose SnO_2 sensors with catalytic material and the thermal cycling of the sensors at different temperatures. Improved performance of metal oxide based sensors by doping with catalysts such as palladium and platinum and introduction of thermal modulation

cycles is well established. Sakai *et al.* (2009) reported improved sensitivity of SnO_2 sensors to VOCs by doping with platinum, palladium and gold. Corcoran *et al.* (1998) reported increased sensitivity of metal oxide sensors with use of a thermal cycling technique. Introduction of a temperature cycle of 250 and 500 °C resulted in improvement of tea classification from 69 % achieved with at fixed temperature to 90 %.

4.5 CONCLUSION

The results obtained from the three electronic nose technologies evaluated indicate potential application of the technology for screening aflatoxin contamination in maize. The three instruments achieved significant correct classification rates across the different sample comparisons that ranged from 68 % to 94 %. The capability of MI as an effective method for selection of features for discrimination of aflatoxin contaminated from uncontaminated maize samples was also demonstrated. The use of MI resulted in improved classification accuracies for all three electronic nose instruments, with fewer number of features compared to the instrument associated data reduction methods hence reduced computational effort and time. The methods developed offer important insights into the potential application of the electronic nose as a diagnostic technique for a preliminary screening of maize samples, with the aim of reducing the number of samples requiring more expensive and time consuming chemical analysis. The DiagNose was the most effective in discriminating between the different classes of samples, while for the Fox 3000 sufficiently high and consistent classification rates were recorded for the different sample comparisons in the two experiments. Significant correct classification rates were recorded for Cyranose 320 in the first experiment, these however, was not consistent in the second experiment, indicating the instrument could be less stable and reliable for detection of aflatoxin contamination. Therefore it can be concluded that the DiagNose and Fox 3000 are more suitable for detecting aflatoxin contamination in maize.

In addition to the ability of the platform to detect aflatoxin contamination, other factors such as cost and possibility of field use need to be considered. The approximate capital cost of Fox 3000 is $ 120,000, while the cost of portable electronic noses such Cyranose 320 and DiagNose is approximately $ 8,000 (Vandeventer and Mallikarjunan, 2002). In addition to the significant cost differences between the Fox 3000 and DiagNose, Fox 3000 is non-portable therefore, more ideal for routine laboratory analysis. The DiagNose on the other hand, due to the low capital investment costs and its portability, could be the most suitable field diagnostic technique for detection of aflatoxin contamination in maize, as it would allow for quick screening of produce at the market entry level. Detection of mycotoxin contamination by an electronic nose is based on analysis of changes in composition of volatile compounds in the sample headspace following infection by mycotoxigenic fungi (Falasconi *et al.*, 2012). However, the production patterns of VOCs have been shown to vary with the environmental conditions of growth and also with the plant genotype/variety (Jurjevic *et al.*, 2008; Busko *et al.*, 2016; Sun *et al.*, 2016). Therefore the future applicability of DiagNose for detection of aflatoxin contamination is dependent on validation of capability to detect contamination in other maize genotypes that are artificially and naturally infected with *A. flavus*.

CHAPTER 5

EVALUATION OF A FIELD PORTABLE ELECTRONIC NOSE FOR DETECTION OF AFLATOXIN CONTAMINATION IN KENYAN MAIZE VARIETIES ARTIFICIALLY INOCULATED AND NATURALLY INFECTED WITH *A. FLAVU*S

5.1 INTRODUCTION

Developments in sensor technology in the last 30 years have facilitated the emergence of electronic nose devices that can potentially be deployed as rapid, non-invasive, sensitive, cost-effective and portable systems for detection of mycotoxin contamination (Logrieco *et al.*, 2005; Turner and Magan, 2004). Successful application of electronic noses in the food industry for quality control in different matrices has been demonstrated (Schaller *et al.*, 1998; Ampuero and Bosset, 2003; Berna, 2010; Baldwin *et al.*, 2011; Loutfi *et al.*, 2015; Peris and Escuder-Gilabert, 2016; Di Rosa *et al.*, 2017). With respect to mycotoxin contamination, several studies have shown the capability of an electronic nose to discriminate between non-infected samples and samples infected with different species or strains of mycotoxigenic fungi in various cereals (Olsson *et al.*, 2002; Paolesse *et al.*, 2006; Cheli *et al.*, 2009; Campagnoli *et al.*, 2011 Gobbi *et al.*, 2011; Lippolis *et al.*, 2014).

The detection of mycotoxin contamination by an electronic nose is based on analysis of changes in the composition of volatile compounds in the sample headspace following infection by toxigenic fungi (Falasconi *et al.*, 2012). Previous studies have documented diverse VOCs associated with infection of cereals with different species of mycotoxigenic fungi that include;

hydrocarbons, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives (Kaminski *et al.*, 1972; Jelen *et al.*, 1995; Pasanen *et al.*, 1996; Jurjevic *et al.*, 2008; De Lucca *et al.*, 2012). Most of the studies have been *in vitro,* with the fungi inoculated on artificial media or sterilised grain. However, biosynthesis of volatile organic compounds is strongly dependant on the environmental conditions of growth. Significant variations in volatile profiles have been observed when mycotoxigenic fungi are grown on different substrates and in different environment (Jurjevic *et al.*, 2008; De Lucca *et al.*, 2012; Sun *et al.*, 2016). Variations in volatile profiles have also been observed between different genotypes of wheat and maize infected with the same mycotoxigenic fungal species. Busko *et al.* (2016) reported significant differences in volatile compounds present in the grains of bread wheat, durum wheat and triticale contaminated with various species of mycotoxigenic fungi. Sherif *et al.* (2016) reported differences in volatiles produced by hybrid and dwarf maize varieties infected with different strains of *F. graminareum* and *F. verticillioides* at flowering stage.

In view of the various factors influencing the production of volatile compounds by toxigenic fungi on grains, deployment of the electronic nose as a diagnostic technique for aflatoxins is contingent on validating its capability to detect contamination in inoculated and naturally infected samples of different maize varieties. The objective of the current study is to evaluate the potential of a field portable electronic nose (DiagNose) to detect aflatoxin contamination in Kenyan maize varieties artificially and naturally infected with *A. flavus.* The DiagNose was selected for further evaluation with Kenyan maize varieties that were artificially inoculated and naturally infected with *A. flavus* based on results from Chapter 4 on evaluation of different sensor technologies for detection of aflatoxin contamination using Australian maize variety (DK703w) artificially inoculated with *A. flavus.* DiagNose in addition to being field portable,

exhibited a greater capacity for discriminating controls from the aflatoxin contaminated samples when compared to Fox 3000 and Cyranose 320.

5.2 MATERIALS AND METHODS

5.2.1 Inoculation of maize with *A. flavus* and collection of naturally contaminated samples

The potential for a field portable electronic nose (DiagNose) to detect aflatoxin contamination was evaluated with two Kenyan maize varieties Pioneer and Duma 43 that were artificially inoculated with *A. flavus*. The two Kenyan maize varieties are the most common cultivars in the Eastern Region where aflatoxin contamination is more prevalent. The varieties were provided by National Irrigation Board- Mwea Irrigation Agriculture Development Centre Kenya. The varieties were inoculated with aflatoxigenic isolate 121365 and non-aflatoxigenic isolate 3VM787 obtained from the strain collection at University of Nairobi School of Biological Sciences as described in Chapter 2 section 2.2.1. Treatments for the experiments included maize cobs contaminated with aflatoxigenic and non-aflatoxigenic *A. flavus* at two, six and ten incisions per cob to achieve different aflatoxin concentration levels, two controls namely; cobs inoculated with 2 % v/v Tween 20 at two, six and ten incisions and un-inoculated controls with no incisions. A set of ten cobs was used for each treatment as described in Chapter 2 section 2.2.1.4. The potential for DiagNose to detect aflatoxin contamination was also investigated with maize samples that had been naturally contaminated with aflatoxin under field conditions in Kenya. A total of 200 maize samples comprising three varieties (Pioneer, Duma 43, and DH04) and market samples were collected from aflatoxin prone areas as described in Chapter 2 section 2.2.2.

5.2.2 Determination of aflatoxin contamination levels

The presence and concentration of aflatoxin in inoculated and naturally contaminated samples was determined by direct competitive ELISA (AgraQuant®total aflatoxin assay 1/20 Romer labs. Inc., Union, MO, USA) as per manufacturer protocol, as described in Chapter 2 section 2.2.3.

5.2.3 Electronic nose analysis

The head space volatile profiles of Kenyan maize varieties experimentally inoculated or naturally contaminated with *A. flavus* as well as the control treatments were analysed with a field portable electronic nose DiagNose (C-it, The Netherlands) using prodecure described in Chapter 4 section 4.2.2 subsection 4.2.2.3. Briefly, the instrument was operated with zero air (20.9 % oxygen, 78.1 % nitrogen and 0.9 % argon (BOC Australia, North Ryde, NSW)) at a flow rate of 400 mL/min carrier gas. A mass of 1.65 g of maize flour was weighed and transferred to a 10 mL glass vial (Supelco, Bellefonte, PA) fitted with a silicon/teflon magnetic autosampler vial cap (Agilent Technologies, Australia). The vials were incubated in a block heater for 30 min at 35 ^{o}C to allow for the release of volatiles into the sample headspace. After incubation, the headspace was carried to and from the electronic nose chamber via Teflon tubing at a flow rate of 40 mL/ min. Headspace was sampled for five minutes. After each analysis, the instrument was purged with zero air at a flow rate of 300 mL/min for 35 min to allow the instrument to return to baseline for subsequent samples. To prevent contamination of the electronic nose chamber with flour or fungal spores, PTFE filters 25 mm x 0.2 μm (Supelco, Bellefonte, PA) were placed at the inlet and outlet sampling ports.

5.2.4 Data pre-processing and analysis

The DiagNose data was extracted and pre-processed using *"e-nose explorer"* algorithms coded in MatLab (Mathworks, USA) by Dr. Rosalind Wang (CSIRO) and Dr. Thomas Nowotny (Sussex University, UK) as described in Chapter 4 section 4.2.3.1. MI was used to select features for contributing to the differences between the different sample classes through a number of class comparisons that included: control maize vs all *A. flavus* infected maize, control maize vs aflatoxigenic *A. flavus* infected maize, control maize vs non-aflatoxigenic *A. flavus* infected maize and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize using the procedure described in Chapter 4 section 4.2.3.2.2. KNN and SVM classification algorithms with leave-one-out cross-validation were used to determine the effectiveness of selected features to discriminate between the different classes of samples as described in Chapter 4 section 4.2.3.3.

5.2.5 External validation

In cross-validation described previously in Chapter 4 section 4.2.3.3, the data is normally split into two sets namely the training set and a validation (test) set. The training set is used to define the model parameters whereas the validation set is used to evaluate the performance of the trained model, measured by the percentage of samples in the validation set that are correctly classified. This is referred to as internal validation. However, it is preferable to have a validation set that is completely independent from the training set, referred to as external validation. External validation of DiagNose data was performed using data from samples artificially inoculated with the aflatoxigenic *A. flavus* as the training set and data obtained from samples naturally infected with *A. flavus* as a test/validation test. This was evaluated for all inoculated samples vs all naturally infected samples, inoculated samples vs naturally infected samples of similar varieties, inoculated samples vs naturally infected samples of different varieties and

inoculated samples vs naturally infected samples of unknown variety (market samples). A summary of the training and validation (test) set combinations evaluated is shown in Table 5.1.

Table 5.1: Summary of training and validation set combinations used for external validation of the potential for DiagNose to detect aflatoxin contamination in maize.

Description	Training set	Validation/test set
All samples	All artificial infected	All field samples
Similar Varieties	Duma artificial infected	Duma naturally infected
	Pioneer artificial infected	Pioneer naturally infected
Different varieties	Duma inoculated	DH04 naturally infected
	Pioneer inoculated	DH04 naturally infected
***Market samples**	Duma inoculated	Market samples - naturally infected
	Pioneer inoculated	Market samples - naturally infected

* Market samples – Mixture of unknown varieties

5.3 RESULTS

5.3.1 Evaluation of potential for DiagNose to detect aflatoxin contamination in Kenyan maize varieties artificially inoculated with *A. flavus*.

5.3.1.1 Aflatoxin contamination levels

The potential of DiagNose to detect experimentally introduced aflatoxin contamination in maize was evaluated using two Kenyan maize varieties, Duma 43 and Pioneer, inoculated with 2 % Tween 20 as control samples and non-aflatoxigenic and aflatoxigenic *A. flavus* isolates. Aflatoxin contamination levels for the different classes of samples are presented and discussed in Chapter 2 section 2.3.1 and 2.4 respectively.

5.3.1.2 DiagNose sensors response

As mentioned previously, DiagNose consist of an array of 12 *n* type metal oxide sensors that comprise of SnO_2 sensors dopped with palladium (SnO_2-Pd), platinum (SnO_2-Pt), silver (SnO_2-Ag), copper (SnO_2-Cu), undoped WO_3 sensors and an experimental sensor (Extype 1). The response of the sensors to the different classes of samples namely control, non-aflatoxigenic *A. flavus* infected maize and aflatoxigenic *A. flavus* infected maize was evaluated with MI across a number of class comparsions. Using MI the sensors containing the most discriminating information on differences between sample classes were identified. The results are presented as a heatmap (Figure 5.1) where cells with high MI are represented by many colour graduations from dark red to cyan which indicates those sensors that contain higher information on the different classes. MI close to or equal to zero indicates sensors that contain no information about the classes and is represented by cells compressed into few shades of blue (Figure 5.1). The sensors response was higher for the control vs non-aflatoxigenic *A. flavus* infected maize in both varieties when compared to the other class comparisons. Information of the differences between the two classes was encoded by both the SnO_2 and WO_3 sensors (Figure 5.1). MI value

for the most responsive sensors was between 0.25 to 0.35 as shown by cells with colour graduations from dark red to cyan (Figure 5.1). Sensor reponse was lowest for the control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* for the two varieties (Figure 5.1). The MI value for the most responsive sensors was between 0.15 to 0.25 and is shown by cells with colour graduations from light yellow to light red (Figure 5.1). Differences in sensor responses were recorded between the two vareties for the two class comparasions. For Duma 43 most information on the difference between the classes for the two class comparsions was contained in the WO_3 sensors (6,7,9) while for Pioneer the information was contained in the SnO_2 sensors (1-4,8 and 11). This could indicate differences in the type of volatile compounds produced by *A. flavus* in the two varieties (Figure 5.1, Appendix 4 and 5). Highest sensor response was recorded between 10 and 25 time index for all the class comparasions for the two varieties (Figure 5.1).

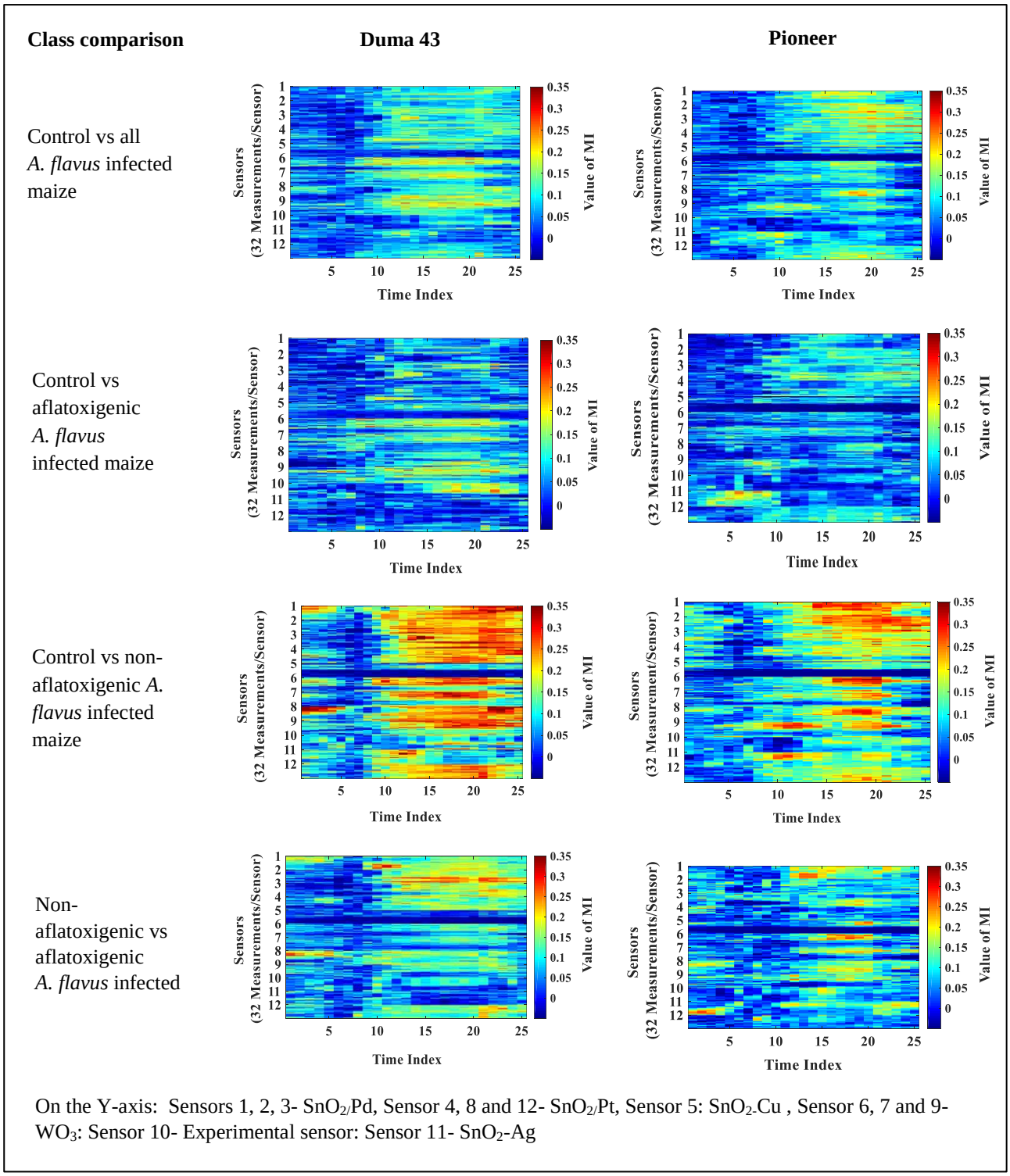
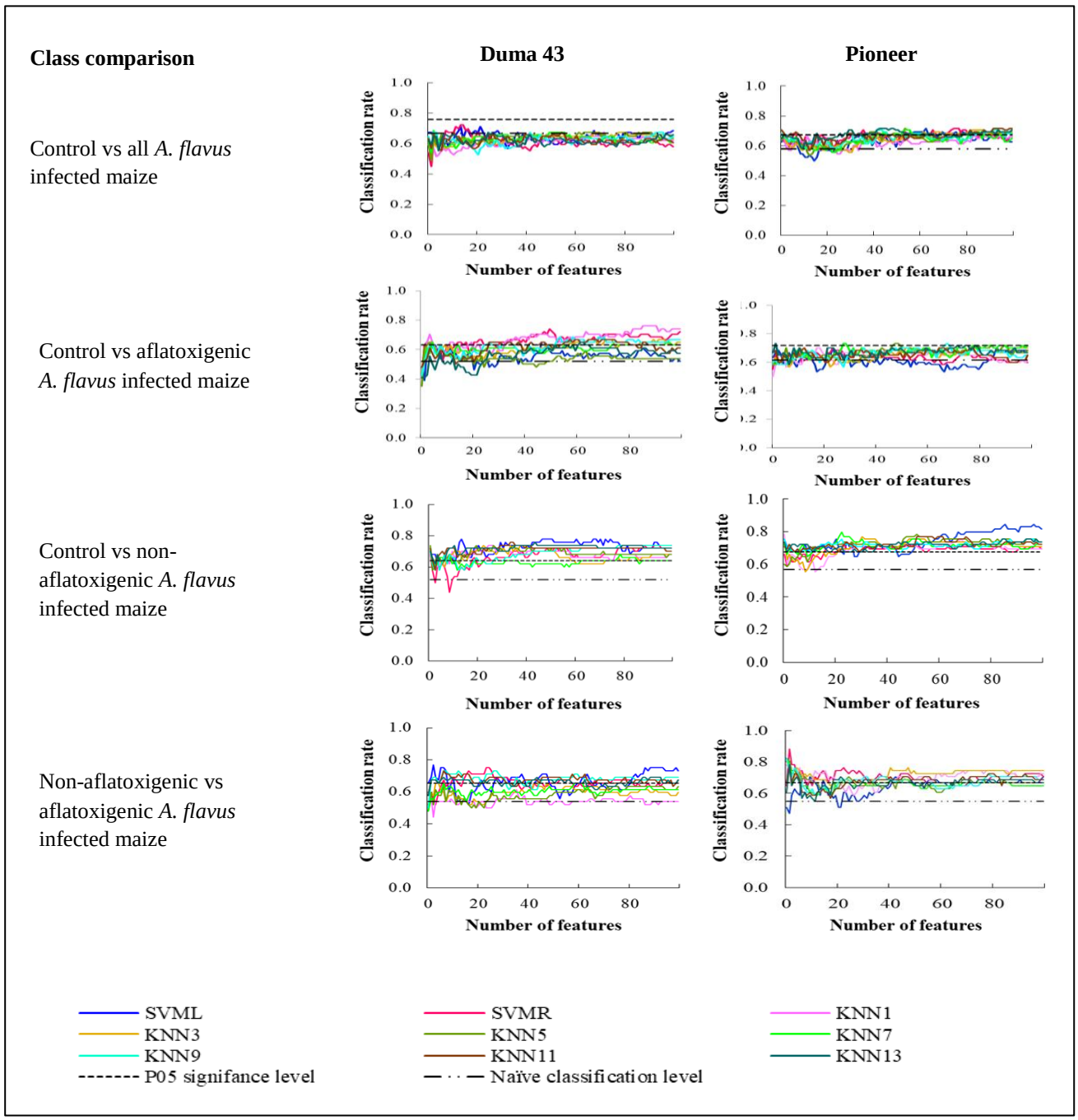


Figure 5.1: DiagNose relative mutual information (MI) between classes for Kenyan Maize varieties Duma 43 and Pioneer; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour .

5.3.1.3 Classification accuracy for experimentally inoculated varieties

The capability of DiagNose to detect aflatoxin contamination was evaluated by the ability to correctly classify different classes of samples through a number of class comparasions. MI was calculated between individual features and the respective classes to determine the combinations of sensors and time indices that contained discriminating information related to the classes. Based on the MI values, up to 100 of the most informative features were selected. The effectiveness of features selected by leave-one out cross-validation to discriminate between the different classes of samples was evaluated with two classifiers SVM and KNN. For each class comparison, a statistical significance level at 95 % confidence interval was determined based on the number of samples. Classification accuracy rates below the significance level value are considered non-significant. The naïve classification level which represents classification rates expected by chance was also determined. The performance of a given classifier is the average correct classification rate it obtained over the *N* fold cross-validation partitions which was plotted against the number of features for each class comparison (Figure 5.2). The number and type (false positive and false negative) of misclassified samples were investigated from the classifier that achieved the best correct classification rate.

The results demonstrate potential for DiagNose to detect aflatoxin contamination in Kenyan maize varieties artificially inoculated with *A. flavus.* Classification accuracies ranged from 72 % to 88 % for the two varieties across the different class comparison (Figure 5.2, Table 5.2.). Classification accuracies across the different class comparisons for both varieties were significant in all but one case and better than chance in all cases (Figure 5.2, Table 5.2). Classification accuracy for the control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* maize which represent the actual situation in the field ranged from 72 % to 76 % (Figure 5.2, Table 5.2).



SVML- Support vector machines-linear function; SVMR- Support vector machines-radial function; KNN-*k*-nearest neighbour with different values of n; n= 1, 3,5,7 9,11,13; Classification rate above or equal to the P05 significance level is significant; Naïve classification level represents classification rates expected by chance.

Figure 5.2: DiagNose cross-validated classification accuracy versus number of features using up to 100 of the most informative features selected by relative mutual information for Kenyan maize varieties Duma 43 and Pioneer; control vs all *A. flavus* infected maize flour, control vs aflatoxigenic *A. flavus* infected maize flour, control vs non-aflatoxigenic *A. flavus* infected maize flour and non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize flour.

Table 5.2: Summary of results for DiagNose analysis of maize flour for Kenyan maize varieties Duma 43 and Pioneer inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Variety	Class comparisons	Best correct classification (%)	P05 significance level (%)	Naïve classification (%)	Number classifiers ≥ P05 significance	Best classifier	Number of features	Most responsive sensors
Duma 43	Control vs all *A. flavus*	72	76	67	0/9	SVM	15	WO_3
	Control vs aflatoxigenic	76	63	52	9/9	KNN	91	SnO_2/Pd
	Control vs non-afla	78	64	52	9/9	SVM	51	SnO_2/Pt
	Non-afla vs aflatoxigenic	77	65	54	7/9	SVM	4	Exptype1
Pioneer	Control vs all *A. flavus*	72	67	58	9/9	SVM	100	SnO_2/Pd
	Control vs aflatoxigenic	73	72	62	3/9	KNN	91	SnO_2/Pd
	Control vs non-afla	85	68	57	9/9	SVM	97	SnO_2/Pd
	Non-afla vs aflatoxigenic	88	67	55	9/9	SVM	2	WO_3

Non-afla- non-aflatoxigenic; Best correct classification (%)-Highest classification rate recorded; P05 significance level (%) - significance level at 95 % confidence level; Naïve classification- classification expected by chance; Number classifiers ≥ P05 significance- Number of classifiers out of the total nine that achieved classification rates equal to or greater than the P05 significance level; Best classifier- Classifier that achieved the highest classification rate, SVML- Support vector machines-linear function; KNN - *k*-nearest neighbour; Number of features- Number of features used to achieve the highest classification rate; Most responsive sensors- Sensors from which features that achieved best classification rates were selected; SnO_2- tin dioxide sensors; WO_3- Tungsten trioxide sensors.

In practice the results imply that DiagNose if deployed as screening technique can correctly detect the presence of aflatoxin or *A. flavus* in up to 76 out of 100 maize samples at best performance and 72 out of 100 samples at the worst performance.

Differences in classification rates were recorded between the Duma 43 and Pioneer for the control vs all *A. flavus* infected maize and the control vs aflatoxigenic *A. flavus* infected maize (Figure 5.2, Table 5.2). For control vs all *A. flavus* infected maize, classification rates for Duma 43 were below the P05 significance level (76 %) for all the nine classifiers tested while for Pioneer all classifiers achived classification rates equal to or above the P05 level (67 %). The classification rate for Pioneer was also more robust as it was sustained across the different number of features (Figure 5.2). Significant classification accuracies of 76 % and 73 % were achieved for Duma 43 and Pioneer respectively, for the control vs aflatoxigenic *A. flavus* infected maize. For Duma 43 all nine classifiers achieved classification rates equal or above the P05 significance level (63 %), while for Pioneer only three classifiers achieved classification rates above or equal to the P05 significant level (72 %). Classification performance was more robust for Duma 43 compared to Pioneer with classification rates sustained across the different number of features (Figure 5.2).

The capability of DiagNose to discriminate between controls and maize inoculated with non-aflatoxigenic *A. flavus* as well between maize samples inoculated with non-aflatoxigenic and aflatoxigenic *A. flavus* was also demonstrated. Classification rates were relatively similar for the two varieties for the two pairwise comparisons (Figure 5.2, Table 5.2). For the control vs non-aflatoxigenic *A. flavus* infected maize classification accuracies of 78 % and 85 % were recorded for Duma 43 and Pioneer respectively, which were significant (P05= 64 % and 68 %

respectively) and sustained across the different number of features for all the classifiers (P05= 64 % and 68 % respectively) (Figure 5.2, Table 5.2). Discrimination of maize samples inoculated with the non-aflatoxigenic and aflatoxigenic *A. flavus* was achieved with 77 % and 88 % accuracy for Duma and Pioneer respectively. In both cases significant classification rates (P05 = 65 % and 67 % respectively) were sustained across the different number of features (Figure 5.2, Table 5.2). Though the non-aflatoxigenic *A. flavus* does not produce aflatoxins, the capability of DiagNose to discriminate between samples infected with non-aflatoxigenic strains from controls and samples inoculated with aflatoxigenic strain indicate its potential application for research purposes.

SVM was superior to KNN in classification of the different sample types. A higher number of percent best correct classification rates were achieved with SVM compared to KNN (Table 5.2). SVM recorded best percent best correct classification rates for three out of the four pairwise comparisons for the two varieties (Table 5.2). In both varieties KNN gave the highest classification rate for the control vs aflatoxigenic *A. flavus* infected maize (Table 5.2). The number of feature sets used to achieve the best classification rates ranged 2 to 100 across the different class comparisons for two varieties. The sensors from which features that achieved best classification rates were selected varied for Duma 43 across the different class comparisons but were consistent for Pioneer (Table 5.2). In the latter features that achieved best classification rates were selected from SnO_2 sensors for three out of the four pairwise comparisons (Table 5.2). For Duma 43 the features that achieved the best classification rates for the control vs aflatoxigenic *A. flavus* infected maize and control vs non-aflatoxigenic *A. flavus* maize were also selected from the SnO_2 sensors (Table 5.2). In general the SnO_2 sensors were more effective in discriminating between the different classes of samples than the WO_3 sensors.

5.3.1.4 Number and type of misclassified samples for experimentally inoculated varieties

The number and type (false positive and false negative) of misclassified samples were investigated from the classifier that achieved the highest classification rate. The sum of false positives and false negatives represents the percentage of predictions that are misclassified out of the total presented to the system. A false positive represents uncontaminated samples classified as contaminated and a false negative is a contaminated sample classified as uncontaminated. Misclassification rates were relatively similar between the two varieties and ranged from 22 % to 28 % for Duma 43 and 12 % to 28 % for Pioneer respectively across the different class comparisons (Table 5.3). Lowest misclassification rate was recorded for Pioneer non-aflatoxigenic vs aflatoxigenic *A. flavus* infected maize at 12 % while the highest misclassification rates was recorded for the control vs. all *A. flavus* infected maize at 28 % for the two varieties (Table 5.3). Apart from the control vs all *A. flavus* infected maize, the number of false positives was higher than the number of false negatives, across the different comparisons for the both varieties (Table 5.3). In the context of this study the higher number of false positives compared to false negatives means the DiagNose when used as an initial screening technique for aflatoxin detection would classify more of the non-contaminated samples as contaminated versus contaminated samples as non-contaminated with latter being more detrimental. False negatives are potentially more concerning than false positives in this case. A high proportion of false negatives indicate a higher chance of releasing contaminated samples for consumption.

Table 5.3: Misclassification errors for DiagNose analysis of maize flour Kenyan varieties inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* across different class comparisons.

Variety	Class comparison	Misclassification (%)	No. of misclassified /total samples	
			False +ves	**False -ves**
Duma 43	Control vs all *A. flavus*	28	10/76	11/76
	Control vs aflatoxigenic	24	7/54	6/54
	Control vs non-afla	22	6/50	5/50
	Non-afla vs aflatoxigenic	23	10/52	2/52
Total			**33/232**	**24/232**
Pioneer	Control vs all *A. flavus*	28	11/88	14/88
	Control vs aflatoxigenic	27	9/60	7/60
	Control vs non-afla	15	7/65	3/65
	Non-afla vs aflatoxigenic	12	3/51	3/51
Total			**30/264**	**27/264**

No.–Number; Non-afla- Non-aflatoxigenic; False +ve - Number of false positive samples (control samples classified as either *A. flavus* infected, aflatoxigenic or non-Aflatoxigenic); False –ve- Number of false negative samples (*A. flavus* infected, aflatoxigenic and non-aflatoxigenic samples classified as control).

5.3.2 Evaluation of the potential of DiagNose to detect aflatoxin contamination in Kenyan maize naturally infected with *A. flavus*

5.3.2 .1 Aflatoxin levels for Kenyan maize naturally infected with *A. flavus*

The potential for DiagNose to detect aflatoxin contamination was investigated with maize samples naturally contaminated with aflatoxins. The samples were purchased from farms in aflatoxin prone areas in Kenya and included the main varieties grown in those respective areas. Three varieties were obtained. Two of these; Duma 43 and Pioneer, were the same as those experimentally inoculated. The third variety was DH04. Market samples were also purchased and used for validation of the technique, but their varieties were not known. Aflatoxin contamination levels as determined with ELISA are described and discused in Chapter 2 section 2.3.2.and 2.4 respectively.

5.3.2.2 DiagNose sensors response to Kenyan maize naturally infected with *A. flavus*

The response of DiagNose sensors to Kenyan maize varieties naturally infected with *A. flavus* was evaluated using MI to identify sensors that contained discriminating information on the difference between uncontaminated (< 6 ppb aflatoxin) and contaminated (≥ 6 ppb aflatoxin) samples. The results are presented as heatmap as described earlier in section 5.3.1.2. Sensors that contained discriminating information on the difference between the control and contaminated samples were relatively similar across the different varieties (Figure 5.3). Information was contained in both the SnO_2 and WO_3 sensors for all the varieties and market samples (Figure 5.3). Higher sensor response was recorded for DH04 and market samples than Duma 43 and Pioneer (Figure 5.3). The MI value for the most responsive sensors for DH04 and market samples was between 0.2 to 0.3 as shown by cells with colour graduation from dark red to cyan (Figure 5.3). This indicates a higher response of the sensors to the DH04 and market samples presumably due to the greater amounts of VOCs in the samples.

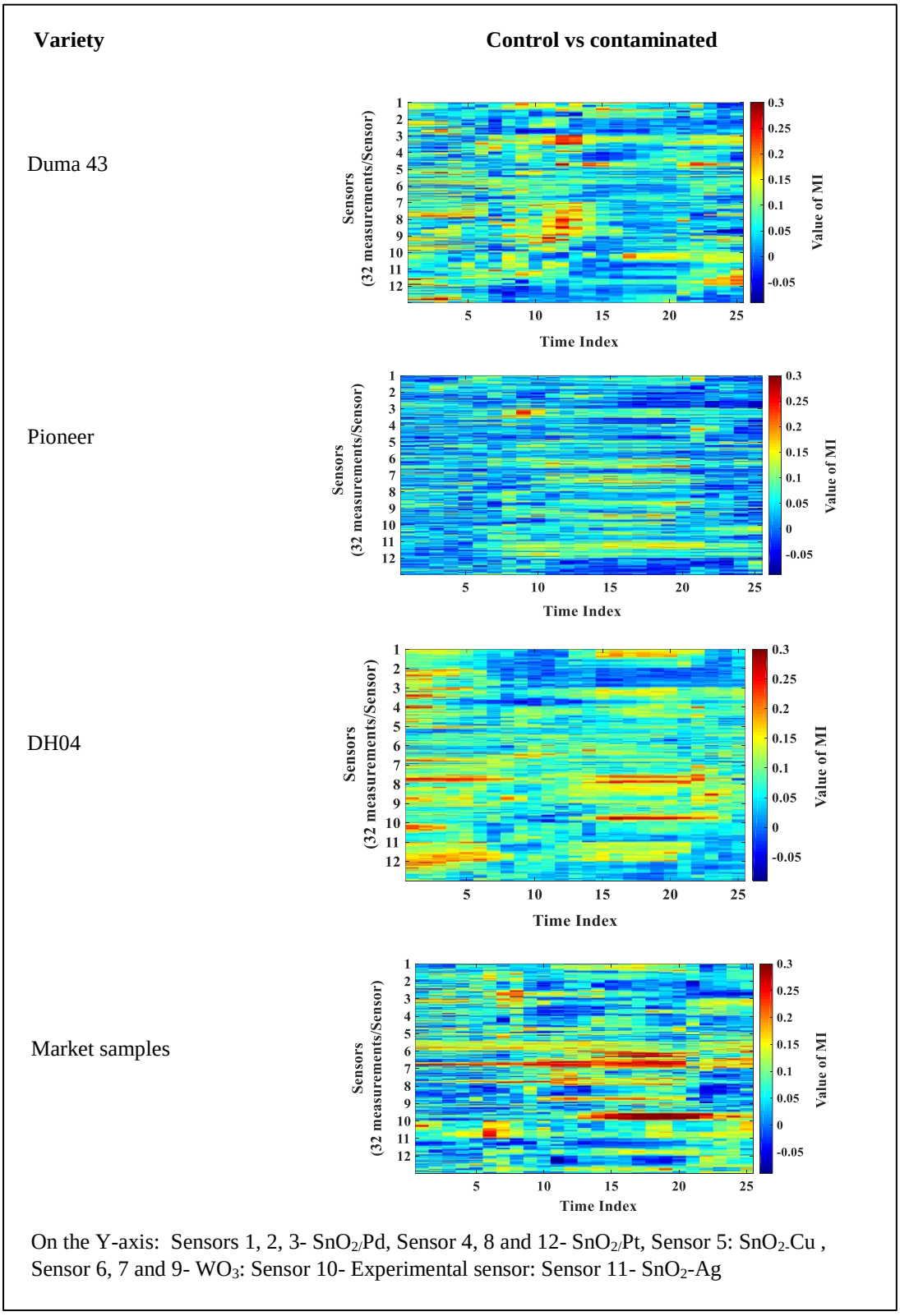


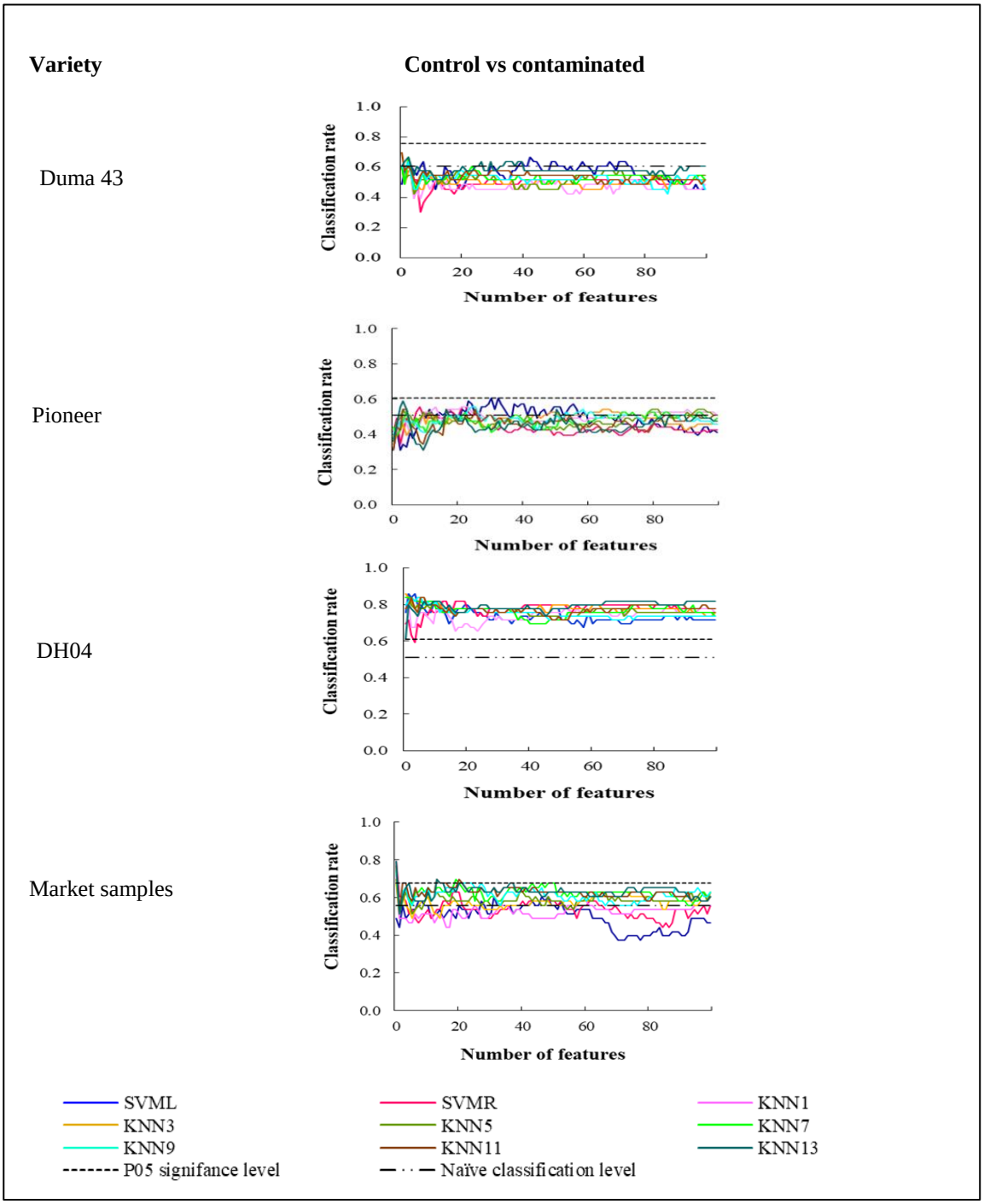
Figure 5.3: DiagNose relative mutual information (MI) between control vs contaminated maize flour for Kenyan varieties Duma 43, Pioneer, DH04 and market samples naturally infected with *A. flavus* under field conditions.

5.3.2.3 Classification accuracy for Kenyan maize naturally infected with *A. flavus*

The capability of DiagNose to detect aflatoxin in naturally contaminated Kenyan maize varieties was evaluated by the ability to correctly differentiate uncontaminated (< 6 ppb aflatoxin) and contaminated (≥ 6 ppb aflatoxin) samples. MI was used to select the most informative features discriminating uncontaminated from contaminated samples. The effectiveness of selected features to discrimate between control and contaminated samples was evaluated with SVM and KNN as detailed in section 5.3.1.3. Results demonstrate capability of DiagNose under some circumstances to detect aflatoxin contamination in maize samples naturally infected with *A. flavus* for both known varieties and market samples with classification accuracies that ranged from 61 % to 86 % (Figure 5.4 , Table 5.4). Capability of DiagNose to detect aflatoxin contamination was not consitent for the three varieties and the market samples (Figure 5.4, Table 5.4). Classification accuracy was highest (86 %) and more robust for DH04 compared to the other varieties and the market samples. All classifiers tested achieved statistically significant (P05 = 61) classification rates which were sustained across the different number of features (Figure 5.4, Table 5.4). A statistically significant classification accuracy of 79 % was also achieved for market samples, which are expected to be the most difficult to classify, considering they are a mixture of different and unknown varieties (Figure 5.4, Table 5.4). The samples represents the actual situation in the market where maize sold to consumers in retail outlets comprise a mixture of different unknown varieties. Though eight out of the nine classifiers tested achieved classification rates above or equal to the P05 significance level (67 %), the rates were achieved with only one feature and were not suistained across the different number of features indicating a weak/non-robust classification performance (Figure 5.4, Table 5.4). DiagNose was least effective in discriminating contaminated from uncontamianted samples for Duma 43 and Pioneer. For Duma 43 the classification rates for all classifiers were below the P05 significance (76 %) and only 70 % of the samples were

accurately classified (Figure 5.4, Table 5.4). For Pioneer only 61 % of the samples were accurately classified, with only one classifier out of the nine tested achieving classificaton rate equal to the P05 significance level (61 %) which was not suitained across the different number of features (Figure 5.4, Table 5.4).

SVM was more effective than KNN in classification of maize samples naturally infected with *A. flavus*. SVM achieved significant best percent correct classification rates for two out of three varieties evaluated and the market samples while KNN gave a non-significant best correct classification rate for Duma 43 (Table 5.4). The number of feature sets used to achieve the best classification rates for the naturally contaminated samples ranged from 1 to 33 across the different varieties (Table 5.4). The features were selected from the WO_3 sensors for all the varieties and market samples (Table 5.4).



SVML- Support vector machines-linear function;SVMR- Support vector machines- radial function; KNN - *k*-nearest neighbour with different values of n; n= 1, 3,5,7 9,11,13; Classification rate above or equal to the P05 significance level is significant; Naïve classification level represents classification expected by chance based on number of samples in each class.

Figure 5.4: DiagNose cross-validated classification accuracy versus number of features using up to 100 of the most informative features selected by relative mutual information for control vs contaminated maize flour for Kenyan maize varieties Duma 43, Pioneer, DH04 and market samples naturally infected with *A. flavus* under field conditions.

Table 5.4: Summary of results for potential of DiagNose to detect aflatoxin contamination in maize flour of Kenya maize varieties and markets samples naturally infected with *A. flavus* under field conditions.

Variety	Best correct classification (%)	P05 significance level (%)	Naïve classification level (%)	No. classifiers ≥ P05 significance level (%)	Best Classifier	Number of features	Most responsive sensors
Duma 43	70	76	60	0/9	KNN	1	WO_3
Pioneer	61	61	51	1/9	SVM	33	WO_3
DH04	86	61	51	9/9	SVM	4	WO_3
Market samples	79	67	56	8/9	SVM	1	WO_3

Best correct classification (%)-Highest classification rate recorded; P05 significance level (%) - significance level at 95% confidence level; Naïve classification- classification expected by chance depending on the number of samples in each class; Number classifiers ≥ P05 significance- Number of classifiers out of the total nine that achieved classification rates equal to or greater than the P05 significance level; Best classifier- Classifier that achieved the highest classification rate; SVM Support Vector Machines; KNN - *k*-nearest neighbour; Number of features- Number of features used to achieve the highest classification rate; Most responsive sensors- Sensors from which features that achieved best classification rates were selected; WO_{3-} Tungsten trioxide sensors.

5.3.2.4 DiagNose number and type of misclassified samples for Kenyan maize naturally infected with *A. flavus*.

The number and type (false positive and false negative) of misclassifications were identified for the classifier that achieved the best correct classification rate as described previously in section 5.3.1.4. Misclassification rates for DiagNose analysis of Kenyan maize vareties naturally contaminated with aflatoxins ranged from 14 % to 39 %. (Table 5.5). The lowest misclassification rate was recorded for DH04 at 14 % while the highest was recored for Pioneer at 39 %. The number of false negatives was higher than false positives across the different varieties and market samples indicating that the DiagNose has a higher chance to misclassify samples that are naturally infected with *A. flavus* as non-contaminated when they are actually contaminated (Table 5.5).

Table 5.5: Misclassification errors for DiagNose analysis of maize flour for Kenyan maize varieties and market samples naturally infected with *A. flavus* under field conditions.

Variety	**Class comparison**	**Misclassification (%)**	**No. of misclassified samples/total sample**	
			False +ves	**False -ves**
Duma 43	Control vs conta	30	2/33	3/33
Pioneer	Control vs conta	39	12/61	12/61
DH04	Control vs conta	14	4/43	5/43
Market samples	Control vs conta	21	2/49	5/49
Total			**20/186**	**25/186**

Conta- contaminated; No. – Number; False +ve - Number of false positive samples (non-contaminated samples classified as contaminated); False –ve- Number of false negative samples (contaminated samples classified as non-contaminated); Conta vs unconta- Contaminated versus contaminated uncontaminated.

5.3.3 External validation of DiagNose data

The potential for DiagNose to detect aflatoxin contamination in maize was externally evaluated by using data from maize samples artificially infected with aflatoxigenic *A. flavus* as a training set and data from maize samples naturally infected with *A. flavus* as a validation set. Percent best correct classification rates achieved with external validation were compared with classification rates achieved previously with internal validation for samples naturally infected with *A. flavus* in a number of training and validation sets combination. The training and validation sets evaluated included; all inoculated samples vs all naturally infected samples, inoculated samples vs naturally infected samples of similar varieties, inoculated samples vs naturally infected samples of different varieties and inoculated samples vs naturally infected samples of unknown variety (market samples). In all except one case (Pioneer inoculated vs Pioneer naturally infected), the external validation was weaker than the leave-one out cross-validation (internal validation) (Table 5.6). Classification rates achieved previously with internal validation showed DiagNose capability to discriminate between uncontaminated and contaminated maize samples naturally infected with *A. flavus* with accuracies ranging from 61 % to 86 % (Table 5.6). External validation of the data yielded classification accuracies that ranged from 62 % to 78 % across the different training and validation set combinations (Table 5.6). Classification accuracies were statistically significant for four out of seven training: validation set combinations (Table 5.6). Statistically significant classification accuracy was achieved with external validation for inoculated samples vs naturally infected samples of similar varieties for Pioneer (64 %, P05 = 61 %). Significant classification accuracy was also achieved for inoculated samples (Duma 43 and Pioneer) vs naturally infected samples of different variety (DH04) naturally infected with *A. flavus* (Table 5.6). Classification accuracies of 76 % (P05 = 61 %) and 78 % (P05 = 61 %) was achieved when data for Duma 43 and Pioneer respectively, were used for external validation of DH04 samples naturally infected with

A .flavus (Table 5.6). A statistically significant classification accuracy of 74 % (P05 = 67 %) was achieved when data from Duma 43 samples artificially inoculated with *A. flavus* was used for external validation of data from market samples naturally infected with *A. flavus* (Table 5.6). Classification accuracies were however, non-significant when, data from all artificially infected samples was used for external validation for all naturally infected samples (62 % P05 = 64 %), Duma 43 artificially inoculated data was used for external validation of Duma 43 naturally infected with *A. flavus* (67 %. P05 = 76 %) and when Pioneer data for artificially inoculated samples was used for external validation of market samples naturally infected with *A. flavus* (58 %, P05= 67) (Table 5.6). Though the classification rates were below the significant level, they were all above the naïve classification level (Table 5.6).

Table 5.6: Cross-validated accuracies for external validation of potential for DiagNose to detect aflatoxin contamination in maize flour using data from maize samples artificially infected with *A. flavus* as a training set and data from maize samples naturally infected with *A. flavus* as validation set.

Description	Training set	Validation/test set	Internal validation (validation set)	External validation (Training: validation set)		
			Best correct classification (%)	Best correct classification (%)	P05 significance level (%)	Naïve classification level (%)
All samples	All AI	All NI	68	62	64	58
Similar Varieties	Duma-AI	Duma-NI	70	67	76	60
	Pioneer-AI	Pioneer-NI	61	64	61	51
Different varieties	Duma-AI	DH04-NI	86	76	61	51
	Pioneer-AI	DH04-NI	86	78	61	51
***Market samples**	Duma-AI	Market samples-NI	79	74	67	56
	Pioneer-AI	Market samples-NI	79	58	67	56

AI – Samples artificially inoculated with *A. flavus*; NI- Samples naturally infected with *A. flavus* under field conditions.

5.4 DISCUSSION

The potential for DiagNose as a field portable diagnostic technique for detection of aflatoxin contamination of maize was evaluated with Kenyan maize varieties that were artificially and naturally infected with *A. flavus*. Artificially infected samples comprised of maize cobs inoculated with 2 % Tween 20 as control samples, non-aflatoxigenic and aflatoxigenic *A. flavus* isolates. The response of DiagNose sensors varied across the different sample types evaluated in a number of class comparisons. The sensors were most responsive to the control vs maize infected with the non-aflatoxigenic *A. flavus* isolates. This could be attributed to the prolific growth of the non-aflatoxigenic *A. flavus* strain compared to the aflatoxigenic strain hence production of more volatile organic compounds detected by the different sensors (Sun *et al.*, 2014).

DiagNose was able to discriminate between the different classes of samples with accuracies that ranged from 72 % to 88 % (Table 5.2). Of particular importance was the potential to discriminate between control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize which represent the actual situation in the field where maize is either contaminated or uncontaminated with aflatoxins. Classification accuracy for these particular samples ranged from 72 % to 76 %, which implies capability for DiagNose to detect aflatoxin contamination in 76 out of 100 at best performance and 72 out of 100 samples at worst performance in a typical field situation. The classification rates were however not consistent for the two varieties. Classification accuracies were significant for both class comparison for Pioneer (control vs all *A. flavus* infected maize (72 %, P05 = 67 %); control vs aflatoxigenic *A. flavus* infected maize (73 %, P05 = 62 %), while for Duma 43, classification accuracies was only significant for the control vs aflatoxigenic *A. flavus* infected maize (76 %, P05 = 63 %).

The desired outcome of a diagnostic technique is to discriminate between different samples with 100 % accuracy, which is rarely the case. Therefore, in addition to classification accuracy the type of misclassified samples are important in determining the applicability of a technique. In the current situation, the preference is to have more false positives than false negatives, which indicate high sensitivity and low specificity. In the context of this study the higher number of false positives compared to false negatives means that the DiagNose when used as an initial screening technique for aflatoxin detection would classify more of the non-contaminated samples as contaminated versus contaminated samples as non-contaminated with later being more detrimental. False negatives are potentially more concerning than false positives in this case. A high proportion of false negatives indicate a higher chance of releasing contaminated samples for consumption. The results of this study corroborate with findings from other studies in maize and other cereals artificially contaminated with different mycotoxins. Gobbi and Locci (2006) reported successful discrimination of maize samples artificially inoculated with toxigenic and non-toxigenic isolates of *F. verticillioides* using an electronic nose equipped with an array of six metal oxide semiconductor sensors. In the study the electronic nose was also able to discriminate between control and samples infected with *F. verticillioides*, irrespective of the toxigenic status. Eifler *et al.* (2011) using an electronic nose with an array of eight quartz microbalances reported discrimination wheat samples infected with *Fusarium cerealis*, *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium redolens* from un-infected samples with 80 % accuracy. In the study the number of false positives was higher than the false negatives.

The potential for DiagNose to detect aflatoxin contamination of maize was tested using maize samples that were naturally infected with *A. flavus* under field conditions in Kenya. Capability of DiagNose to correctly discriminate between contaminated and uncontaminated samples for

both known varieties and market samples was variable with accuracies that ranged from 61 % to 86 %. This indicates potential for DiagNose to accurately detect aflatoxin contamination in actual field samples with an accuracy up to 86 % or as low as 61 %. In addition to the classification accuracies achieved, the misclassification rates ranged from 14 % to 39 % for the known varieties and market samples, with a higher number of false negatives (25/186) compared to false positives (20/186) (Table 5.6). As mentioned previously, the preference is to have more false positives compared to false negatives, which minimizes the likelihood of contaminated samples being released for consumption. A high proportion of false negatives compared to false positive as is the case for DiagNose for detection of aflatoxin in maize naturally infected *A. flavus* is unsatisfactory. This would lead to classification of contaminated samples as uncontaminated hence release of contaminated lots for consumption. The results are in line with findings from other studies evaluating the potential application of electronic nose to detect mycotoxin contamination in cereals naturally infected with mycotoxigenic fungi. Campagnoli *et al.* (2011) reported successful classification of durum wheat samples naturally contaminated with deoxynivalenol as either non-contaminated, contaminated below the legal limit or contaminated above the legal limit using a metal oxide semiconductor based electronic nose and classification and regression trees. Lippolis *et al.* (2014) using metal oxide semiconductors based electronic nose and Discriminant Function Analysis reported 86.7 % classification accuracy for durum wheat varieties naturally contaminated with deoxynivalenol at different levels. Similar results were also reported by Olsson *et al.* (2002) using an electronic nose consisting of 10 metal oxide semiconductor field effect transistor sensors and six SnO_2 sensors to classify barley samples naturally contaminated with ochratoxin A. The electronic nose misclassified seven out of the total thirty seven samples of which six were false negatives.

The highest classification accuracy was recorded with DH04 at 86 % while the lowest was for Pioneer at 61 %. Similar observations were recorded for GC-MS in combination with statistical techniques for detection of aflatoxin in maize naturally infected with *A. flavus*. (Chapter 3, section 3.3.2). This could be an indication that potential for DiagNose to discriminate between aflatoxin contaminated and uncontaminated field samples could also be variety dependent. These could be attributed to inherent genetic differences between the varieties which could have an influence on the type and quantity of VOCs produced and hence on the capability of DiagNose to detect contamination. Variations in VOCs profile among maize and wheat varieties infected with the same species of mycotoxigenic fungi has been previously reported (Busko *et al.*, 2016; Sherif *et al.*, 2016; Fauguel *et al.*, 2017). The maize samples were collected from farmers in different agro-ecological zones in Kenya and were therefore presumably produced under different ecological, agronomic and climatic conditions which could also account for the variability in VOCs produced hence the classification accuracies. Gouinguene´ and Turlings (2002) reported significant variations in intensity and variability of VOCs produced by maize under different soil humidity, air humidity, temperature, light, and fertilization rate. DH04 was grown under irrigated conditions hence high soil moisture and better crop management practices compared to all the other varieties and market samples that were grown under rain fed conditions and individual farmer management. Therefore the future applicability of DiagNose for detection of aflatoxins in maize naturally infected with *A. flavus* is dependent on validation with a wide range of varieties grown in field trials under similar and different environmental conditions. These would a facilitate selection of DiagNose sensors that are responsive across a wide range of samples hence more effective and accurate in detection of aflatoxin contamination.

Performance of DiagNose as a field portable instrument for detection of aflatoxins was further tested by external validation performed by analysing samples naturally infected with *A. flavus* using data obtained from samples that were artificially inoculated with aflatoxigenic *A. flavus*. External validation is conducted to evaluate the predictive performance of a classification model using datasets that were not used to develop the model. The objective is to quantify the predictive accuracy of the model with different samples and its potential for generalizability to future settings (Consonnia *et al.*, 2010). External validation is a much stronger test for model performance compared to internal validation procedures such as cross-validation because it provides a measure of the model reproducibility as well as transportability. Reproducibility refers to the ability of a model to give valid predictions with samples that are very similar to ones used for its development whilst transportability refers to the ability to give valid predictions with samples that are related to but different from the ones used for development (Justice *et al.*, 1999).

In the context of this study, a laboratory-location validation was conducted to ascertain the possibility of training an electronic nose with laboratory generated samples and achieving correct classification with field samples. Though the external validation was generally weaker than leave-one out cross-validation (Table 5.6), statistically significant classification accuracies were achieved with external validation in some circumstances. Statistically significant classification accuracy were achieved when data obtained from artificially infected samples was used to classify; naturally contaminated varieties similar varieties to artificially infected varieties for Pioneer, naturally infected variety that was different from artificially infected varieties for both Duma 43 and Pioneer and market samples that comprise a mixture of unknown varieties for Duma 43. The results indicate the possibility that DiagNose could be trained with maize samples artificially contaminated with aflatoxins in the laboratory and be

used to classify field samples that are naturally contaminated with aflatoxins for similar varieties to one used for training, different varieties from one used for training and mixture unknown varieties/market samples. However, there is need to validate the findings with a wider range of maize varieties and training-validation combinations than used in this study. To the best of our knowledge, this is the first study reporting external validation of the potential for electronic nose to detect aflatoxin contamination in maize. It is however, in line with previous studies on external validation of the potential application of electronic nose as a diagnostic technique. Lippolis *et al.* (2016) externally validated the potential of an electronic nose equipped with metal oxide semiconductors sensors to detect ochratoxin A in dry-cured meat. In the study a classification model was developed using sausages inoculated with ochratoxin producing strains of *Penicillium nordicum* and two non-ochratoxin producing strains of *Penicillium nalgiovense* and *Penicillium salamii*. The model was successfully used to classify dry-cured meat samples produced at the industrial scale. External validation of the potential of electronic nose as diagnostic techniques for detection of human pathogens and diseases has also been reported (Collins *et al.*, 2014; Leopold *et al.*, 2015; Nieboer *et al.*, 2016).

The effectiveness of features selected to discriminate between the different classes of samples was evaluated by two classification algorithms SVM and KNN. For maize samples artificially inoculated with *A. flavus*, SVM achieved the highest classification accuracies for three out of the four class comparisons with the two varieties evaluated while for naturally infected samples, SVM recorded highest classification accuracies of the two out three farm bought varieties and the market samples of unknown varieties (Table 5.2 and 5.4). The superior performance of SVM compared to KNN for classification of maize samples artificially inoculated or naturally infected with *A. flavus* could be attributed to the inherent ability to deal with non-linear complex separation problems through application of the kernel function that enables transformation of

the data from a low dimension space to a higher dimensional space where the classes can be separated (Xu *et al.*, 2006). Similar results were reported by Chen *et al.* (2011) while evaluating the potential of an electronic nose for discrimination of green tea quality. The study compared SVM, KNN and artificial neural network for discrimination of four grades of green tea. Optimum discrimination was achieved with SVM in comparison to KNN and artificial neural network.

5.5 CONCLUSION

Availability of a rapid, sensitive, inexpensive and field portable diagnostic technique for detection of aflatoxin contamination would greatly reduce the risk of their introduction into the food and feed chain. The possibility of electronic nose to detect contamination with simplified sample preparation would allow for rapid, efficient and cost-effective initial screening on large number of samples on a 'Yes' or 'No' criterion basis with an objective of reducing the number of samples to undergo more expensive and time-consuming quantitative analyses.

The current study aimed at evaluating the potential application of field portable, metal oxide semiconductor sensors based electronic nose (DiagNose) as a diagnostic technique for screening maize to detect aflatoxin contamination. The results indicate potential for DiagNose to detect aflatoxin contamination in two Kenyan maize varieties (Duma 43 and Pioneer) that were artificially infected with *A. flavus* with accuracies that ranged from 72 % to 76 %. The classification accuracies though acceptable for a screening technique are relatively low to justify deployment of DiagNose as a field diagnostic for detection of aflatoxin contamination at this stage. The preference would be to have classification accuracies closer to 100 %.

Capability of DiagNose to detect aflatoxin contamination in Kenyan maize naturally infected with *A. flavus* was variable with accuracies that ranged from 61 % to 86 %. This could indicate that the potential for DiagNose to discriminate between aflatoxin contaminated and uncontaminated field samples could also be variety dependent. Despite the relatively low classification accuracies recorded for maize samples artificially inoculated with *A. flavus* and the variable classification accuracy achieved with maize naturally infected with *A. flavus*, the results provide a proof of concept on the potential application of metal oxide sensors with thermal modulation for detection of aflatoxin contamination of maize. Further studies are however required to improve on the classification performance of the sensors. A more extended study needs to be performed with a wider range of maize varieties grown under similar and different agronomic and environment conditions, to validate the response of sensors to maize infected with *A. flavus*. These would allow for selection of the most responsive sensors for detection of aflatoxin contamination across a wide range of conditions. The selected sensors could be utilized for formulation of an optimized instrument with fewer sensors that are more selective hence more effective and accurate in detecting aflatoxin contamination. The optimized instrument when available would be an innovative, cost-effective and easy to use technique for detection of aflatoxin contamination. It would therefore not require highly skilled operators, hence well suited for rural areas where there is very little or non-existent laboratory infrastructure.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Among the mycotoxins, aflatoxins are of the greatest concern for food safety and security due to their impact on human health and the agriculture economy. This is especially true in developing countries, where proposed management strategies have not been effective at the subsistence farming level (Hell and Mutegi, 2011; Weidenbörner, 2017). As a result, regulatory control to prevent entry of aflatoxin contaminated produce into the food supply chain is of paramount importance to protect consumers (Hell *et al.*, 2008). Implementation is however, dependant on the availability of a well-established regulatory mechanism and analytical capacity to monitor food samples frequently, at different points along the value chain. Various analytical methods, including, chromatographic methods, such as HPLC and LC-MS, and immunochemical methods, such as ELISA are widely used for analysis of aflatoxins in food (Wacoo *et al.*, 2014). Although these methods are effective in detecting aflatoxins, they are cumbersome, requiring extensive sample preparation, expensive equipment and/or trained personnel, which makes their routine use confined to laboratories. This limits their use in developing countries where the majority of farmers are located in remote villages without access to laboratories located in major cities (Mbithi and VanHuylenbroeck, 2000). There is, therefore, a need to develop simple, rapid and cost effective field portable diagnostic tool, well adapted to the regulatory and surveillance systems operating in developing countries.

The use of VOCs produced by mycotoxigenic fungi upon plant infection has been identified as a potential novel diagnostic technique for detection of mycotoxins, to circumvent the drawbacks associated with current techniques. Detection is based on changes in the composition of VOCs in the headspace of samples, as a result of infection by mycotoxigenic fungi (Cheli *et al.*, 2009;

Sankaran *et al.*, 2010; Martinelli *et al.*, 2014). The VOCs produced are species and sometimes strain specific and can therefore be used as potential taxonomic markers for fungal activity and differentiation of fungal species, as well as contaminated and un-contaminated produce (Keshri and Magan, 2000; Magan and Evans, 2000). Use of VOCs for detection of mycotoxin contamination offer considerable advantages over other diagnostic techniques. The method is non-destructive and eliminates the need for long and cumbersome extraction procedures. The VOCs are sampled from the entirety of the sample, overcoming representativeness problems associated with sampling produce due to the heterogeneous distribution of toxins within contaminated bodies of grain.

The GC-MS is the most widely used technique for separation and identification of VOCs. Successful discrimination between contaminated and uncontaminated samples is often dependent on finding specific marker compounds. It has already been demonstrated to be successful for a number of plant diagnostic applications, including mycotoxin analysis (Zeringue *et al.*, 1993; De Lacy *et al.*, 2001; Vikram *et al.*, 2004; Jurjevic *et al.*, 2008; De Lucca *et al.*, 2010; Cellini *et al.*, 2017). The practical application for diagnostic purposes is, however limited by high operational costs in terms of equipment and training of personnel. Additionally, due to the nature of the GC-MS equipment, its use is mainly confined to the laboratory, limiting its application as a field diagnostic. The electronic nose on the other hand, although they do not allow for identification and quantification of VOCs, they are rapid, cost-effective, portable and easy to operate. Hence they represent a potential diagnostic for detection of aflatoxin contamination in the field.

The present study was centred on two main objectives. The first objective was to evaluate the potential for GC-MS based analysis of VOCs to discriminate between aflatoxin contaminated

and uncontaminated maize flour. To meet this objective, the use of VOCs to discriminate between *A. flavus* infected and uninfected Australian and Kenyan maize varieties under laboratory and field conditions was investigated. In addition, the study aimed to identify unique VOCs as markers for both *A. flavus* infection and aflatoxin contamination of maize. The second objective was to evaluate the potential of the electronic nose to detect aflatoxin contamination in maize. In this case, the performance of three electronic nose instruments based on different sensor technologies were compared using an Australian maize variety (DK703w) artificially inoculated with *A. flavus* under laboratory conditions. The sensors were metal oxide semiconductor sensors represented by Fox 3000, metal oxide semiconductor sensors with thermocycling represented by DiagNose and conducting polymers represented by Cyranose 320. The performance of data reduction method for each instrument and feature selection with MI was also compared. Subsequently, the best performing electronic nose was selected for further evaluation, using Kenyan maize varieties artificially and naturally infected with *A. flavus* under laboratory and field conditions.

The application of GC-MS and electronic nose for discrimination of toxigenic and non-toxigenic isolates of *A. flavus* as well as contaminated and uncontaminated intact maize grains has been previously evaluated (Zeringue *et al.*, 1993; Jonsson *et al.*, 1997; Olsson *et al.*, 2002; Paolesse *et al.*, 2006; Jurjevic *et al.*, 2008; De Lucca *et al.*, 2010; Gobbi *et al.*, 2011; De Lucca *et al.*, 2012). The studies have, however, been based on *in vitro* procedures with fungus cultured on artificial media or sterile grains. These conditions do not reflect natural conditions, where infection of maize with *A. flavus* occurs at the pre-harvest stage followed by aflatoxin accumulation through the post-harvest stage. In this study, the potential application of VOCs analysis was evaluated with intact maize kernels artificially inoculated with *A. flavus* in the laboratory and maize samples naturally infected with *A. flavus* under field conditions. This

study documents methods that can be used to achieve artificial colonization of intact maize kernels by *A. flavus* and subsequent contamination with aflatoxins. Unsurprisingly, determination of aflatoxin levels by ELISA showed higher contamination levels in samples inoculated with aflatoxigenic isolates in comparison to control samples inoculated with 2 % Tween 20 or maize samples inoculated with the non-aflatoxigenic *A. flavus* isolates. Aflatoxin levels for the Australian and Kenya maize varieties ranged from 0.1 to 1.7 ppb for the control samples, 0 to 5.1 ppb for the non-aflatoxigenic *A. flavus* infected maize and 65.4 to 1064.7 ppb for the aflatoxigenic *A. flavus* infected maize (Figure 2.5). Contamination levels varied widely in samples inoculated with the aflatoxigenic isolates which, replicates the heterogeneous distribution of aflatoxins in naturally contaminated food matrices.

The results indicate that GC-MS in combination with statistical techniques may offer potential for detection of *A. flavus* infection and aflatoxin contamination in maize. The GC-MS was effective in discriminating maize samples inoculated under laboratory conditions with aflatoxigenic and non-aflatoxigenic *A. flavus* isolates from control samples inoculated with 2 % Tween 20. GC-MS also discriminated maize infected with aflatoxigenic *A. flavus* from maize infected with non-aflatoxigenic *A. flavus*. The performance was consistent for both the Australian maize variety DK703w and Kenyan maize varieties Duma 43 and Pioneer with classification accuracies ranging from 81 % to 100 %. The capability of GC-MS in combination with statistical techniques to detect aflatoxin contamination was further evaluated with Kenyan maize varieties that were naturally infected with *A. flavus* under field conditions. The classification accuracies achieved for the naturally contaminated samples were, however, much lower than for the artificially inoculated samples, ranging from only 48 % to 80 %. To the best of our knowledge, this is the first study to evaluate the potential application of GC-MS for detection of aflatoxin on maize naturally infected with *A. flavus.* Previous studies have only

used controlled laboratory conditions which could have overestimated the potential for GC-MS to detect contamination.

A number of VOCs in the headspace of maize flour artificially or naturally infected with *A. flavus* were identified as potential biomarkers of infection. The predictive VOCs appeared to be variety dependent. For the Australian maize variety DK703w, tetramethyl pyrazine was significantly ($p < 0.0001$) and consistently effective in discriminating controls from all *A. flavus* infected maize; maize infected with aflatoxigenic *A. flavus* and maize infected with the aflatoxigenic *A. flavus* from maize infected with non-aflatoxigenic *A. flavus*. The compound was not identified as important in discriminating controls from maize infected with the non-aflatoxigenic *A. flavus* isolate. Additionally, the concentration of this compound was significantly higher ($p < 0.0001$) in aflatoxigenic *A. flavus* inoculated samples when compared to the controls and samples inoculated with the non-aflatoxigenic isolates and could therefore be a potential marker for aflatoxigenic *A. flavus* infection and aflatoxin contamination in DK703w. Tetramethyl pyrazine is a fermentation product of microorganisms including *Aspergillus* species on food, synthesised by the condensation reaction between acetoin and ammonia (Kempler, 1983; Jelen and Wasowicz, 1998).

A similar pattern was observed for p-xylene for Kenyan varieties, Pioneer artificially infected with *A. flavus*, DH04 naturally infected with *A. flavus* and market samples. P-xylene was effective in discriminating controls from maize infected with aflatoxigenic *A. flavus* as well as non-aflatoxigenic *A. flavus* infected maize from aflatoxigenic *A. flavus* infected maize. P-xylene is an aromatic hydrocarbon produced as a secondary metabolite during the conversion of glucose to pyruvate in the glycolytic pathway (Magan and Evans, 2000; Li *et al.*, 2013; Show

et al., 2015). Production of p-xylene by *Aspergillus* species and its effectiveness to discriminate between mycotoxin contaminated and uncontaminated cereals has been previously demonstrated (Jelen and Grabarkiewicz-szczeüsna, 2005; Jurjevic *et al.*, 2008; De Lucca *et al.*, 2012; Perkowski *et al.*, 2012; Laddomada *et al.*, 2014; Busko *et al.*, 2016). What is important about tetramethyl pyrazine and p-xylene in this study, is the significantly ($p < 0.0001$) high concentration in *A. flavus* infected maize than in controls and maize infected with the non-aflatoxigenic *A. flavus* and capability to discriminate between aflatoxin contaminated and uncontaminated maize samples.

The results obtained from maize samples artificially inoculated with *A. flavus* provide evidence that GC-MS in combination with statistical techniques could be a rapid and cost-effective diagnostic technique for initial screening of maize to detect aflatoxin contamination. This would allow for separation of presumptively contaminated and uncontaminated lots prior to trade or human consumption, potentially reducing the number of samples to undergo further analysis with more complicated and expensive techniques. However, under field conditions variations associated with the samples or 'noise' obscures the signal and hence capability to detect contamination. The field samples were collected from different farmers therefore were presumably produced under different, agronomic and climatic conditions, in addition to the inherent genetic variability between varieties. These factors could have had an effect on the type and quantity of VOCs produced, hence the capability of GC-MS to detect contamination. The influence of these sort of factors on type and quantity of VOCs produced upon infection of maize by mycotoxigenic fungi has been previously documented (Gouinguene´ and Turlings, 2002).

The classification accuracies achieved for the naturally contaminated samples, which represents the actual situation in practice was, though better than chance in most instances, not good enough for practical use. The VOC profile varied widely across the different varieties. The practical and future application of this technique is therefore dependent on identifying specific biomarker compounds that are consistent over a wide range of growth conditions and varieties. Further studies are required with a wide range of maize varieties grown under similar and different agronomic and environmental conditions to identify and compensate for above variabilities. In future it will be useful to evaluate the production of identified marker compounds over time, possibly on live plants to verify their production and association with the aflatoxin biosynthesis cycle. These will aid in identification of specific marker compounds correlated with aflatoxin contamination. These has previously been demonstrated for *F. graminearum* and *F. verticillioides* inoculated simultaneously on ears of a hybrid and dwarf maize varieties in the green house. The study documented useful volatile biomarkers for early detection of *Fusarium* infection and associated mycotoxin contamination (Sherif *et al.*, 2016).

Additionally, in this study the storage time from milling to analysis was identified as possibly having an effect on the concentration of VOCs in the sample headspace and hence on the ability of GC-MS to detect contamination. The level of VOCs decreased with increase in time from milling to analysis from 14 days to 30 days. A 61 % to 65 % decrease in level of VOCs was recorded for controls, maize infected with non-aflatoxigenic *A. flavus* and maize infected with aflatoxigenic *A. flavus* with increase in time from milling to analysis from 14 days to 30 days (Figure 3.4). The temperature at which samples are stored prior to analysis has also been shown to have an effect on the total number and concentrations of VOCs produced by grains infected with mycotoxigenic fungi (Garcia-Cela *et al.*, 2018). Further studies on changes in concentration of VOCs in the head space of maize infected with *A. flavus* are required with

storage times longer than the four weeks used in this study (probably up to 16 weeks) with varying temperatures probably 15 to 30 ^{o}C (Garcia-Cela *et al.*, 2018; Wicklow *et al.*, 2018) to determine the duration of storage and temperature at which GC-MS detection of aflatoxins remains viable. There is also the need to develop a system that standardizes the process of field sampling for maize in terms of variety, sampling time after harvest, temperature and duration of storage prior to analysis and the sampling conditions to minimize the variabilities associated with naturally contaminated samples. This is however, more applicable in a large scale corporate setting as opposed to individual small scale farmers.

A detailed study was conducted to evaluate the potential application of the electronic nose for detection of aflatoxin contamination of maize. Advances in the field of electronic noses have resulted in development of a wide range of sensor technologies with varied response to various VOCs. It is therefore important to identify sensor types that can be adapted for specific applications. This study aimed to determine the most appropriate sensor technology for detection of aflatoxin contamination in maize by comparing three different electronic technologies namely metal oxide semiconductor sensors (Fox 3000), metal oxide semiconductor sensors with thermocycling (DiagNose) and conducting polymer sensors (Cyranose 320). Due to the high dimensionality of sensor response data, the choice of the appropriate feature selection method to identify a small set of parameters that are most informative was crucial for managing data analysis. Results demonstrated MI as a more effective method for selecting useful features for discrimination of aflatoxin contaminated and uncontaminated samples compared to instrument based data reduction methods. The use of MI resulted in improved classification performance from 32 % to 84 % achieved with instrument associated data reduction methods to 58 % to 91 % for the three electronic nose instruments across the different sample class comparisons (Tables 4.1, 4.2 and 4.3). MI allows for selection

of a few features with high relevance but low redundancy by ranking them according to their predictive power based on the feature's relevance and non-redundancy. It eliminates the contributions of redundant and irrelevant sensors/features that may contain incomplete and inconsistent information which degrades the classification accuracy. Therefore higher classification accuracies are achieved with fewer features hence reduced time and computational effort required to manage electronic nose data in the field (Bennasar *et al.*, 2015). The two classifiers evaluated, SVM and KNN were equally effective in determining the capability of features selected with MI to discriminate between the different classes of samples. This indicates that the choice of feature selection method is more critical than classification algorithm in achieving good classification results for an electronic nose instrument.

Results demonstrate the capability of the three electronic nose instruments/sensor technologies to discriminate between *A. flavus* infected and uninfected maize and subsequently aflatoxin contaminated and uncontaminated maize, with varying accuracy levels. The study documents higher discrimination capability between aflatoxin contaminated and uncontaminated maize with the metal oxide based sensors when compared to the conducting polymer sensors. Classification accuracies ranged from 75 % (P05 = 70 %) to 94 % (P05 = 71 %) for the metal oxide based electronic noses (Fox 3000 and DiagNose) compared to 59 % (P05 = 68 %) to 79 % (P05 = 72 %) for conducting polymer sensors (Cyranose 320) across the different sample class comparisons. The results indicate that the metal oxide sensors could be more responsive to the type of VOCs produced by maize upon infection by *A. flavus.* The better performance of metal oxide sensors could be attributed to their higher selectivity for non-polar compounds and their ability to operate at high temperatures which makes them more responsive to a wide variety of VOCs produced by *A. flavus* that may be non-polar and non-reactive at room temperature (Bai *et al.*, 2007).

The performances of two different types of metal oxide sensors were compared; metal oxide sensors with thermocycling (DiagNose) and metal oxide sensors without thermocycling (Fox 3000). The DiagNose, equipped with an array of 12 metal oxide sensors with cyclical thermal modulation exhibited slightly higher ability to discriminate aflatoxin contaminated from uncontaminated samples than the Fox 3000. Classification accuracies ranged from 81 % to 94 % for DiagNose and 75 % to 91 % for Fox 3000 across the different sample class comparisons. The Fox 3000 consists of six standard SnO_2 and six CTO sensors, while the DiagNose consists of doped and undoped SnO_2 and WO_3 sensors. Since, in both cases, the SnO_2 were the most responsive, the marginal difference in performance between the two instruments could be attributed to the thermal modulating cycles and doping of DiagNose SnO_2 sensors with catalytic materials that include palladium, platinum and silver. Based on these results, and considering the potential for field use and capital investments associated with each instrument, the DiagNose was selected for further evaluation with Kenyan maize varieties that were artificially inoculated and naturally infected with *A. flavus*.

Results indicate the potential for DiagNose as a field portable instrument for detection of aflatoxin contamination of maize. The DiagNose was able to discriminate between controls and maize samples artificially inoculated with *A. flavus* under laboratory conditions for two Kenyan varieties, Duma 43 and Pioneer. Classification accuracies ranged from 72 % to 88 %, across different sample class comparisons. Of particular interest was its ability to discriminate between control vs all *A. flavus* infected maize and control vs aflatoxigenic *A. flavus* infected maize which represents the actual situation in the field where maize is either contaminated or uncontaminated with aflatoxins. Classification accuracy for these particular samples ranged from 72 % to 76 % for the two varieties. In practice the result implies that if DiagNose was to be deployed as a rapid technique for detection of aflatoxin contamination in the field, it might

be able to correctly determine contamination in 76 out every 100 at best performance and 72 out of every 100 samples at worst performance. Apart from the classification accuracy, the types of misclassifications are important in determining the practical utility of the technique. The preference is to have more false positives than false negatives, which was the case in this study. The higher proportion of false positives implies that the DiagNose would classify more of the uncontaminated samples as contaminated. This, although undesirable, is less concerning because few genuinely contaminated samples could be released for consumption. A high proportion of false negatives would be less safe because more contaminated samples would be released for consumption.

The DiagNose was also able to discriminate between aflatoxin contaminated and uncontaminated Kenyan maize varieties that were naturally infected with *A. flavus* under field conditions with accuracies that ranged from 61 % to 86 %. Additionally, external validation of the potential for DiagNose to detect aflatoxin contamination was achieved by analysing samples naturally contaminated with *A. flavus* using the classification model generated using samples that had been artificially inoculated with the aflatoxigenic *A. flavus.* Classification accuracies achieved with external validation, were relatively consistent with accuracies obtained with internal validation. The accuracies were consistent when the classification model for artificially inoculated varieties was used to classify similar naturally infected varieties, naturally infected variety that was different from artificially infected varieties and market samples that comprised a mixture of unknown varieties. The results demonstrate the applicability of classification models generated with artificially inoculated maize samples to a practical field classification of aflatoxin contamination.

The DiagNose was able to discriminate between aflatoxin contaminated and uncontaminated samples naturally infected with *A. flavus* with accuracies that ranged from 61 % to 86 %. In ideal circumstances, the goal is to have a diagnostic technique that can perfectly discriminate between aflatoxin contaminated and uncontaminated samples. However, 100 % accuracy is rare for any diagnostic, values close to 100 % are considered acceptable (van Stralen *et al.*, 2009). The classification accuracies achieved in this study, though statistically significant in some cases and better than chance, are too low to justify deployment of DiagNose as a field diagnostics for detection aflatoxin contamination at this stage. Additionally, as mentioned earlier, the type of misclassified samples plays an important role in determining the applicability of a diagnostic technique. Unlike the maize samples artificially inoculated with *A. flavus*, a higher proportion of false negatives compared to false positives were recorded for the maize samples naturally infected with *A. flavus* under field conditions. This is unacceptable for a field diagnostic technique.

The results do, however, provide important initial insights and a proof of concept of the potential application of DiagNose and in general the electronic nose technology for detecting aflatoxin contamination in maize. In practice, results indicate that the DiagNose on average can correctly discriminate between aflatoxin contaminated and uncontaminated samples in 70 out of 100 samples. This, coupled with the field portability and possibility of electronic nose to detect contamination without sample preparation, would allow for rapid, efficient and cost-effective initial screening on a large number of samples on a 'Yes' or 'No' criterion basis. The objective would be to reduce the number of samples to undergo more expensive and time-consuming quantitative analyses. This could translate into reduced cost and time of analysis, which would facilitate timely removal of contaminated lots from the food or feed chain. However, there is need for further research to improve the classification accuracy and

performance of electronic noses before they could be adopted for practical use. A number of challenges related to the performance of the electronic nose need to be addressed before the technology can be transferred to the industry. These include selectivity and redundancies of sensors, where sensors with poor selectivity affect adversely the discriminating power of the array (Cellini *et al.*, 2017).

CONCLUSIONS AND FUTURE DIRECTIONS

The metal oxide semi-conductor sensors based electronic noses were found to be more effective than conducting polymer sensors in detecting aflatoxin contamination of maize. The electronic noses comprise an array of sensors with broad and overlapping specificities which has an impact on the discriminatory ability of the system. Some sensors in the array lack sensitivity to the target VOCs, which increases variance/noise. Others have very similar sensitivities to the target VOCs (cross-sensitivity) hence providing redundant information. Therefore improvement in performance of the electronic nose for detection of aflatoxin contamination will require selection of optimal sensors or features with the aim of developing an application-specific instrument. Through feature selection, redundant and irrelevant sensors, as well as other variables that introduce noise, could be eliminated to create an optimised sensor array with improved discrimination capability for the samples of interest. This could potentially result in improvement of the system performance in terms of predictive accuracy as well as reduced cost and time for data acquisition and management (Gardner and Hines, 2005).

In this study the performance of different feature selection methods were evaluated. The results, show the MI approach to be an effective method for selecting optimal features/sensors to discriminate aflatoxin contaminated from uncontaminated samples. By use of MI, the sensors

most responsive to the different classes of samples were identified for each electronic nose instrument. In the case of metal oxide semiconductor sensors without thermocycling, represented by Fox 3000, the SnO_2 sensors contained more discriminatory information for the different classes of samples when compared to the CTO sensors. Of particular interest were sensors that contained discriminating information for the control vs all *A. flavus* infected maize and control vs. aflatoxigenic *A. flavus* infected maize which represent the critical tests in the field. Responsive sensors included CTO sensor 4 (Chromium–titanium oxide sensor with H-LTA zeolite overlayer, pore size 3.5A) and SnO_2 sensors 8 (for detection of hydrocarbons and methane), 9 (for detection of methane, propane and aliphatic non polar molecules), 10 (for detection of chlorinated and fluorinated compounds) and 12 (for detection of low concentrations of hydrogen, ammonia, amines) (Nowotny *et al.*, 2013). The performance of metal oxide semi-conductor sensors with thermocycling (represented by DiagNose) was evaluated with Australian and Kenyan maize varieties artificially inoculated with *A. flavus* and Kenyan maize varieties that were naturally infected with *A. flavus* under field conditions. The sensor response varied with the different sample types. However, across the different sample types, the WO_3 (WO_3-3 and WO_3-2) sensors contained the most information for discrimination of aflatoxin contaminated and uncontaminated maize samples, followed by the SnO_2 doped with palladium (SnO2/Pd/2-3), SnO_2 doped with silver (SnO2-Ag) and SnO_2 doped with platinum (SnO2/Pt/2-1, SnO2/Pt/2-2). In future, a smaller subset can be selected and optimised for detection of aflatoxin contamination of maize.

As a whole, this study illuminates the potential use of GC-MS based analysis of VOCs and the electronic nose for detection of *A. flavus* infection and aflatoxin contamination in artificially and naturally contaminated maize samples. GC-MS demonstrated variations in volatile profiles

between *A. flavus* infected and uninfected samples that are indicative of aflatoxin contamination. This provides insights into the potential use of electronic nose as a portable device for detection of aflatoxin. Both techniques offer potential advantages to industry and public health by providing tools for rapid, non-destructive and cost-effective assessment of maize for aflatoxin contamination that can be performed prior to trade or human consumption However, there is a performance deficit that must be addressed before the two techniques can be adapted routinely. Significant and development research is needed before their use can become a routine practice for regulatory purposes. For GC-MS, a useful future direction is to identify specific biomarker compounds for aflatoxin contamination that are consistent over a wide range of conditions by screening a wide variety of maize genotypes. These could initially include more maize varieties from aflatoxin prone areas other the ones surveyed in this study, such as local landraces (Kikamba) and the hybrids (H5 and H6 series). Additionally, it will be worthwhile to conduct studies on the effect of storage time on concentration of VOCs in the head space of maize infected with *A. flavus* to determine duration of storage at which different VOCs remain viable for both GC-MS and electronic nose analysis. The studies could be extended to a period of up to 16 weeks, which corresponds to the longest duration a famer would store maize prior to trade or consumption (Hella *et al.*, 2000; Kaaya and Kyamuhangire, 2006).

For the electronic nose, there is need to develop an appropriate system for detecting aflatoxins by assessment of the selectivity and sensitivity range of individual sensor arrays for particular target VOCs related to aflatoxin contamination as identified by GC-MS. The metal oxide semi-conductor sensors that contained most information for discriminating between samples with and without aflatoxin contamination can be used as a reference. The sensor's accuracy, reproducibility, response speed, recovery rate, robustness, and overall performance need to be

evaluated in order to identify a smaller optimal array for aflatoxin detection. This will lead to creation of a highly sensitive, selective, smaller and cheaper device which can potentially be miniaturised into a probe that is easy to use at the different points of the maize supply chain. To implement this there is need to optimize the VOC sampling protocol to detect contamination in intact maize kernels rather than milled flour. This could eliminate the milling stage prior to analysis, hence reducing time and resources required for analysis. These improvements can simplify the operation of the electronic nose and allow analysis of maize samples without the need for expensive laboratory equipment and highly skilled personnel.

A probe at the small scale farmer level could go a long way in ensuring the safety of maize before trade and consumption, hence reducing the negative impact of aflatoxins. At the industry and regulatory level, the use of such a probe could be integrated with other technologies such as optical sorters and NIR-based analysis to create a technological platform for monitoring aflatoxin contamination along the maize value chain (Cheli *et al.*, 2016). Considering the sensitivity associated with analysis of produce for mycotoxin contamination due to potential loss of trade, the implementation of this technology would require a cooperative, multidisciplinary approach, involving farmers, the industry, regulators and researchers.

CHAPTER 7

REFERENCES

Abbas, H.K., Zablotowicz, R.M., Arnold, B., Abel, C.A., 2006. Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. *Biocontrol Science and Technology 16*, 437-449.

Abramson, D., Sinha, R.N., Mills, J.T., 1980. Mycotoxin and odor formation in moist cereal grain during granary storage. *Cereal Chemistry 57,* 346-351.

Achieng, J., Ouma, G., Odhiambo, G., 2013. Use of organic inputs in management alfisols and ultisols for sustainable maize production in Western Kenya. *American Journal of Experimental Agriculture 3*, 884-895.

Alam, H., Saeed, S.H., 2013. Modern applications of electronic nose: A review. *International Journal of Electrical and Computer Engineering 3*, 52-63.

Alaniz, M.S., Barros, G.G., Chulze, S.N., 2016. Non-aflatoxigenic *Aspergillus flavus* as potential biocontrol agents to reduce aflatoxin contamination in peanuts harvested in Northern Argentina. *International Journal of Food Microbiology 231,* 63-68.

Albert, K.J., Lewis, N.S., 2000. Cross reactive chemical sensor arrays. *Chemical Reviews 100,* 2595-2626.

Aldred, D., Magan, N., 2004. The use of HACCP in the control of mycotoxins: The case of cereals. In: Magan, N., Olsen, M. (Eds.), Mycotoxins in Food: Detection and Control. CRC Press, Boca Raton, FL, pp. 139-173.

Ampuero, S., Bosset, J.O., 2003. The electronic nose applied to dairy products: A review. *Sensors and Actuators B: Chemical 94,* 1-12.

Anfossi, L., Baggiani, C., Giovannoli, C., Gilda, D., Giraudi, G., 2013. Lateral-flow immunoassays for mycotoxins and phytotoxins: A review. *Analytical Bioanalytical Chemistry 405*, 467-480.

Anklama, E., Stroka, J., Boenkeb, A., 2002. Acceptance of analytical methods for implementation of EU legislation with a focus on mycotoxins. *Food Control 13*, 173-183.

Arriola, K.G., 2006. Effect of stay-green ranking, maturity and moisture concentration of corn hybrids on silage quality and the health and productivity of lactating dairy cows. Graduate School. University of Florida, pp. 1-114.

Arshak, K., Moore, E., Lyons, G.M., Harris, J., Clifford, S., 2004. A review of gas sensors employed in electronic nose applications. *Sensor Review 24,* 181-198.

Artursson, T., Eklov, T., Lundstrom, I., Martensson, P., Sjostrom, M., Holmberg, M., 2000. Drift correction for gas sensors using multivariate methods. *Journal of. Chemometrics 14,* 711-723.

Asao, T., Büchi, G., Abdel-Kader, M.M., Chang, S.B., Wick, E.L., 1965. The structures of aflatoxins B and G. *Journal of the American Chemical Society,* 87, 882–886.

Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Schurz, H., Kieszak, R.S., Njapau, H., Schleicher, R., McCoy, L.F., Misore, A., DeCock, K., Rubin, C., Slutsker, L., 2004. Case-control - Study of an acute aflatoxicosis outbreak, Kenya. *The Aflatoxin Investigative Group,* 1779-1783.

Bag, A.K., Tudu, B., Bhattacharyya, N., Bandyopadhyay, R., 2014. Dealing with redundant features and inconsistent training data in electronicnose: A rough set based approach. *Institute of Electrical and Electronics Engineers Sensors Journal 14*, 758-767.

Bai, H., Li, C., Chen, F.E., Shi, G., 2007. Aligned three-dimensional microstructures of conducting polymer composites. *Polymer 48,* 5259-5267.

Balasubramanian, S., Panigrahi, S., Kottapalli, B., Wolf-Hall, C.E., 2007. Evaluation of an artificial olfactory system for grain quality discrimination. *Food Science and Technology 40,* 1815-1825.

Baldwin, E.A., Jinhe, B., Plotto, A., Dea, S., 2011. Electronic noses and tongues: Applications for the food and pharmaceutical industries. *Sensors 11,* 4744-4766.

Bankole, S., Schollenberger, M., Drochner, W., 2006. Mycotoxins in food systems in sub- Saharan Africa. *Mycotoxin Research 22,* 163-169.

Barsan, N., Koziej, D., Weimar, U., 2007. Metal oxide-based gas sensor research: How to? *Sensors and Actuators B: Chemical 121,* 18-35.

Battiti, R., 1994. Using mutual information for selecting features in supervised neural net learning. *Institute of Electrical and Electronics Engineers Transactions on Neural Networks 5,* 537-550.

Baumann, K., 2003. Cross-validation as the objective function for variable-selection techniques. *Trends in Analytical Chemistry 22,* 395-406.

Bennasar, M., Hicks, Y., Setchi, R., 2015. Feature selection using joint mutual information maximisation. *Expert Systems with Applications 42,* 8520-8532.

Berardo, N., Pisacane, V., Battilani, P., Scandolara, A., Pietri, A., Marocco, A., 2005. Rapid detection of kernel rots and mycotoxins in maize by near-infrared reflectance spectroscopy. *Journal of Agricultural and Food Chemistry 53,* 8128-8134.

Berna, A., 2010. Metal oxide sensors for electronic noses and their application to food analysis. *Sensors 10,* 3882-3910.

Berrueta, L.A., Alonso-Salces, R.M., Héberger, K., 2007. Supervised pattern recognition in food analysis. *Journal of Chromatography A 1158,* 196-214.

Berthiller, F., Sulyok, M., Krska, R., Schuhmacher, R., 2007. Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. *International Journal of Food Microbiology 119,* 33-37.

Bii, F., Wanyoike, W., Nyende, A.B., Gituru, R., Bii, C., 2012. Fumonisin contamination of maize (*Zea mays*) in aflatoxin 'hot' zones in Eastern province of Kenya. *African Journal of Health Science 20,* 28-36.

Borjesson, T., Stollman, U., Schnurer, J., 1992. Volatile metabolites produced by six different fungal species compared to other indicators of fungal growth on cereal grains. *Applied and Enviromental Microbiology 58,* 2599–2605

Boilot, P., Hines, E.L., Gongora, M.A., Folland, R.S., 2003. Electronic noses inter-comparison, data fusion and sensor selection in discrimination of standard fruit solutions. *Sensors and Actuators B: Chemical 88,* 80-88.

Borjesson, T., Eklov, T., Jonsson, A., Sundgren, H., Schnurer, J., 1996. Electronic nose for Odor classification of grains. *Cereal Chemistry 73,* 457-461.

Bothast, R., Hesseltine, C., 1975. Bright greenish-yellow fluorescence and aflatoxin in agricultural commodities. *Applied Microbiology 30,* 337–338.

Brown, G., Pocock, A., Zhao, M., Lujan, M., 2012. Conditional likelihood maximisation: A unifying framework for information theoretic feature selection. *Journal of Machine Learning Research 13,* 27-66.

Bruins, M., Rahim, Z., Bos, A., Sande, W., Endtz, H.P., Belkum., 2013. Diagnosis of active tuberculosis by e-nose analysis of exhaled air. *Tuberculosis 93,* 232-238.

Busko, M., Stuper , K., Jelen´, H., Góral, T., Chmielewski, J., Tyrakowska, B., Perkowski, J., 2016. Comparison of volatiles profile and contents of trichothecenes group B, ergosterol, and ATP of bread wheat, durum wheat and triticale grain naturally contaminated by mycobiota. *Frontiers in Plant Science 7,* 1-16.

Campagnoli, A., Cheli, F., Polidori, C., Zaninelli, M., Zecca, O., Savoini, G., Dell'Orto, V., 2011. Use of the electronic nose as a screening tool for the recognition of durum wheat naturally contaminated by deoxynivalenol: A preliminary approach. *Sensors 11,* 4899-4916.

Campagnoli, A., Cheli, F., Savoini, G., Crotti, A., Pastori, A.G.M., Dell'Orto, V., 2009. Application of an electronic nose to detection of aflatoxins in corn. *Veterinary Research Communication 33 (1),* 273-275.

Casalinuovo, I.A., Donato, D.P., Coletta, M., Di Francesco, P., 2006. Review: Application of electronic noses for disease diagnosis and food spoilage detection. *Sensors 6,* 1428-1439.

CDC, 2004. Outbreak of aflatoxin poisoning-Eastern and Central provinces Kenya. *Morbidity Mortal Weekly Report 53,* 3-5.

CDC, 2005. Aflatoxicosis Outbreak, Kenya. *Morbidity and Mortality Weekly Report 53, (34),* 790-793.

Cellini, A., Blasioli, S., Biondi, E., Bertaccini, A., Braschi, I., Spinelli, F., 2017. Potential applications and limitations of electronic nose devices for plant disease diagnosis: Review. *Sensors 17,* 1-13.

Chebon, S., Wanyoike, W., Bii, C., Gathumbi, J., Ogoyi, D., 2016. Incidence of aflatoxigenic fungi and aflatoxins in maize cultivated in highland and mid-altitude agro-ecological zones in Kenya. *Journal of Scientific Research and Reports 9,* 1-12.

Cheli, F., Campagnoli, A., Pinotti, L., Dell'Orto, V., 2012. Rapid methods as analytical tools for food and feed contaminant evaluation. In: Aladjadjiyan, A. (Ed.), Methodological Implications for Mycotoxin Analysis in Cereals, Food Production - Approaches, Challenges and Tasks. InTech, pp. 185-204.

Cheli, F., Campagnoli, A., Pinotti, L., Savoini, G., Dell'Orto, V., 2009. Electronic nose for determination of aflatoxins in maize. *Biotechnology Agronomy Social Environment 13,* 39-43.

Cheli, F., Pinotti, L., Ottoboni, M., Tretola, M., Dell'Orto, V., 2016. Cereal industry: Electronic nose for real time and online quality and safety control and management. *Sensors and Transducers 201,* 52-58.

Chen, Q., Zhao, J., Chen, Z., Lin, H., Zhao, D., 2011. Discrimination of green tea quality using the electronic nose technique and the human panel test, comparison of linear and nonlinear classification tools. *Sensors and Actuators B: Chemical 159,* 294-300.

Codex, 2010. Codex general standard for contaminants and toxin in food and feed. *Codex Standard 193-1995,* 1-39.

Collins, G.S., Groot, J.A.d., Dutton, S., Omar, O., Shanyinde, M., Tajar, A., Voysey , M., Wharton, R., Yu, M., Moons, K.G., Altman, D.G., 2014. External validation of multivariable prediction models: A systematic review of methodological conduct and reporting. *Medical Research Methodology 14(40),* 1-11.

Consonnia, V., Ballabioa, D., Todeschini, R., 2010. Evaluation of model predictive ability by external validation techniques. *Journal of Chemometrics 24,* 194-201.

Corcoran, P., Lowery, P., Anglesea, J., 1998. Optimal configuration of a thermally cycled gas sensor array with neural network pattern recognition. *Sensors and Actuators B: Chemical 48,* 448-455.

Cortes, C., Vapnik, V., 1995. Support-vector networks. *Machine Learning 20,* 273-297.

Cotty, Jaime-Garcia, R., 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *International Journal of Food Microbiology, 119,* 109–115.

Cover, T., Thomas, J.A., 2006. Elements of information theory. John Wiley & Sons, Inc., New York, pp. 12-29.

Cucullu, A., Lee, L., Mayne, R., Goldblatt, L., 1966. Determination of aflatoxins in individual peanuts and peanut sections. *Journal of the American Oil Chemists Society 43,* 89-92.

Cui , X., Muhammad, I., Li, R., Jin, H., Guo, Z., Yang, Y., Hamid, S., Li, J., Cheng, P., Zhang, X., 2017. Development of a UPLC-FLD method for detection of Aflatoxin B1 and M1 in animal tissue to study the effect of curcumin on mycotoxin clearance rates. *Frontiers in Pharmacology 8,* 1-10.

Daniel, J., Lewis, L., Redwood, Y., Kieszak, S., Breiman, R., Flanders, W., Bell, C., Mwihia, J., Ogana, G., Likimani, S., Straetemans, M., McGeehin, 2011. Comprehensive assessment of maize aflatoxin levels in Eastern Kenya. *Environmental Health Perspectives, 119 (12),* 1794-1799.

de Castro, L., Vargas, E., 2001. Determining Aflatoxins B1, B2, G1 and G2 in maize using florisil clean up with thin layer chromatography and visual and densitometric quantification. *Food Science and Technology 21,* 115-122.

De Lacy, C., Evans, P., Gunson, R., Jones, H., Ratcliffe, P., Spencer, N., Phillips, P., 2001. Gas chromatography-mass spectrometry analyses of volatile organic compounds from potato tubers inoculated with *Phytophthora infestans* or *Fusarium coeruleum*. *Plant Pathology 50,* 489–496.

De Lucca, A., Boue, S.M., Carter-Wientjes, C., Bhatnagar, D., 2012. Volatile profiles and aflatoxin production by toxigenic and non-toxigenic isolates of *Aspergillus flavus* grown on sterile and non-sterile cracked corn. *Annals of Agricultural and Environmental Medicine 19,* 91-98.

De Lucca, A., Boue, S.M., Carter-Wientjes, C.H., Bland, J.M., Bhatnagar, D.B., Cleveland, T.E., 2010. Volatile profiles of toxigenic and non-toxigenic *Aspergillus flavus* using SPME for solid phase extraction. *Annals of Agricultural and Enviromental Medicine 17,* 291-298.

De Maesschalck, R., Jouan-Rimbaud, D., Massart, D.L., 2000. The Mahalanobis distance. *Chemometrics and Intelligent Laboratory Systems 50,* 1-18.

Demyttenaere, J., Moriña, R., De Kimpe, N., Sandra, P., 2004. Use of headspace solid-phase microextraction and headspace sorptive extraction for the detection of the volatile metabolites produced by toxigenic *Fusarium* species. *Journal of Chromatography A 1027,* 147-154.

Dermarne, V., Sanjine, S.R., 1992. Thin film semiconducting metal oxide gas sensors. In: Sbverveglieri, G. (Ed.), Gas Sensors. Kluwer Academic Publishers, Dordrecht, pp. 89-116.

Di Carlo, S., Falasconi, M., 2012. Drift correction methods for gas chemical sensors in artificial olfaction systems. In: Wang, W. (Ed.), Techniques and Challenges, Advances in Chemical Sensors. InTech Europe, pp. 305-326.

Di Natale, C., Macagnano, A., Paolesse, R., D'Amico, A., 2001. Artificial olfaction systems: principles and applications to food analysis. *Biotechnology Agronomy Social Environment 5,* 159-165.

Di Rosa, A.R., Leone, F., Cheli, F., Chiofalo, V., 2017. Fusion of electronic nose, electronic tongue and computer vision for animal source food authentication and quality assessment: A review. *Journal of Food Engineering 210,* 62-75.

Dickinson, T.A., White, J., Kauer, J.S., Walt, D.R., 1998. Current trends in `artificial-nose' technology. *Trends in Biotechnology 16,* 250-258.

Dixon, S.J., Brereton, R.G., 2009. Comparison of performance of five common classifiers represented as boundary methods: Euclidean distance to centroids, Linear discriminant analysis, Quadratic discriminant analysis, Learning vector quantization and Support vector machines, as dependent on data structure. *Chemometrics and Intelligent Laboratory Systems 95,* 1-17.

Doleman, B.J., Lonergan, M.C., Severin, E.J., Vaid, T.P., Lewis, N.S., 1998. Quantitative study of the resolving power of arrays of carbon black-polymer composites in various vapor-sensing tasks. *Analytical Chemistry 70,* 4177-4190.

Dorner, J.W., Cole, R.J., Diener, U.L., 1984. The relationship of *Aspergillus flavus* and *Aspergillus parasiticus* with reference to production of aflatoxins and cyclopiazonic acid. *Mycopathologia, 87 (1-2),* 13-15.

Dors, G.C., Caldas, S.S., Feddern, V., Bemvenuti, R.H., Hackbart, H.C., Moraes de Souza, M., Oliveira, M.S., Ednei, J.F., Primel, G., Badiale-Furlong, E., 2011. Aflatoxins: Contamination, analysis and control. In: Guevara-Gonzalez, R.G. (Ed.), Aflatoxins- Biochemistry and Molecular Biology. InTech., pp. 415-438.

Ehrlich, K.C., 2014. Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops:Advantages and limitations. *Frontiers in Microbiology 5,* 1-9.

Eifler, J., Martinelli, E., Santonico, M., Capuano, R., Schild, D., Di Natale, C., 2011. Differential detection of potentially hazardous *Fusarium* species in wheat grains by an electronic nose. *PLoS One 6,* 1-6.

Eisert, R., Pawliszyn, J., 1997. New trends in solid-phase microextraction. *Critical Reviews in Analytical Chemistry 27,* 103-135.

El Akadi, A., El Ouardighi, A., Aboutajdine, D., 2008. A powerful feature selection approach based on mutual information. *International Journal of Computer Science and Network Security 8 (4),* 116-121.

Entezari-Maleki, R., Rezaei, A., Minaei-Bidgoli, B., 2009. Comparison of classification methods based on the type of attributes and sample size. *Journal of Convergence Information Technology 4,* 1-9.

Espinosa-Calder, A., Contreras-Medina, L., Huerta, R.F., Almaraz, J.R., Gonzlez, R., Torres-Pacheco, I., 2011. Methods for detection and quantification of aflatoxins. In: Torres-Pacheco, I. (Ed.), Aflatoxins: Detection, Measurement and Control. InTech, pp. 109-128.

Falasconi, M., Gobbi, C.E., Sberveglieri, V., Pulvirenti, A., Sberveglieri, G., 2012. Review article: Electronic nose for microbiological quality control of food products. *International Journal of Electrochemistry 2012,* 1-12.

Falasconi, M., Gobbi, E., Pardo, M., Della Torre, M., Bresciani, A., Sberveglieri, G., 2005. Detection of toxigenic strains of *Fusarium verticillioides* in corn by electronic olfactory system. *Sensors and Actuators B: Chemical 108,* 250-257.

Fang, L., Zhao, H., Wang, P., Yu, M., Yan, J., Cheng, W., Chen, P., 2015. Feature selection method based on mutual information and class separability for dimension reduction in multidimensional time series for clinical data. *Biomedical Signal Processing and Control 21,* 82-89.

Fauguel, C.M., Bermudez, V.A., Fernandeza, M., Farronia, A., Andreob, C.S., Preselloa, D.A., 2017. Volatile compounds released by maize grains and silks in response to infection by *Fusarium verticillioides* and its association with pathogen resistance. *Plant Pathology 66,* 1128-1138.

Fennell, D.I., Bothast, R.J., Lillehoj, E.B., Peterson, R.E., 1973. Bright greenish-yellow fluorescence and associated fungi in white corn naturally contaminated with aflatoxin. *Cereal Chemistry. 50,* 404-414.

Filazi, A., Sireli, U.T., 2013. Occurrence of aflatoxins in food. In: Razzaghi-Abyaneh, M. (Ed.), Aflatoxins - Recent Advances and Future Prospects. InTech, pp. 143-169.

Fleuret, F.O., 2004. Fast binary feature selection with conditional mutual information. *Journal of Machine Learning Research 5,* 1531–1555

Fountain, J.C., Scully, B.T., Ni, X., Kemerait, R.C., Lee, R.D., Chen, Z., Guo, B., 2014. Environmental influences on maize-*Aspergillus flavu*s interactions and aflatoxin production. *Frontiers in Microbiology 5,* 1-7.

Garcia-Cela, E., E, K., Medina, A., Sulyok, M., Krska, R., Magan, N., 2018. Interacting environmental stress factors affects targeted metabolomic profiles in stored natural wheat and that inoculated with *Fusarium graminearum*. *Toxins 10,* 1-14.

Gardner, J.W., Hines, P.B., E.L, 2005. Enhancing electronic nose performance by sensor selection using a new integer-based genetic algorithm approach. *Sensors and Actuators B 106,* 114–121.

Gobbi, E., Falasconi, M., Torelli, E., Sberveglieri, G., 2011. Electronic nose predicts high and low fumonisin contamination in maize cultures. *Food Research International 44,* 992-999.

Gobbi, E., Locci, R., 2006. An electronic nose to evaluate fungal contamination. *Gjornal Furlan des Siencis 7,* 71-82.

Gou, J., Du, L., Zhang, Y., Xiong, T., 2012. A new distance-weighted *k*-nearest neighbor classifer. *Journal of Information and Computational Science 9,* 1429–1436.

Gouinguene, S.P., Turlings, T.C., 2002. The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiology 129,* 1296-1307.

Grace, D., Mahuku, G., Hoffmann, V., Atherstone, C., Upadhyaya, H.D., Bandyopadhyay, R., 2015. International agricultural research to reduce food risks: Case studies on aflatoxins. *Food Security 7,* 569–582.

Gromski, P.S., Correa, E., Vaughan,.A., Wedge, D., Turner, L., Goodacre, R., 2014 A comparison of different chemometrics approaches for the robust classification of electronic nose data. *Analytical Bioanalytical Chemistry 406,* 7581–7590.

Gujar, A., Anderson, T., Cavagnino, D., Patel, A., 2018. Comparative analysis of mass spectral matching for confident compound identification using the advanced electron ionization source for GC-MS. *Thermo Scientific Technical Note 10598,* 1-7.

Gutierrez-Osuna, R., 2002. Pattern Analysis for Machine Olfaction: A Review. *Institute of Electrical and Electronics Sensors Journal 3,* 189-202.

Gutierrez, J., Getino, J., Horrillo, M.C., Arés, L., Robla, J., Garcıa, C., Sayago, I., 1998. Electrical characterization of a thin film tin oxide sensor array for VOCs detection. *Thin Solid Films 317,* 429-431.

Guyon, I., Elisseeff, A., 2003. An introduction to variable and feature selection. *Journal of Machine Learning Research 3,* 1157-1182.

Harsanyi, G., 2000. Polymer films in sensor applications: A review of present uses and future possibilities. *Sensor Review 20,* 98-105.

Harvey, J., Gnonlonfin, B., Fletcher, M., Fox, G., Trowell, S., Berna, A., Nelson, R., Darnell, R., 2013. Improving diagnostics for aflatoxin detection. In: Unnevehr, L., Grace, D. (Eds.), Aflatoxins: Finding Solutions for Improved Food Safety. International Food Policy Research Institute Washington, DC USA.

Hassan, R.M., 1996. Planting strategies of maize farmers in Kenya: A simultaneous equations analysis in the presence of discrete dependent variables. *Agricultural Economics 15,* 137-149.

Hassanshahian, M., Mohamadi, H., Abbaspour, H., 2012. Comparisons of different methods for detection of aflatoxin in grain and grain products. *Acta Horticulturae International Symposium on Mycotoxins in Nuts and Dried Fruits 963(45),* 275-280.

Hella, K., Cardwella, K.F., Setamoub, M., Poehling, H.M., 2000. The influence of storage practices on aflatoxin contamination in maize in four agroecological zones of Benin, West Africa. *Journal of Stored Products Research 36,* 365- 382.

Heilig, A., Bârsan, N., Weimar, U., Schweizer-Berberich, M., Gardner, J.W., Göpel, W., 1997. Gas identification by modulating temperatures of SnO_2-based thick film sensors. *Sensors and Actuators B: Chemical 43,* 45-51.

Hell, K., Bandyopadhyay, P., Kiewnick, S., 2008. Pre-and post harvest management of aflatoxin in maize. An African perspective. In: Leslie, J.F., Bandyopadhyay, R., Visconti, A. (Eds.), Mycotoxin, Methods, Management, Public Health and Agriculture Trade. CAB International, pp. 219-243.

Hell, K., Mutegi, C., 2011. Aflatoxin control and prevention strategies in key crops of Sub-Saharan Africa. *African Journal of Microbiology Research 5,* 459-466.

Hines, E.L., Llobet, E., Gardner, J.W., 1999. Electronic noses: A review of signal processing techniques. *Institute of Electrical and Electronics Proc-circuits Devices Systems,146,* 297-310.

Hodgins, D., Sirnmonds, D., 1995. The electronic nose and its application to manufacture of food products. *Journal of Automatic Chemistry 17,* 179-185.

Hoffmann, V., Gatobu, K.M., 2014. Growing their own: Unobservable quality and the value of self-provisioning. *Journal of Development Economics 106,* 168–178.

Hoffmann, V., Mutiga, S., Harvey, J., Milgroom, M., Nelson, R., 2013. A Market for Lemons: Maize in Kenya. Working Paper,pp 1-41

Holcomb, M., Wilson, D.M., Trucksess, M.W., Thompson, H.C., 1992. Determination of aflatoxins in food products by chromatography. *Journal of Chromatography A 624,* 341-352.

Hung, R., Lee, S., Bennett, J.W., 2015. Mini review: Fungal volatile organic compounds and their role in ecosystems. *Applied Microbiology and Biotechnology 99(8),* 3395-3405.

Hussein, H.S., Brasel, J.M., 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology 167,* 101-134.

IARC, 1993. Monograph on the evaluation of carcinogenic risk to humans. *International Agency for Research on Cancer, Lyon, 56,* 257-263.

Insam, H., Seewald, M.S.A., 2010. Review: Volatile organic compounds (VOCs) in soils. *Biology and Fertility of Soils 46,* 199-213.

Jaetzold, R., Schmidt, H., Hornetz, B., Shisanya, C., 2007. Farm management handbook of Kenya: part C, East Kenya. Ministry of Agriculture Nairobi.

James, D., M., S., Scott, Z., William, T., 2005. Review: Chemical sensors for electronic nose systems. *Microchimica. Acta 149,* 1-17.

Janata, J., Josowicz, M., 2003. Conducting polymers in electronic chemical sensors. *Nature materials 2,* 19-24.

Jayaram, M.A., Karegowda, A.G., Manjunath, A.S., 2010. Feature subset selection problem using wrapper approach in supervised learning. *International Journal of Computer Applications 1,* 13-17.

Jelen, H., Grabarkiewicz-szczeüsna, J., 2005. Volatile compounds of *Aspergillus* strains with different abilities to produce ochratoxin A. *Journal of Agricultural and Food Chemistry 53,* 1678-1683.

Jelen, H., Wasowicz, E., 1998. Volatile fungal metabolites and their relation to the spoilage of agricultural commodities. *Food Reviews International 14,* 391-426.

Jelen, H., Wasowicz, E., 2000. Application of solid phase micro extraction (SPME) for the determination of fungal volatile metabolites. In: Bielecki, S., Tramper, J., Polak, J. (Eds.), Food Biotechnology. Elsevier Science Bv, Amsterdam, pp. 369-377.

Jelen, H., Mirocha, C.J., Wasowicz, E., Kaminski, E., 1995. Production of volatiles sesquiterpenes by *Fusarium sambucinum* strains with different abilities to synthesize trichothecens. *Applied and Environmental Microbiology 61,* 3815−3820.

Jiacheng, M., Tinglin, Z., You , W., Guang , L., 2015. Optimal sensor selection for classifying a set of ginsengs using metal-oxide sensors. *Sensors 15,* 16027-16039.

Jinap, R.S., Soleimany, F., 2009. Qualitative and quantitative analysis of mycotoxins. *Comprehensive Reviews in Food Science and Food Safety 8,* 202-251.

Johansson, A.S., Whitaker, T.B., Hagler, W.M., Giesbrecht, F.G., Young, J.H., Bowman, D.T., 2000. Testing shelled corn for aflatoxin, part I: estimation of variance components. *Journal of the Association of Official Agricultural Chemists 83,* 1264–1269.

Jolly, P.E., Jiang, Y., Ellis, W.O., Awuah, R.T., Appawu, J., Nnedu, O., Stiles, J.K., Wang, J., Adjei, O., Jolly, C.M., Williams, J.H., 2007. Association between aflatoxin exposure and health characteristics, liver function, hepatitis and malaria infections in Ghanaians. *Journal of Nutritional and Environmental Medicine 16,* 242-257.

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

Jurjevic, Z., Rains, G.C., Wilson, D.M., Lewis, J., 2008. Volatile metabolites associated with one aflatoxigenic and one non-toxigenic *Aspergillus flavus* strain grown on two different substrates. *Phytopathology Mediterranean 47,* 266-271.

Justice, A.C., Covinsky, K.E., Berlin, J.A., 1999. Assessing the generalizability of prognostic information. *Annals of Internal Medicine 130,* 515-524.

Kaaya, A.N., Kyamuhangire, W., 2006. The effect of storage time and agroecological zone on mould incidence and aflatoxin contamination of maize from traders in Uganda. *International Journal of Food Microbiology 110,* 217-223.

Kalkana, H., Gunesa, A., Durmusa, E., Kuscu, A., 2014. Non-invasive detection of aflatoxin-contaminated figs using fluorescence and multispectral imaging. *Food Additives and Contaminants: Part A 31,* 1414–1421.

Kaminski, E., Libbey, L.M., Stawicki, I.S., Wasowicz, E., 1972. Identification of the predominant volatile compounds produced by *Aspergillus flavus*. *Applied Microbiology 24,* 721-726.

Kangethe, E., Langa, K.A., 2009. Aflatoxin B1 and M1 contamination of animal feeds and milk from urban centers in Kenya. *African Health Sciences 9,* 218-226.

Karlshoi, K., Larsen, T.O., 2005. Differentiation of species from the *Penicillium roqueforti* group by volatile metabolic profiling. *Journal of Agricultural and Food Chemistry 53,* 708-715.

Kataoka, H., Lord, H.L., Pawliszyn, J., 2000. Applications of solid-phase microextraction in food analysis. *Journal of Chromatography A 880,* 35-62.

Kempler, G.M., 1983. Production of flavor compounds by microorganisms. In: Laskin, A.I. (Ed.), Advances in Applied Microbiology. Academic Press, pp. 29-51.

Kershi, G., Magan, N., Voysey, P., 1988. Use of an electronic nose for the early detection and differentiation between spoilage fungi. *Letters in Applied Microbiology 27,* 261–264.

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

Kim, H., Lee, J., 2014. Highly sensitive and selective gas sensors using p-type oxide semiconductors: Overview. *Sensors and Actuators B: Chemical 192,* 607-627.

Kimatu, J.N., McConchie, R., Xie, X., Nguluu, S.N., 2012. Review article: The significant role of post-harvest management in farm management, aflatoxin mitigation and food security in sub-Saharan Africa. *Greener Journal of Agricultural Sciences 2,* 279-288.

Kinderlerer, J.L., 1989. Volatile metabolites of filamentous fungi and their role in food flavour. *Journal of Applied Bacteriology Symposium Supplement 1989,* 133–144.

Kiunga, S.K., 2015. Climate change and agriculture in Kenya: Possible impacts on maize production. *Asian Journal of Multidisciplinary Studies 3,* 56-69.

Klich, A.A., 2007. Pathogen profile. *Aspergillus flavus*: The major producer of aflatoxin. *Molecular Plant Pathology 8,* 713–722.

Klich, M.A., Pitt, J.I., 1988. Differentiation of *Aspergillus flavus* from *Aspergillus. parasiticus* and other closely related species. *Transactions of the British Mycological Society 91,* 99–108.

Koech, O.K., Kinuthia, R.N., Mureithi, S.M., Karuku, G.N., Wanjogu, R., 2014. Effect of different soil water content and seed storage on quality of six range grasses in the semi-arid ecosystems of Kenya. *Environment and Ecology Research 2,* 261-271.

Korpi, A., Järnberg, J., Pasanen, A.-L., 2009. Microbial volatile organic compounds. *Critical Reviews in Toxicology 39,* 139-193.

Kralj, I.C., Prosen, H., 2009. An overview of conventional and emerging analytical methods for the determination of mycotoxins. *International Journal of Molecular Science 10,* 62-115.

Kraskov, A., Stogbauer H., 2004. Estimating mutual information. *Physical Review Statistical, Nonlinear, and Soft Matter Physics 69,* 1-16.

Kumar, V., Basu, M.S., Rajendran, T.P., 2008. Mycotoxin research and mycoflora in some commercially important agricultural commodities. *Crop Protection 27,* 891-905.

Kurtzman, C.P., Horn, B.W., Hesseltine, C.W., 1987. *Aspergillus nomiu*s, a new aflatoxin producing species related to *Aspergillus flavus* and *Aspergillus tamarii. Antonie van Leeuwenhoek 53,* 147-158.

Kuske, M., Romain, A., Nicolas, J., 2005. Microbial volatile organic compounds as indicators of fungi: Can an electronic nose detect fungi in indoor environments?. *Building and Environment 40,* 824–831.

Laddomada, B., Del Coco, L., Durante, M., Presicce, D.S., Siciliano, P.A., Fanizzi, F.P., Logrieco, A.F., 2014. Volatile metabolite profiling of durum wheat kernels contaminated by *Fusarium poae*. *Metabolites 17,* 932-945.

Lee, N., Wang, S., Allan, R.D., Kennedy, A., 2004. Rapid aflatoxin B1 ELISA: Development and validation with reduced matrix effects for peanuts, corn, pistachio and soybeans. *Journal of Agricultural and Food Chemistry 52,* 2746-2755.

Leopold, J.H., Bos, L.D.J., Sterk, P.J., Schultz, M.J., Fens, N., Horvath, I., Bikov, A., Montuschi, P., Di Natale, C., Yates, D.H., Abu-Hanna, A., 2015. Comparison of classification methods in breath analysis by electronic nose. *Journal of Breath Research 9,* 1-12.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, M.A., Misore, A., DeCock, K., Rubin, C., 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. *Environmental Health Perspective 113,* 1763-1767.

Li, A., Caspers, M., Punt, P., 2013. A systems biology approach for the identification of target genes for the improvement of itaconic acid production in *Aspergillus* species. *BioMedCentral Research Notes 6,* 1-5.

Li, P., Zhang, Q., Zhang, W., 2009. Immunoassays for aflatoxins. *Trends in Analytical Chemistry 28,* 1115-1126.

Lippolis, V., Ferrara, M., Cervellieri, S., Damascelli, A., Epifani, F., Pascale, M., Perrone, G., 2016. Rapid prediction of ochratoxin A-producing strains of *Penicillium* on dry-cured meat by MOS-based electronic nose. *International Journal of Food Microbiology 218,* 71-77.

Lippolis, V., Pascale, M., Cervellieri, S., Damascelli, A., Visconti, A., 2014. Screening of deoxynivalenol contamination in durum wheat by MOS-based electronic nose and identification of the relevant pattern of volatile compounds. *Food Control 37,* 263-271.

Lizier, J.T., 2014. An information-theoretic toolkit for studying the dynamics of complex systems. *Frontiers in Robotics 1,* 1-37.

Logrieco, A., Arrigan, D.W.M., Brengel-Pesce, K., Siciliano, P., Tothill, I., 2005. DNA arrays, electronic noses and tongues, biosensors and receptors for rapid detection of toxigenic fungi and mycotoxins: A review. *Food Additives and Contaminants 22,* 335-344.

Loutfi, A., Coradeschi, S., Mani, G.K., Shankar, P., Rayappan, J.B., 2015. Electronic noses for food quality: A review. *Journal of Food Engineering 144,* 103-111.

Macías, M.M., Agudo, J.E., Manso, A.G., Orellana, C.J.G., Velasco, H.M.G., Caballero, R.G., 2014. Improving short term instability for quantitative analyses with portable electronic noses. *Sensors 14,* 10514-10526.

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

Maina, A.W., Wagacha, J.M., Mwaura, F.B., Muthomi, J.W., Woloshuk, C.P., 2016. Post harvest practices for farmers in Kaiti district Kenya and the impact of hermatic storage on population of *Asperigillus* spp and aflatoxin contamination. *Journal of Food Research 5,* 53-66.

Manetta, A.C., 2011. Aflatoxins: Their measure and analysis. In: Guevara-Gonzalez, R.G. (Ed.), Aflatoxins- Biochemistry and Molecular Biology. InTech, pp. 93 -108.

Maragos, C.M., 2009. Fluorescence polarization immunoassay of mycotoxins: A review. *Toxins 1,* 196-207.

Martinelli , F., Scalenghe , R., Davino, S., Panno, S., Scuderi, G., Ruisi , P., Villa, P., Stroppiana , D., Boschetti, M., Goulart , L.R., Davis , C.E., Dandekar, A.M., 2014. Advanced methods of plant disease detection. A review. *Agronomy for Sustainable Development 35,* 1-25.

Mati, B.M., 2000. The influence of climate change on maize production in the semi-humid-semi-arid areas of Kenya. *Journal of Arid Environments 46,* 333-334.

Maxim, L.D., Niebo, R., Utell, M.J., 2014. Screening tests: A review with examples. *Inhalation toxicology 26,* 811-828.

Mbithi, L.M., VanHuylenbroeck, L., 2000. A comparison of maize production in Kenya before and during liberalisation period. *Tropicultura, 18 (4),* 181-188.

Mielle, P., 1996. Electronic noses: Towards the objective instrumental characterization of food aroma. *Trends in Food Science and Technology 7*, 432-438.
Migwi, B., G, 2016. Assessment of farmers' perceptions of and willingness to pay for aflasafe KE01, a biological control for aflatoxins in Kenya. Agricultural Economics. University of Nairobi, p. 107.
Morath, S.U., Hung, R., Bennett, J.W., 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biology Reviews 26*, 73-83.
Mostafa, A., Armin, A., Hamid, P., Reza, M.A., 2012. Rapid detection methods for analysis of fungi and mycotoxins in agriculture products. *Research Journal of Recent Sciences 1*, 90-98.
Murphy, P.A., Hendrich, S., Bryant, C.M., 2006. Food Mycotoxins: An update. *Journal of Food Science 71*, 51-65.
Mutegi, C., Wagacha, M., Kimani, J., Otieno, G., Wanyama, R., Hell, K., Christie, M.E., 2013. Incidence of aflatoxin in peanuts (*Arachis hypogaea*, Linnaeus) from markets in Western, Nyanza and Nairobi Provinces of Kenya and related market traits. *Journal of Stored Products Research, 52*, 118-127.
Muthomi, J.W., Njenga, L.N., Gathumbi, J.K., Chemining'wa, P., 2009. The occurrence of mycotoxin-producing fungi in Eastern Kenya. *Plant Pathology Journal 8*, 113-119.
Mutiga, S.K., Hoffmann, V., Harvey, J.W., Milgroom, M.G., Nelson, R., J., 2015. Assessment of aflatoxin and fumonisin contamination of maize from Western Kenya. *Phytopathology, Post Harvest Pathology and Mycotoxins 105*, 1251-1261.
Mutiga, S.K., Morales, L., Angwenyi, S., Wainaina, J., Harvey, J., Das, B., Nelson, R.J., 2017. Association between agronomic traits and aflatoxin accumulation in diverse maize lines grown under two soil nitrogen levels in Eastern Kenya. *Field Crops Research 205*, 124-134.
Mutiga, S.K., Were, V., Hoffmann, V., Harvey, J.W., Milgroom, M.G., Nelson, R.J., 2014. Extent and drivers of mycotoxin contamination: Inferences from a survey of Kenyan maize mills. *Phytopathology Postharvest Pathology and Mycotoxins 104*, 1221-1231.
Nangindu, A., Johnson, B.K., Kenya, P.R., Ngira, J.A., Ochieng, D.M., Nandwa, H., Omondi, T.N., Janse N, A.J., Ngare, W, Kaviti, P.R., Gatei, D., Siongok, T.A., 1982. Outbreak of acute hepatitis caused by aflatoxin in Kenya. *Lancet 319*, 1346-1348.
Ngeno, V., Mengist, C., Langat, B.K., Nyangweso, P.M., Serem, A.K., Kipsat, M.J., 2011. Technical efficiency of maize farmers in Uasin Gishu district, Kenya. *African Crop Science Conference Proceedings 10*, 41-47.
Nieboer, D., Ploeg, T., Steyerberg, E.W., 2016. Assessing discriminative performance of external validation of clinical prediction models. *PLoS One 11*, 1-10.
Niederbacher, B., Winkler, J.B., Schnitzler, J.P., 2015. Review Paper: Volatile organic compounds as non-invasive markers for plant phenotyping. *Journal of Experimental Botany 66*, 5403–5416.
Nielsen, K., Lin, M., Gall, D., Jolley, M., 2000. Fluorescence polarization immunoassay: detection of antibody to *Brucella abortus*. *Methods 22*, 71-76.
Nowotny, T., Berna, A.Z., Binions, R., Trowell, S., 2013. Optimal feature selection for classifying a large set of chemicals using metal oxide sensors. *Sensors and Actuators B: Chemical 187*, 471-480.
Nyaga, P.N., 2010. Report on aflatoxin contamination in maize. In: Agriculture, M.O. (Ed.), Retrieved on 14th February 2018 from http://www.fao.orghttp://www.fao.org.
Nyoro, J.K., Ayieko, M., Muyanga, M., 2007. The compatibility of trade policy with domestic policy interventions affecting the grains sector in Kenya. In: FAO (Ed.), FAO's workshop Trade and Policy for Food Products Conducive to Development, Rome, Italy.
Odhiambo, B.O., Murage, H., Wagara, I.N., 2013. Isolation and characterisation of aflatoxigenic *Aspergillus* species from maize and soil samples from selected counties of Kenya. *African Journal of Microbiology Research 7*, 4379-4388.

Okoth, S., De Boevre , M., Vidal , A., Di Mavungu, J.D., Landschoot , S., Kyallo, M., Njuguna, J., Harvey, J., De Saeger, S., 2018. Genetic and toxigenic variability within *Aspergillus flavus* population isolated from maize in two diverse environments in Kenya. *Frontiers in Microbiology 9,* 1-14.

Okoth, S., Nyongesa, B., Ayugi, V., Kang'ethe, E., Korhonen, H., Joutsjoki, V., 2012. Toxigenic potential of *Aspergillus* species occurring on maize kernels from two Agro-ecological zones in Kenya. *Toxins 4,* 991-1007.

Okoth, S.A., Kola, M.A., 2012. Market samples as a source of chronic aflatoxin exposure in Kenya. *African Journal of Health Sciences 20,* 56-61.

Olsson, J., Borjesson, T., Lundstedt, T., Schnürer, J., 2002. Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose. *International Journal of Food Microbiology 72,* 203-214.

Onono, A.P., Wawire, N., Ombuki, C., 2013. The response of maize production in Kenya to economic incentives. *International Journal of Development and Sustainability 2,* 1-14.

Otunge, D., Muchiri, N., Wachoro, G., Gethi, J., Agili, G., 2010. Reducing maize insecurity in Kenya: The WEMA project. African Advanced Technology Foundation Policy Brief, pp. 1-2.

Owaga, E., Muga, R., Mumbo, H., Aila, F., 2011. Chronic dietary aflatoxins exposure in Kenya and emerging public health concerns of impaired growth and immune suppression in children. *International Journal of Biological and Chemical Science 5,* 1325-1336.

PACA, 2013. Aflatoxin impacts and potential solutions in agriculture, trade, and health. A background paper for the PACA strategy development stakeholder consultation workshop. 1-11.

Padilla, M., Perera, A., Montoliu, I., Chaudry, A., Persaud, K., Marco, S., 2010. Drift compensation of gas sensor array data by orthogonal signal correction. *Chemometrics and Intelligent Laboratory Systems 100,* 28-35.

Paolesse, R., Alimelli, A., Martinelli, E., Natale, C.D., D'Amico, A., D'Egidio, M.G., Aureli, G., Ricelli, A., Fanelli, C., 2006. Detection of fungal contamination of cereal grain samples by an electronic nose. *Sensors and Actuators B: Chemical 119,* 425-430.

Pasanen, A., Lappalainen, S., Pasanen, P., 1996. Volatile organic metabolites associated with some toxic fungi and their mycotoxins. *Analyst 121,* 1949–1953.

Pascale, M.N., 2009. Detection methods for mycotoxins in cereal grains and cereal products. *Proc Nat Sci, Matica Srpska Novi Sad, 117,* 15-25.

Pearce, T.C., Gardner, J.W., Gopel, W., 1998. Strategies for mimicking olfaction: The next generation of electronic noses. *Sensors Update 3,* 61-130.

Pearson, C.T., Wicklow, T.D., Maghirang, B.E., Xie, F., Dowell, E.F., 2001. Detecting aflatoxin in single corn kernels by transmittance and reflectance spectroscopy. T*ransactions of the American Society of Agricultural Engineers 44,* 1247-1254.

Peris, M., Escuder-Gilabert, L., 2016. Electronic noses and tongues to assess food authenticity and adulteration. *Trends in Food Science and Technology 58,* 40-54.

Perkowski, J., Stuper, K., Buśko, M., Góral, T., Kaczmarek, A., Jelen, H., 2012. Differences in metabolomic profiles of the naturally contaminated grain of barley, oats and rye. *Journal of Cereal Science 56,* 544-551.

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

Philip, T.D., Breverly, A.C., Douglas, L.P., 1994. Approaches to reduction of aflatoxins in foods and feeds. In: Eaton, L.D.G., J. D (Ed.), The Toxicology of Aflatoxins. Academic Press, INC, San Diego. New York., pp. 383-406.

Presicce, D.S., Forleo, A., Taurino, A.M., Zuppa, M., Siciliano, P., Laddomada, B., Logrieco, A., Visconti, A., 2006. Response evaluation of an electronic nose towards contaminated wheat by *Fusarium poae* fungi. *Sensors and Actuators B: Chemical 118,* 433-438.

Prieto-Simo, B., Noguer, T., Campas, M., 2007. Emerging biotools for assessment of mycotoxins in the past decade. *Trends in Analytical Chemistry 26,* 689-702.

Probst, C., Bandyopadhyay, R., Price, L.E., Cotty, P.J., 2011. Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. *Plant Disease 95,* 212-218.

Probst, C., Njapau, H., Cotty, P.J., 2007. Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. *Applied and Environmental Microbiology 73,* 2762–2764.

Rahmani, A., Jinap, S., Soleimany, F., 2009. Qualitative and quantitative analysis of mycotoxins. *Comprehensive Reviews in Food Science and Food Safety 8,* 202-251.

Rai, M.K., Bonde, S.R., Ingle, P.A., Gade, A.K., 2012. Mycotoxin: Rapid detection, differentiation and safety. *Journal of Pharmacy Education and Research 3,* 22-34.

Rashid, S., Kurt.Rosentrater, Carl, B., 2013. Effects of deterioration parameters on storage of maize: A review. *Journal of Natural Sciences Research 13,* 2224- 3186.

Richard-Molard, D., Cahgnier, B., Poisson, J., Drapron, R., 1976. Comparative evolutions volatile constituents and microflora in maize stored under different temperature conditions and moisture. *Annals of Agricultural Technology 25,* 29-44.

Rock, F., N, B., U, W., 2008. Electronic nose: Current status and future trends. *Chemical Reviews 108,* 705–725.

Rodriguez-del-Bosque, L.A., 1996. Impact of agronomic factors on aflatoxin contamination in preharvest field corn in North-eastern Mexico. *Plant Disease 80,* 988-993.

Roseanu, A., Jecu, L., Badea, M., Evans, R.W., 2010. Mycotoxins: An overview on their quantification methods. *Roman Journal of Biochemistry 47,* 79-86.

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

Sakai, Y., Kadosaki, M., Matsubara, I., Itoh, T., 2009. Preparation of total VOC sensor with sensor-response stability for humidity by noble metal addition to SnO_2. *Journal of the Ceramic Society of Japan 117,* 1297-1301.

Salano, N.E., Obonyo, A.M., Jebet, T.F., Omondi, B., Aman, O., B, 2016. Diversity of putatively toxigenic *Aspergillus* species in maize and soil samples in an aflatoxicosis hotspot in Eastern Kenya. *African Journal of Microbiology Research 10,* 172-184.

Samson, R.A., Hong, S.B., Frisvad, J.C., 2006. Old and new concepts of species differentiation in *Aspergillus*. *Medical Mycology 44,* 133-148.

Sankaran, S., Mishra, A., Ehsani, R., Davis, C., 2010. A review of advanced techniques for detecting plant diseases. *Computers and Electronics in Agriculture 72,* 1-13.

Santra, A.K., Christy, J.C., 2012. Genetic algorithm and confusion matrix for document clustering. *International Journal of Computer Science Issues 9,* 322-328.

Schaller, E., Bosset, J.O., Escher, F., 1998. 'Electronic noses' and their application to food. *Food Science and Technology 31,* 305-316.

Schmidt, C.W., 2013. Breaking the mold: New strategies for fighting aflatoxins. *Environmental Health Perspectives 121,* 271-275.

Schnurer, J., Olsson, J., Börjesson, T., 1999. Fungal volatiles as indicators of food and feeds spoilage. *Fungal Genetics and Biology 27,* 209-217.

Schroeder, C., Onyango, K.O., Nar Bahadur, R., Jick, N.A., Parzies, H.K., Gemenet, D.C., 2013. Potentials of hybrid maize varieties for small-holder farmers in Kenya: A review based on swot analysis. *African Journal of Food Agriculture Nutrition and Development 13,* 7562-7586.

Schroeder, H.W., Boller, R.A., 1973. Aflatoxin production of species and strains of the *Aspergillus flavus* group isolated from field crops. *Applied Microbiology 25,* 885-889.

Schulz-Bohm, K., Martín-Sánchez, L., Garbeva, P., 2017. Microbial volatiles: Small molecules with an important role in intra- and inter-kingdom interactions. *Frontiers in Microbiology 8,* 1-10.

Scott, S.M., James, D., Ali, Z., 2007. Review: Data analysis for electronic nose systems. *Microchim Acta 156,* 183-207.

Shao, Y., Lunetta, R.S., 2012. Comparison of support vector machine, neural network, and CART algorithms for the land-cover classification using limited training data points. *Journal of Photogrammetry and Remote Sensing 70,* 78-87.

Shepard, G.S., 2003. Aflatoxin and food safety: Recent African perspectives. *Journal of Toxicology 22,* 267-286.

Sherif, M., Becker, E.M., Herrfurth, C., Feussner, I., Karlovsky, P., Splivallo, R., 2016. Volatiles emitted from maize ears simultaneously infected with two *Fusarium* species mirror the most competitive fungal pathogen. *Frontiers in Plant Science 7,* 1-11.

Shotwell, O.L., Hesseltine, C.W., 1981. Use of bright greenish yellow fluorescence as a presumptive test for aflatoxin in corn. *Cereal Chemistry 58,* 124–127.

Show, L.P., Oladele, K.O., Siew, Q.Y., Zakry, F.A., Lan , J.C., Ling, T.C., 2015. Overview of citric acid production from *Aspergillus niger*. *Frontiers in Life Science 8,* 271-283.

Sliwinska, M., Wisniewska, P., Dymerski, T., Namiesnik, J., Wardencki, W., 2014. Food analysis using artificial senses. *Journal of Agricultural and Food Chemistry 62,* 1423-1448.

Smith, L., Stasiewicz, M., Hestrin, R., Morales, L., Mutiga, S.K., Nelson, R., J., 2016. Examining environmental drivers of spatial variability in aflatoxin accumulation in Kenyan maize: Potential utility in risk prediction models. *African Journal of Food Agriculture and Nutritional Development 16,* 11086-11105.

Smith, S.D., Eremin, S.A., 2008. Fluorescence polarization immunoassays and related methods for simple, high-throughput screening of small molecules. *Analytical Bioanalytical Chemistry 391,* 1499-1507.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., Schuhmacher, R., 2010. Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma viride* by HS-SPME-GC-MS. *Journal of Microbiological Methods 81,* 187–193.

Stroka, J., Anklam, E., 2002. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. *Trends in Analytical Chemistry 21,* 90-95.

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R.V., Breiman, R., Brune, M.N., DeCock, K., Dilley, A., Groopman, J., Hell, K., Henry, S. H, Jeffers, D., Jolly, C., Jolly, P., Kibata, G.N., Lewis, L., Liu, X., Luber, G., McCoy, L., Mensah, P., Miraglia, M., Misore, A., Njapau, H., Ong, C.N., Onsongo, M.T., Page, S.W., Park, D., Patel, M., Phillips, T., Pineiro, M., Pronczuk, J., Rogers, H.S., Rubin, C., Sabino, M., Schaafsma, A., Shephard, G., Stroka, J., Wild, C., Williams, J.T., Wilson, D., 2006. Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. *Environmental Health Perspective 114,* 1898-1903.

Sun, D., She, J., Gower, J.L., Stokes, E.C., Windham, G.L., Baird, R.E., Mlsna, T.E., 2016. Effects of growth parameters on the analysis of *Aspergillus flavus* volatile metabolites. *Separations 3,* 1-20.

Sun, D., Wood-Jones, A., Wang, W., Vanlangenberg, C., Jones, D., Gower, J., Simmons, P., Baird, R.E., Mlsna, T.E., 2014. Monitoring MVOC profiles over time from isolates of *Aspergillus flavus* using SPME GC-MS. *Journal of Agricultural Chemistry and Environment 3,* 48-63.

Taleuzzaman, M., Ali, S., Gilani, S.J., Imam, S.S., Hafeez, A., 2015. Ultra performance liquid chromatography: A review. A*ustin Journal of Analytical and Pharmaceutical Chemistry 2,* 1-5.

Tarca, A.L., Carey, V.J., Chen, X., Romero, R., Draghici, S., 2007. Machine learning and its applications to biology. *Plos Computational Biology 3,* 953-963.

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

Turner, W.N., Subrahmanyam, S., Piletsky, S.A., 2009. A review analytical methods for determination of mycotoxins. *Analytica Chimica Acta 632,* 168–180.

USAID, 2010. Staple Foods Value Chain Analysis. Country Report-Kenya.

Vadlapudi, V., Borah, N., Yellusani, K.R., Gade, S., Reddy, P., Rajamanikyam, M., Vempati, L., Gubbala, S., Chopra, P., Upadhyayula, S.M., Amanchy, R., 2017. *Aspergillus* secondary metabolite database, a resource to understand the secondary metabolome of *Aspergillu*s genus. *Scientific Reports 7,* 1-10.

van Egmond, H., Jonker, M.A., 2004. Current situation on regulations for mycotoxins. *Japanese Society of Mycotoxicology 2003,* 1-15.

van Stralen, K.J., Stel, V.S., Reitsma, J.B., Dekker, F.W., Zoccali, C., Jager, K.J., 2009. Diagnostic methods: Sensitivity, specificity, and other measures of accuracy. *Kidney International 75,* 1257-1263.

Vandeventer, D., Mallikarjunan, P., 2002. Comparative performance analysis of three electronic nose systems using different sensor technologies in odor analysis of retained solvents on printed packaging. *Journal of Food Science 67,* 3170-3183.

Vergara, J.R., Estevez, P.A., 2014. A review of feature selection methods based on mutual information. *Neural Computng and Applications 24,* 175-186.

Vikram, A., Prithiviraj, B., Hamzehzarghani, Kushalappa, A.C., 2004. Volatile metabolite profiling to discriminate diseases of mcIntosh apple inoculated with fungal pathogens. *Journal of Science, Food and Agriculture 84,* 1333–1340.

Wacoo, A.P., Wendiro, D., Vuzi, P.C., Hawumba, J.F., 2014. Review article: Methods for detection of aflatoxins in agricultural food crops. *Journal of Applied Chemistry 2014,* 1-15.

Wagacha, J.M., Muthomi, J.W., 2008. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology 124,* 1-12.

Waliyar, F., Reddy, S.V., Lava-Kumar, P., 2009. Review of immunological methods for the quantification of aflatoxins in peanut and other foods. *Peanut Science 36,* 54-59.

Wang, X., Lizier, J., Nowotny, T., Berna, A., Prokopenko, M., Trowell, S.C., 2014. Feature selection for chemical sensor arrays using mutual information. *PLoS One 9(3),* 1-17.

Weidenbörner, M., 2017. Mycotoxin contamination of cereals and cereal products. Mycotoxins in Plants and Plant Products. Springer International Publishing, Cham, pp. 729-744.

Weinberger, K., Saul, L.K., 2009. Distance metric learning for large margin nearest neighbor classification. *Journal of Machine Learning Research 10,* 207-244.

Wicklow, T., Angelovic, M., Kristof, K., Jobbagy, J., Findura, P., Krizan, M., 2018. The effect of conditions and storage time on course of moisture and temperature of maize grains. *Contemporary Research Trends in Agricultural Engineering 10,* 1-6.

Williams, D.E., 1999. Semiconducting oxides as gas-sensitive resistors. *Sensors and Actuators B: Chemical 57,* 1-16.

Williams, J.H., Phillip, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M., Aggarwal, D., 2004. Human aflatoxicosis in developing countries. A review of toxicology, exposure, potential health consequences and interventions. *Journal of Clinical Nutrition 80,* 1106-1122.

Wilson, A.D., 2013. Review: Diverse applications of electronic nose technologies in agriculture and forestry. *Sensors 13,* 2295- 2348.

Wilson, A.D., Baietto, M., 2009. Review: Applications and advances in electronic nose technologies. *Sensors 9,* 5099- 5148.

Wilson, D.M., 1989. Analytical methods for aflatoxins in corn and peanuts. *Archives of Environmental Contamination and Toxicology 18,* 308-314.

Wogan, G.N., 1966. Chemical nature and biological effects of the aflatoxins. *Bacteriological Reviews 30,* 460-470.

WorldBank, FAO, NRI, 2011. Missing food: The case of post-harvest grain losses in sub-Saharan Africa.Economic Sector Work Report No. 60371-AFR. World Bank, Washington, DC.

Wu, F., 2004 Mycotoxin risk assessment for the purpose of setting international regulatory standards. *Environmental Science and Technology 38,* 4049-4055.

Wu, F., Guclu, H., 2012. Aflatoxin regulations in a network of global maize trade. *PLoS One 7,* 1-8.

Wu, F., Khlangwiset, P., 2010. Evaluating the technical feasibility of aflatoxin risk reduction strategies in Africa. *Food Additives Contamination Part A Chemical Analytical Control Exposed Risk Assessment 27,* 658–676.

Xiaolong, S., Hui, L., Nan, W., Qiang, Z., 2015. Comparison of different classification methods for analyzing electronic nose data to characterize sesame oils and blends. *Sensors 15,* 26726-26742.

Xu, Y., Zomer, S., Brereton, R.G., 2006. Support vector machines: A recent method for classification in chemometrics. *Critical Reviews in Analytical Chemistry 36,* 177-188.

Yarda, E.E., Daniela, J.H., Lewisa, L.S., Rybaka, M.E., Paliakova, E.M., Kimb, A.A., Montgomeryb, J.M., Rebecca, B., Abudoc, M.U., Akhwalec, W., Breimanb, R.F., Sharifc, S.K., 2013. Human aflatoxin exposure in Kenya, a cross-sectional study. *Food Additives and Contaminants: Part A 30,* 1322–1331.

Yoon, H., 2013. Review: Current trends in sensors based on conducting polymer nanomaterials. *Nanomaterials 3,* 524-549.

Yu, J., 2012. Review: Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. *Toxins 4,* 1024-1057.

Zeringue, H.J., 2000. Identification and effects of maize silk volatiles on cultures of *Aspergillus flavus*. *Journal of Agricultural and Food Chemistry 48,* 921-925.

Zeringue, H.J., Bhantnagar, D., Cleveland, T.E., 1993. $C_{15}H_{24}$ volatile compounds unique to aflatoxigenic strains of *Aspergillus flavus*. *Applied and Environmental Microbiology 59,* 2264-2270.

Zhang, G., Xie, C., 2015. A novel method in the gas identification by using WO_3 gas sensor based on the temperature-programmed technique. *Sensors and Actuators B: Chemical 206,* 220-229.

Zhang, H., Wang, J., 2007. Detection of age and insect damage incurred by wheat, with an electronic nose. *Journal of Stored Products Research 43,* 489-495.

Zhang, L., Tian, F., Nie, H., Dang, L., Li, G., Ye, Q., Kadri, C., 2012. Classification of multiple indoor air contaminants by an electronic nose and a hybrid support vector machine. *Sensors and Actuators B: Chemical 174,* 114-125.

Zhang, Z., Li, G., 2010. A review of advances and new developments in the analysis of biological volatile organic compounds. *Microchemical Journal 95,* 127–139.

Zhang, Z., Yang, M.J., Pawliszyn, J., 1994. Solid-phase microextraction. A solvent-free alternative for sample preparation. *Analytical Chemistry 66,* 844–853.

APPENDICES

Appendix 1: Sample questionnaire for collection of Kenyan maize samples naturally contaminated with aflatoxins.

UNIVERSITY OF CANBERRA
AUSTRALIA'S CAPITAL UNIVERSITY

SAMPLE COLLECTION QUESTIONNARE

Project title: Development of diagnostics techniques for detection of aflatoxin contamination of maize in Kenya

1.0 General background of respondent (to be filled in by the researcher)

Unique sample identifier: …….. Type of Premise: ……..

Name of District: …… Division: …… Location: ……

Sub-Location: …… Village: ……

GPS Co-ordinates: ……..

2.0 Sample Information

Variety: …… Source:……

Planting date: …….. Weeding practice: ……..

Fertilizer application:……..

Remarks

……..

……

……

Appendix 2: Human ethics approval letter for collection of maize samples naturally contaminated with aflatoxins in Kenya.

UNIVERSITY OF CANBERRA
AUSTRALIA'S CAPITAL UNIVERSITY

10 December 2014

APPROVED - Project number 14-251

Ms Catherine Machungo
Faculty of Education, Science, Technology & Maths
University of Canberra
Canberra ACT 2601

Dear Catherine,

The Human Research Ethics Committee has considered your application to conduct research with human subjects for the project titled **Development of diagnostics techniques for detection of aflatoxin contamination of maize in Kenya**.

Approval is granted until 1 December 2016.

The following general conditions apply to your approval.

These requirements are determined by University policy and the ***National Statement on Ethical Conduct in Human Research*** (National Health and Medical Research Council, 2007).

Monitoring:	You must, in conjunction with your supervisor, assist the Committee to monitor the conduct of approved research by completing and promptly returning project review forms, which will be sent to you at the end of your project and, in the case of extended research, at least annually during the approval period.
Discontinuation of research:	You must, in conjunction with your supervisor, inform the Committee, giving reasons, if the research is not conducted or is discontinued before the expected date of completion.
Extension of approval:	If your project will not be complete by the expiry date stated above, you must apply in writing for extension of approval. Application should be made before current approval expires; should specify a new completion date; should include reasons for your request.
Retention and storage of data:	University policy states that all research data must be stored securely, on University premises, for a minimum of five years. You must ensure that all records are transferred to the University when the project is complete.
Contact details and notification of changes:	All email contact should use the UC email address. You should advise the Committee of any change of address during or soon after the approval period including, if appropriate, email address(es).

Yours sincerely
Human Research Ethics Committee

www.canberra.edu.au

Postal Address:
University of Canberra ACT 2601 Australia
Location:
University Drive Bruce ACT

Australian Government Higher Education Registered Provider Number (CRICOS): 00212K

Appendix 3: VOCs identified from the head space of maize flour for variety DK703w inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* isolates.

S/No.	Retention time	Compound	Chemical formula	Relative peak areas (%)
1	1.6 ± 0.1	Methylamine,N,N- dimethyl	C_3H_9N	0.0007
2	1.7 ± 0.2	Trimethylamine	C_3H_9N	0.0008
3	1.8 ±0.2	4-Penten-2-ol	$C_5H_{10}O$	0.212
4	1.9 ± 0.1	Dimethyl ether	C_2H_6O	2.95
5	1.9 ± 0.1	Dimethyl sulfide borane complex	$C_2H_6SBH_3$	0.017
6	2.2 ± 0.1	Acetic acid	CH_3COOH	0.39
7	2.3 ± 0.2	2,3 Butanedione	$C_4H_6O_2$	0.033
8	2.4 ±0.2	Ethyl Acetate**	$C_4H_8O_2$	0.40
9	2.9 ± 0.2	3-methyl butanol	$C_5H_{10}O$	0.04
10	3.1 ± 0.1	Methoxy 2- propanol	$C_4H_{10}O_2$	0.41
11	3.1 ±0.2	2,3 Butanediol	$C_4H_{10}O_2$	0.28
12	3.4 ± 0.1	Methoxy-3-methyl butane	$C_6H_{14}O$	0.0006
13	3.8 ±0.2	3-hyrodxy-2- butanone (Acetoin)**	$C_4H_8O_2$	0.749
14	4.5 ± 0.3	1-pentanol	$C_5H_{12}O$	0.010
15	4.6 ± 0.2	Dimethyl sulfide	C_2H_6S	0.065
16	5.0 ± 0.1	2 methyl propionic acid	$C_4H_8O_2$	0.052
17	5.4 ± 0.1	Oxirane, 2-methyl-3- (1,1,- methyl ethyl)	$C_6H_{12}O$	0.063
18	5.3 ± 0.2	Pentaflluoropropionic acid	$C_3HF_5O_2$	0.331
19	6.6 ± 0.1	Hexanal	$C_6H_{12}O$	0.013
20	8.3 ± 0.1	2,4 Dimethyl-1-heptene	C_9H_{18}	0.0012
21	9.9 ± 0.1	1-Hexanol	$C_6H_{14}O$	0.323
22	10.8 ± 0.2	P-xylene**	C_8H_{10}	0.002
23	10.9 ± 0.1	2-Heptanone	$C_7H_{14}O$	0.04
24	11.6 ± 0.2	Oxime-methoxy-phenyl	$C_8H_9NO_2$	0.037
25	14.6 ± 0.2	4- Hydroxymandelic acid	$C_8H_8O_4$	0.003
26	17.4 ± 0.1	Hexanoic acid, ethyl ester	$C_8H_{16}O_2$	0.077
27	18.6 ± 0.1	Limonene	$C_{10}H_{16}$	0.101
28	21.1 ±0.2	Tetramethyl pyrazine,	$C_8H_{12}N_2$	0.009
29	21.4 ± 0.1	2- Nonadecanone	$C_{19}H_{38}O$	0.059
30	21.8 ±0.2	2,6,7,trimethyl decane	$C_{13}H_{28}$	0.05
31	22.2 ± 0.1	2-ethyl-5-methyl phenol	$C_9H_{12}O$	0.06
32	22.3 ± 0.2	4- ethyl- 3- methyl phenol	$C_9H_{12}O$	0.07
33	23.5 ± 0.1	Camphor	$C_{10}H_{16}O$	0.179
34	24.7 ± 0.3	Naphthalene**	$C_{10}H_8$	0.138
35	25.3 ± 0.1	Octatonic acid, ethyl ester	$C_{10}H_{20}O_2$	0.075
36	27.7 ±0.1	4- ethyl-2- methoxy phenol**	$C_9H_{12}O_2$	0.58
37	27.7 ± 0.1	Eicosane	$C_{20}H_{42}$	0.517
38	27.6 ± 0.1	Hexadecane	$C_{16}H_{34}$	0.507
39	28.0 ± 0.1	2,4,6- trimethyl decane	$C_{13}H_{28}$	0.047
40	28.4 ± 0.1	Isotridecanol	$C_{13}H_{28}O$	0.106
41	28.4 ± 0.2	2- butyl Octanol	$C_{12}H_{26}O$	0.004

Appendix 3 continued

S/No.	Retention time	Compound	Chemical formula	Relative peak areas (%)
42	28.7 ± 0.1	Ethanone-1(2-hydroxy-5-methylphenyl)	$C_9H_{10}O_2$	0.0012
43	28.8 ± 0.1	Pentadecane	$C_{15}H_{32}$	0.071
44	28.7 ± 0.2	2-Isopropyl-5-methyl-1-hexanol	$C_{10}H_{22}$	0.063
45	28.9 ± 0.1	Heptafluorobutyric acid	$C_4HF_7O_2$	0.367
46	29.0 ± 0.1	3- methoxy-phenol	$C_7H_8O_2$	0.082
47	29.0 ± 0.1	Heptadecane	$C_{17}H_{36}$	0.508
48	29.0 ± 0.1	2,6,10 trimemethyl tetradecane	$C_{17}H_{36}$	0.50
49	33.4 ± 0.2	Tritetracontane	$C_{43}H_{88}$	0.016
50	33.4 ± 0.1	Octadecane	$C_{18}H_{38}$	0.647
51	33.4 ± 0.1	Heneicosane	$C_{21}H_{44}$	0.625
52	33.5 ± 0.1	Decosane	$C_{22}H_{46}$	0.399
53	33.7 ± 0.1	Heptacosane	$C_{27}H_{56}$	0.339
54	34.0 ± 0.1	Nonadecane	$C_{19}H_{40}$	0.331
55	34.1 ± 0.1	4,6,8 trimethyl nonene	$C_{12}H_{24}$	0.021
56	34.1 ± 0.2	2,6,10- trimethyl dodecane	$C_{15}H_{32}$	0.010
57	34.1 ± 0.1	2-hexyl octanol	$C_{14}H_{30}O$	0.009
58	39.3 ± 0.2	Tetracosane	$C_{24}H_{50}$	0.035
59	44.4 ± 0.1	Pentadecanoic acid, ethyl ester	$C_{17}H_{34}O_2$	0.161
60	45.7 ± 0.2	9,12- octadecanoic acid ester	$C_{19}H_{34}O_2$	0.824
61	47.6 ± 0.1	Linoleic acid, ethyl ester	$C_{20}H_{36}O_2$	0.113

S/No- Serial number: ******- Compounds identified with authentic standards: All other compounds identified by comparison with NIST library mass spectra- Relative match factor ≥650.

Appendix 4: VOCs identified from the head space of maize flour for variety Duma 43 inoculated with 2% Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* isolates**.**

S/No.	Retention time	Compound	Chemical formula	Relative peak areas (%)
1	1.7 ± 0.2	4-Penten-2-ol	$C_5H_{10}O$	0.395
2	1.8 ± 0.1	Isopropyl Alcohol	C_3H_8O	0.294
3	1.8 ± 0.2	Cyclobutanol	C_4H_8O	0.030
4	1.9 ± 0.1	Butanal	C_4H_8O	0.031
5	2.0 ± 0.2	2,3-dimethyl oxirane	C_4H_8O	0.254
6	2.1 ± 0.1	Acetic acid	CH_3COOH	0.747
7	2.7 ± 0.1	3-methyl- butanal	$C_5H_{10}O$	0.050
8	2.8 ± 0.3	Pentanal, 2,4-dimethyl-	$C_7H_{14}O$	0.046
9	3.0 ± 0.3	1-methoxy-2-propanol	$C_4H_{10}O_2$	0.011
10	3.2 ± 0.2	2-Pentanone	$C_5H_{10}O$	0.065
11	3.2 ± 0.1	3-Aminopyrrolidine	$C_4H_{10}N_2$	0.648
12	4.2 ± 0.1	1-Pentanethiol	$C_5H_{12}S$	0.260
13	4.4 ± 0.1	Propylene Glycol	$C_3H_8O_2$	0.007
14	5.0 ± 0.1	Toluene **	C_7H_8	0.0004
15	5.1 ± 0.3	2-methyl propionic acid	$C_4H_8O_2$	0.015
16	5.1 ± 0.1	1-Pentanol	$C_5H_{12}O$	0.039
17	5.2 ± 0.1	3-Hexanol	$C_6H_{14}O$	0.224
18	5.5 ± 0.1	2,3-Butanediol	$C_4H_{10}O_2$	0.156
19	6.0 ± 0.1	2,4-dimethyl heptane	C_9H_{20}	0.019
20	6.0 ± 0.1	Hexanal	$C_6H_{12}O$	0.423
21	7.5 ± 0.1	2,2, dimethyl-3- pentanone	$C_7H_{14}O$	0.068
22	8.7 ± 0.1	1-Hexanol	$C_6H_{14}0$	0.070
23	9.4 ± 0.1	Styrene	C_8H_8	0.118
24	9.5 ± 0.1	4-methyl-2-Hexanone	$C_7H_{14}O$	0.200
25	10.0 ± 0.1	Heptanal	$C_7H_{14}O$	0.100
26	10.1 ± 0.1	3-ethyl-2-pentanol	$C_7H_{16}O$	0.002
27	10.5 ± 0.1	4-hydroxy butanoic acid	$C_4H_8O_3$	0.053
28	12.7 ± 0.2	Benzaldehyde**	C_7H_6O	0.142
29	14.1 ± 0.1	alpha.-methyl styrene	C_9H_{10}	0.063
30	14.2 ± 0.1	1-Octen-3-ol	$C_8H_{16}O$	0.152
31	15.2 ± 0.1	Pentanoic acid	$C_5H_{10}O_2$	0.0001
32	15.2 ± 0.2	Undecane	$C_{11}H_{24}$	0.016
33	15.2 ± 0.1	Decane	$C_{10}H_{22}$	0.017
34	15.2 ± 0.3	Hexanoic acid	$C_6H_{12}O_2$	0.082
35	16.3 ± 0.1	Pyrazine, 2-ethyl-6-methyl	$C_7H_{10}N_2$	0.063
36	17.5 ± 0.1	4-methyl- tridecane	$C_{14}H_{30}$	0.838
37	17.8 ± 0.2	3-Octen-2-one	$C_8H_{14}O$	0.152
38	18.9 ± 0.1	4-methyl-1-undecene	$C_{12}H_{24}$	0.323
39	18.8 ± 0.2	1-chlorododecane	$C_{12}H_{25}Cl$	0.078
40	19.1 ± 0.1	Dodecane	$C_{12}H_{26}$	0.928
41	19.1 ± 0.1	Tridecane	$C_{13}H_{28}$	0.205

Appendix 4 continued

S/No.	Retention time	Compound	Chemical formula	Relative peak areas (%)
42	19.9 ± 0.3	2-methy-2-undecanethiol	$C_{12}H_{26}S$	0.793
43	19.9 ± 0.1	3-Tetradecene, (E)	$C_{14}H_{28}$	0.563
44	20.5 ± 0.1	2-Nonanone	$C_9H_{18}O$	0.913
45	20.6 ± 0.1	6-ethyl-2-methyloctane	$C_{11}H_{24}$	0.341
46	20.8 ± 0.2	2,6,8-trimethyldecane	$C_{13}H_{28}$	0.721
47	21.1 ± 0.1	Nonanal	$C_9H_{18}O$	0.913
48	21.3 ± 0.1	Phenyl ethyl alcohol	$C_8H_{10}O$	0.085
49	21.4 ± 0.2	2,3,6,7-tetramethyl octane	$C_{12}H_{26}$	1.253
50	21.5 ± 0.1	2-Butyl-1-decene	$C_{14}H_{28}$	0.001
51	24.8 ± 0.1	Pentadecane	$C_{15}H_{32}$	0.517
52	25.2 ±0.2	2-butyl-1-octanol	$C_{12}H_{26}O$	1.051
53	27.3 ±0.1	1-Octadecyne	$C_{18}H_{34}$	3.777
54	28.1 ± 0.1	4,6,8-trimethyl-1-nonene	$C_{12}H_{24}$	0.264
55	28.1 ± 0.1	2,6,10-trimethyl dodecane	$C_{15}H_{32}$	1.575
56	29.3 ± 0.6	Heptadecane	$C_{17}H_{36}$	1.214
57	30.2 ± 0.1	Copaene	$C_{15}H_{24}$	0.041
58	44.4± 0.1	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	0.240

S/No- Serial number: ******- Compounds identified with authentic standards: All other compounds identified by comparison with NIST library mass spectra- Relative match factor ≥650.

Appendix 5: VOCs identified from the head space of maize flour for varities Pioneer, DH04 and market samples (unknown varieties) artificially inoculated and naturally infected with *A. flavus.*

S/No.	Retention time	Compound	Chemical formula	Relative peak areas (%)
1	1.4 ± 0.1	Dl-alanyl-l-alanine	$C_6H_{12}N_2O_3$	0.025
2	1.5 ± 0.1	Methanethiol	CH_4S	0.017
3	1.5 ± 0.2	4-penten-2-ol	$C_5H_{10}O$	0.002
4	1.6 ± 0.2	4-methyl-2-pentanamine	$C_6H_{15}N$	0.085
5	1.7 ± 0.1	Isopropyl alcohol	C_3H_8O	0.067
6	1.7 ± 0.1	Dimethyl sulfide	C_2H_6S	0.661
7	1.7 ± 0.2	Cyclobutanol	C_4H_8O	1.729
8	1.7 ± 0.2	n-Hexylmethylamine	$C_7H_{17}N$	0.661
9	2.0 ± 0.3	Butanal	C_4H_8O	0.072
10	2.0 ± 0.2	Acetic acid	CH_3COOH	0.040
11	2.0 ± 0.1	2,3-dimethyl oxirane	C_4H_8O	0.045
12	2.1 ± 0.1	Hydroxyurea	$CH_4N_2O_2$	0.0002
13	2.2 ± 0.1	3-Buten-2-ol, 2-methyl	$C_5H_{10}O$	0.009
14	2.4 ± 0.1	1-Methyldodecylamine	$C_{13}H_{29}N$	0.038
15	2.7 ± 0.1	3-methyl butanal	$C_5H_{10}O$	1.02
16	2.8 ± 0.1	3-propoxy-1-propene	$C_6H_{12}O$	5.04
17	2.8 ± 0.1	2,4-dimethylpentanal	$C_7H_{14}O$	0.163
18	3.0 ± 0.1	1-methoxy-2-propanol	$C_4H_{10}O_2$	0.154
19	3.0 ± 0.1	4-methoxy-1-butene	$C_5H_{10}O$	0.159
20	3.2 ± 0.2	2-Pentanone	$C_5H_{10}O$	0.108
21	3.2 ± 0.1	3-Aminopyrrolidine	C4H10N2	0.187
22	3.3 ± 0.2	3-methyl hexane	C_7H_{16}	0.186
23	4.2 ± 0.1	1-Pentanethiol	$C_5H_{12}S$	0.260
24	4.3 ± 0.1	Butane, 1-chloro-2-methy	$C_5H_{11}Cl$	0.184
25	4.5 ± 0.2	Propylene glycol	$C_3H_8O_2$	0.131
26	5.0 ± 0.1	Toluene**	C_7H_8	0.092
27	5.1 ± 0.1	2-methyl propionic acid	$C_4H_8O_2$	0.098
28	5.2 ± 0.2	3-Hexanol	$C_6H_{14}O$	0.224
29	5.5 ± 0.1	2,3-Butanediol	$C_4H_{10}O_2$	5.70
30	5.7 ± 0.1	1,2-dimethyl cyclobutane,	C_6H_{12}	0.128
31	5.7 ± 0.2	1-Nonene	C_9H_{18}	3.892
32	6.0 ± 0.2	2,4-dimethyl hexane	C_8H_{18}	0.0002
33	6.0 ± 0.2	2,4-dimethy heptane	C_9H_{20}	4.48
34	6.0 ± 0.1	Hexanal	$C_6H_{12}O$	0.158
35	6.5 ± 0.1	3-Octene, (E)-	C_8H_{16}	0.083
36	7.5 ± 0.3	2,2-dimethy-3-pentanone	$C_7H_{14}0$	0.035
37	8.1 ± 0.1	p-Xylene**	C_8H_{10}	6.400
38	8.1 ± 0.1	Ethylbenzene	C_8H_{10}	0.091
39	8.3 ± 0.1	4-methyloctane	C_9H_{20}	0.073
40	8.6 ± 0.1	1,3-dimethyl benzene	C_8H_{10}	0.097

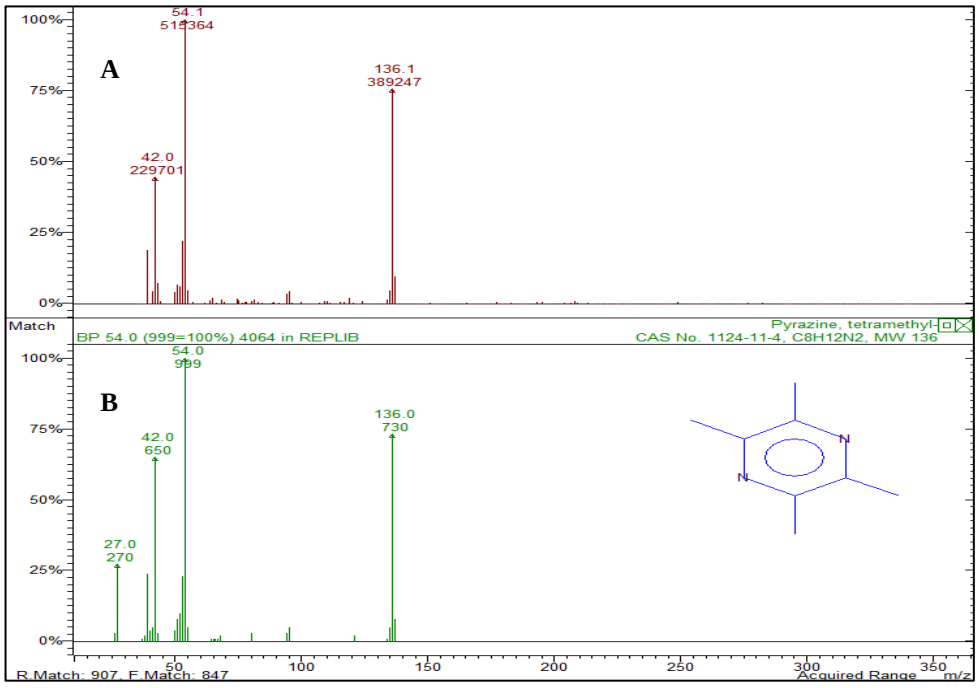
Appendix 5 Continued

S/No.	Retention time	Compound	Chemical formula	Relative peak areas (%)
41	8.5 ± 0.1	o-Xylene	C_8H_{10}	0.086
42	8.9 ± 0.1	4-methyl-3-hexanol	$C_7H_{16}O$	0.119
43	9.3 ± 0.1	Styrene	C_8H_8	0.118
44	9.5 ± 0.1	2-n-butyl furan	$C_8H_{12}O$	0.743
45	10.0 ± 0.1	Heptanal	$C_7H_{14}O$	0.145
46	10.0 ± 0.1	3-ethyl-2-pentanol	$C_7H_{16}O$	0.005
47	10.4 ± 0.1	4-hydroxy butanoic acid	$C_4H_8O_3$	0.049
48	11.5 ± 0.1	2,7-dimethyl oxepine	$C_8H_{10}O$	0.174
49	12.7 ± 0.2	4-chloro-2,4-dimethylhexane	$C_8H_{17}Cl$	0.094
50	12.7 ± 0.1	Benzaldehyde**	C_7H_6O	0.142
51	14.1 ± 0.3	1-Octen-3-ol	$C_8H_{16}O$	0.198
52	14.5 ± 0.1	Isopropyl benzene	C_9H_{12}	1.383
53	15.2 ± 0.1	Decane	$C_{10}H_{22}$	1.436
54	15.2 ± 0.1	2,4,6-trimethyl octane	$C_{11}H_{24}$	0.004
55	17.3 ± 0.1	2,6,7- trimethyl decane	$C_{13}H_{28}$	0.174
56	17.8 ± 0.1	3-Octen-2-one	$C_8H_{14}O$	0.016
57	18.7 ± 0.1	Pentadecane	$C_{15}H_{32}$	0.042
58	18.8 ± 0.1	1,9-Dichlorononane	$C_9H_{18}Cl_2$	0.071
59	19.0 ± 0.2	E-2-Hexenyl benzoate	$C_{13}H_{16}O_2$	0.053
60	19.0 ± 0.1	Tridecane	$C_{13}H_{28}$	0.094
61	19.8 ± 0.1	1-Octanol, 2-nitro-	$C_8H_{17}NO_3$	0.188
62	20.4 ± 0.1	1-Octadecyne	$C_{18}H_{34}$	0.021
63	20.5 ± 0.2	6-ethyl-2-methyl octane	$C_{11}H_{24}$	0.004
64	21.3 ± 0.1	Phenyl ethyl alcohol	$C_8H_{10}O$	0.085
65	21.6 ± 0.2	9-Octadecenal	$C_{18}H_{34}O$	0.049
66	23.6 ± 0.3	3,7-dimethyl-1-octene	$C_{10}H_{20}$	0.240
67	23.8 ± 0.1	2,3-Dimethoxytoluene	$C_9H_{12}O_2$	0.068
68	23.9 ± 0.1	Naphthalene**	$C_{10}H_8$	0.05
69	24.4 ± 0.1	6-Dodecene, (E)-	$C_{12}H_{24}$	0.128
70	25.1 ± 0.1	2-butyl-1-octanol	$C_{12}H_{26}O$	0.629
71	25.1 ± 0.1	Hexadecane	$C_{16}H_{34}$	0.227
72	25.8 ± 0.1	2-ethy 2-propenoic acid	$C_4H_6O_2$	0.243
73	27.1 ± 0.1	2-hexyl-1-octanol	$C_{14}H_{30}0$	0.054
74	28.1 ± 0.3	2,3-dimethyldecane	$C_{12}H_{26}$	0.062
75	28.6 ± 0.1	5-ethyl-1-nonene	$C_{11}H_{22}$	0.094
76	30.2 ± 0.1	Copaene	$C_{15}H_{24}$	0.041
77	32.1 ± 0.1	Dichloroacetic acid, tetradecyl ester	$C_{16}H_{30}Cl_2O_2$	0.061

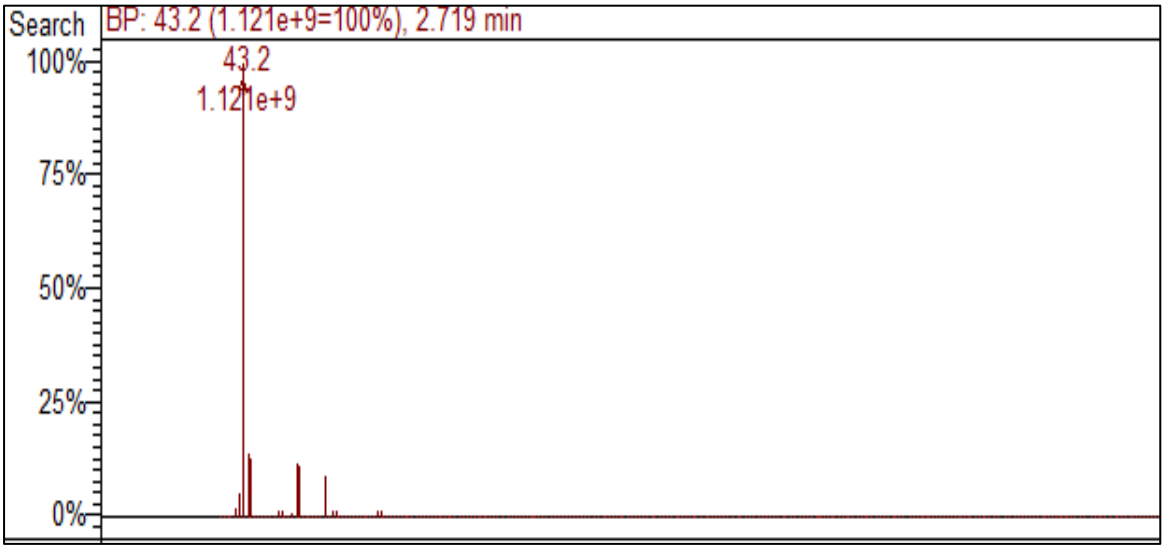
S/No- Serial number: ******- Compounds identified with authentic standards: All other compounds identified by comparison with NIST library mass spectra- Relative match factor ≥650.

Appendix 6: Mass Spectra of diagnostic VOCs identified from the headspace of maize flour for varieties artificially inoculated and naturally infected with *A. flavus* in comparison to the NIST library spectra/ authentic standards (Relative match factor >650 indicate close similarity between the identified VOCS and the NIST library database).

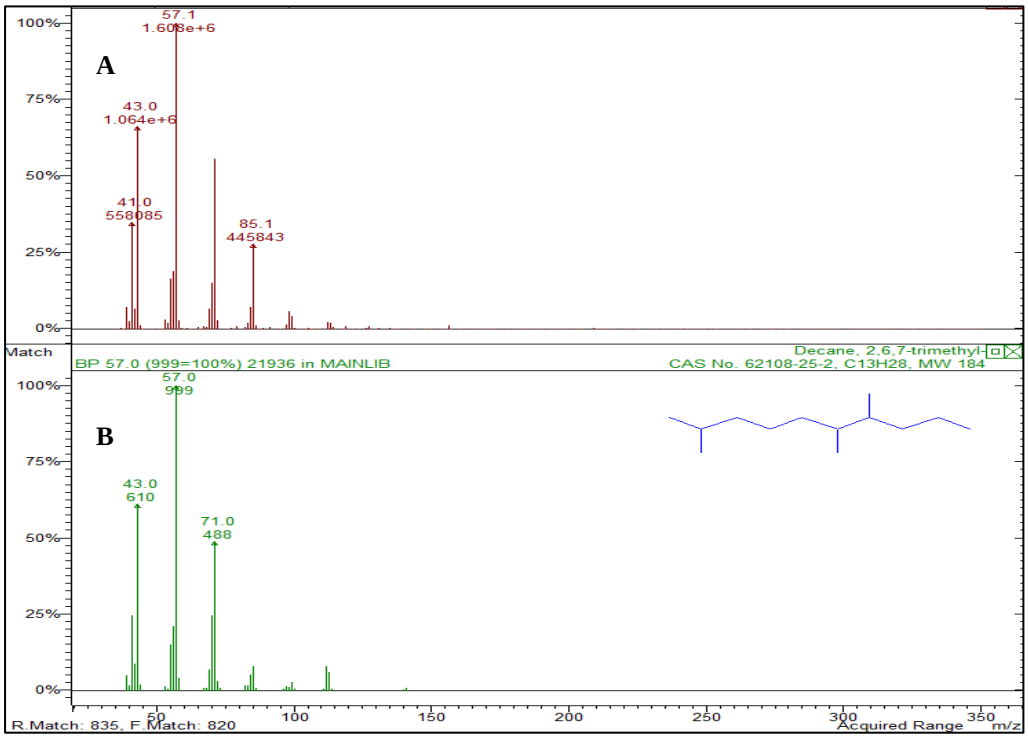
1.0 Tetramethyl pyrazine (Major ion- 54, Retention time-21.1 ± 0.2, Relative match factor-907). **A)** Sample mass spectra, **B)** NIST library mass spectra.



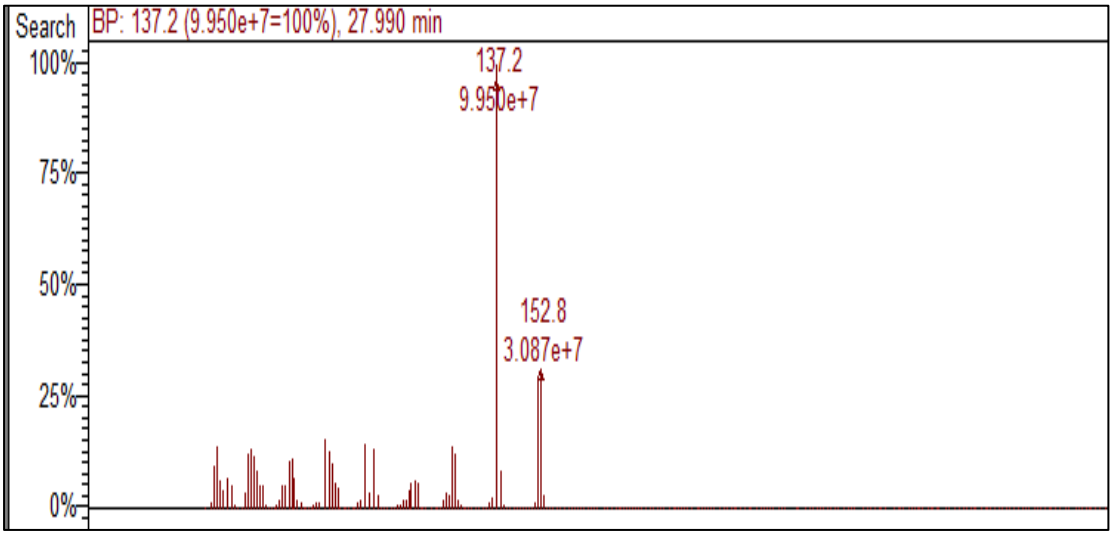
2.0 Ethyl acetate authentic standard (Major ion- 43, Retention time-2.7 ± 0.1)



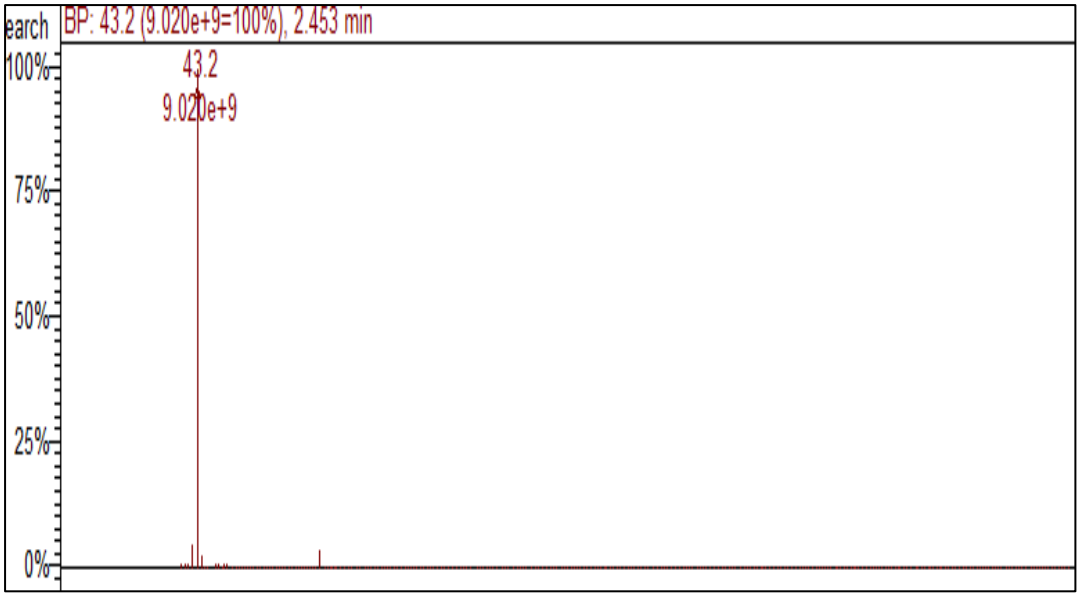
3.0 2, 6, 7 trimethyl decane (Major ion-57, Retention time-21.9 ± 0.1, Relative match factor- 835). **A)** Sample mass spectra, **B)** NIST library spectra.



4.0 4-ethyl-2- methoxy phenol authentic standard (Major ion-137, Retention time-27.9 ± 0.1)

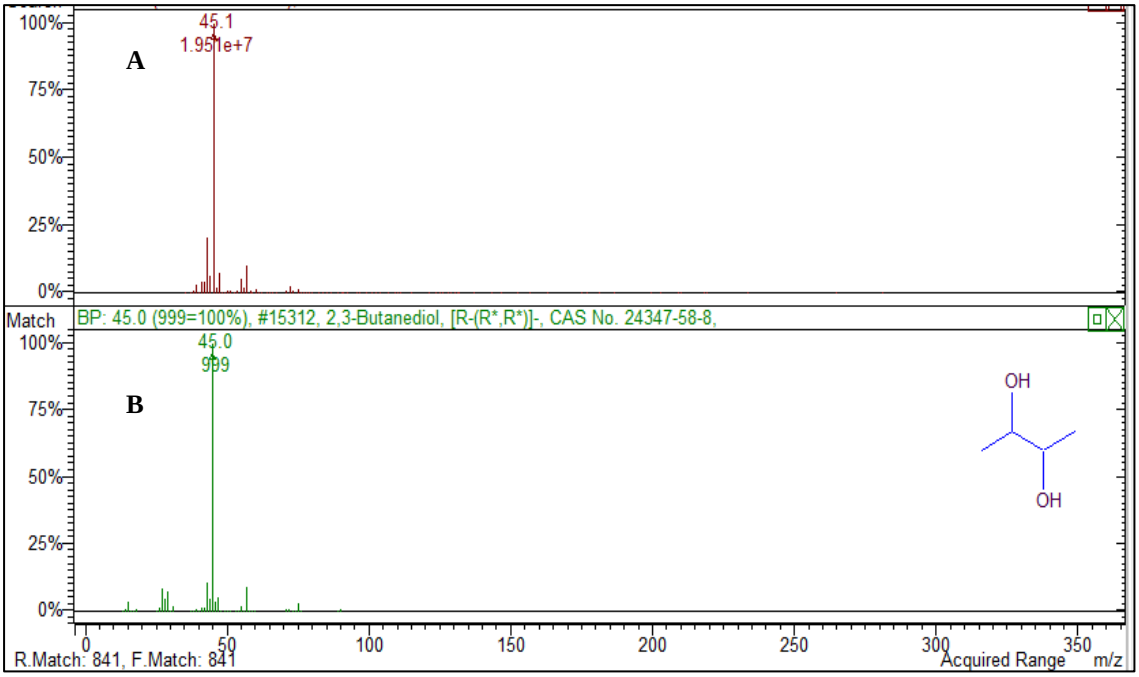


5.0 2,3 butanedione authentic standard (Major ion- 43, Retention time-2.5 ± 0.1

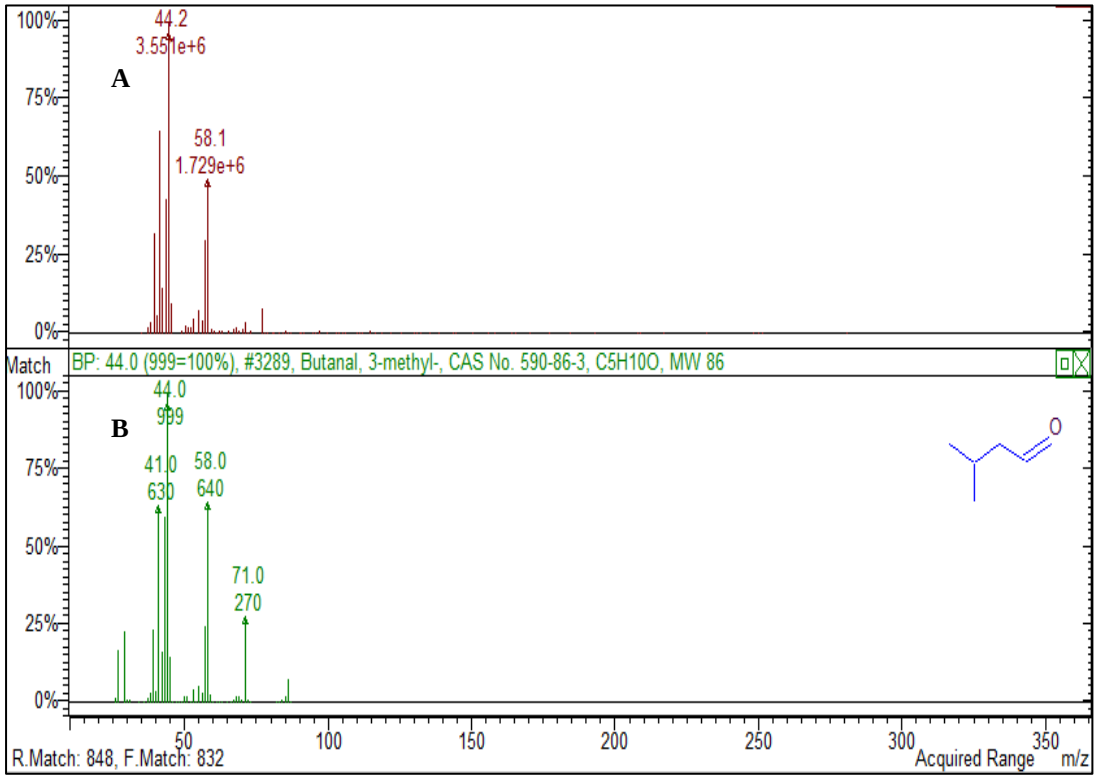


6.0 2,3-hyrodxy butanone (Acetoin) authentic standard (Major ion- 45, Retention time-4.2 ± 0.2).

7.0 2, 3 butanediol (Major ion – 45**,** Retention time-5.8 ± 0.1, Relative match factor- 841). **A)** Sample mass spectra, **B)** NIST library mass spectra.

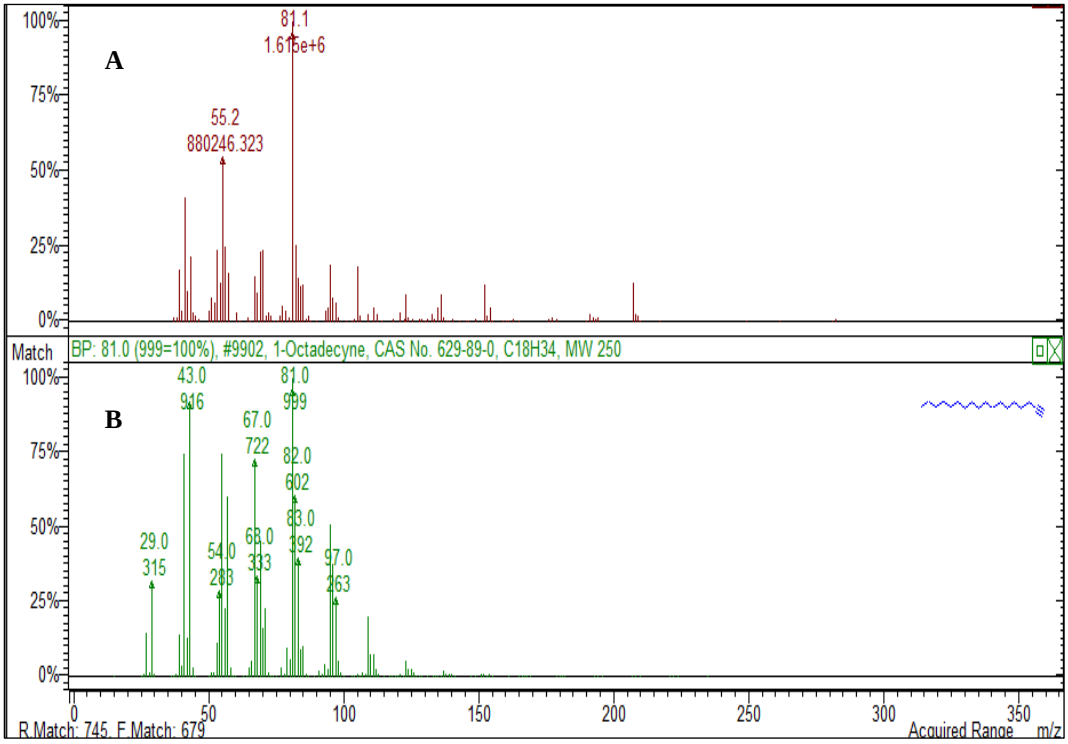


8.0 3-methyl butanol (Major ion- 44, Retention time 3.3 ± 0.4, Relative match factor- 848). **A)** Sample mass spectra, **B)** NIST library mass spectra.

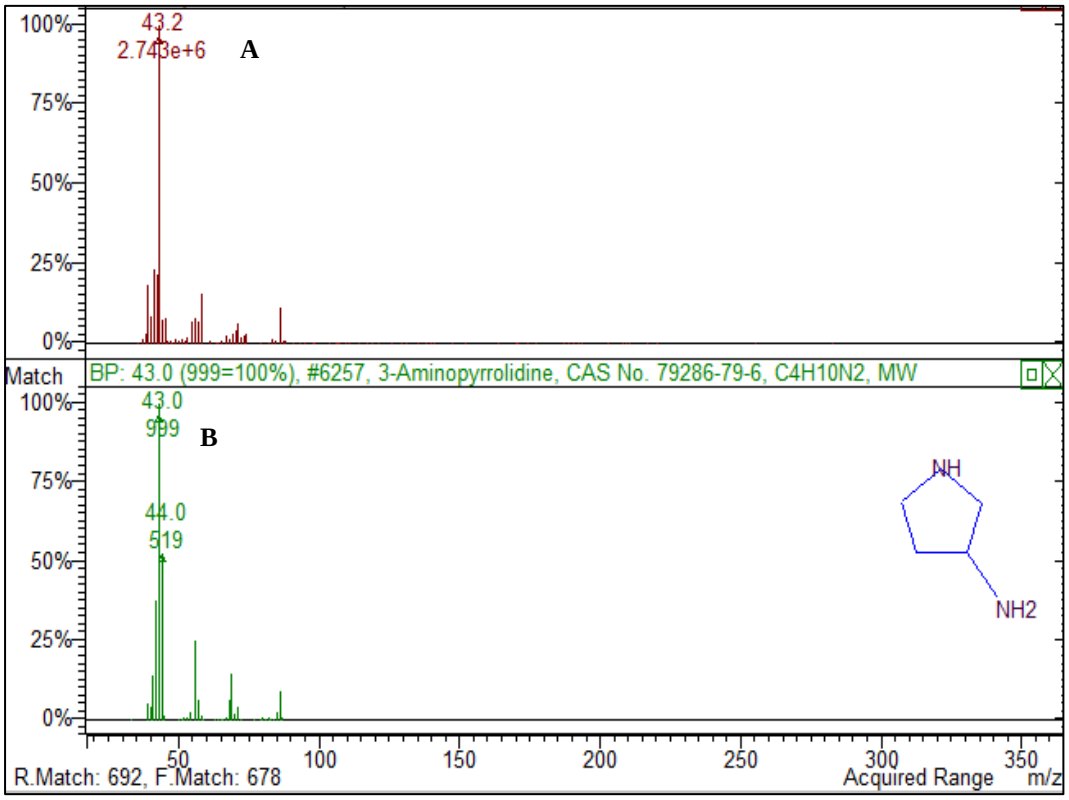


9.0 1-octadecyne (Major ion- 81, Retention time 20.4 ± 0.1, Relative match factor-745).

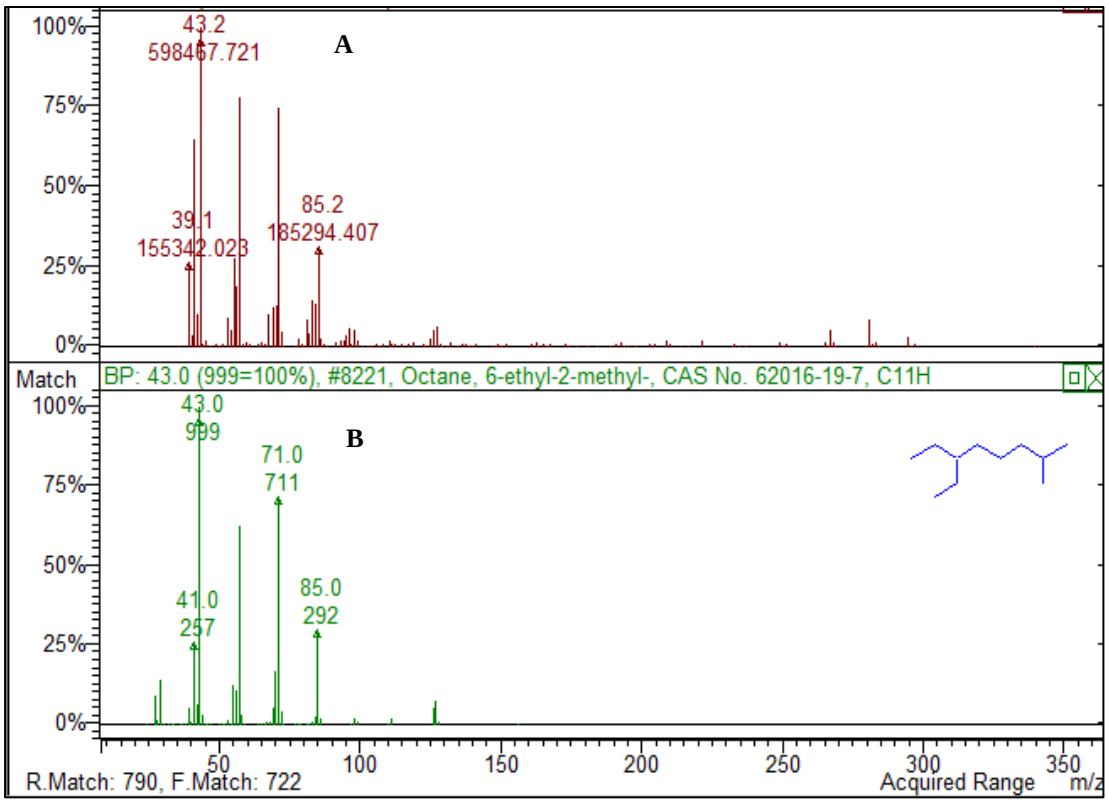
A) Sample mass spectra, **B)** NIST library mass spectra.



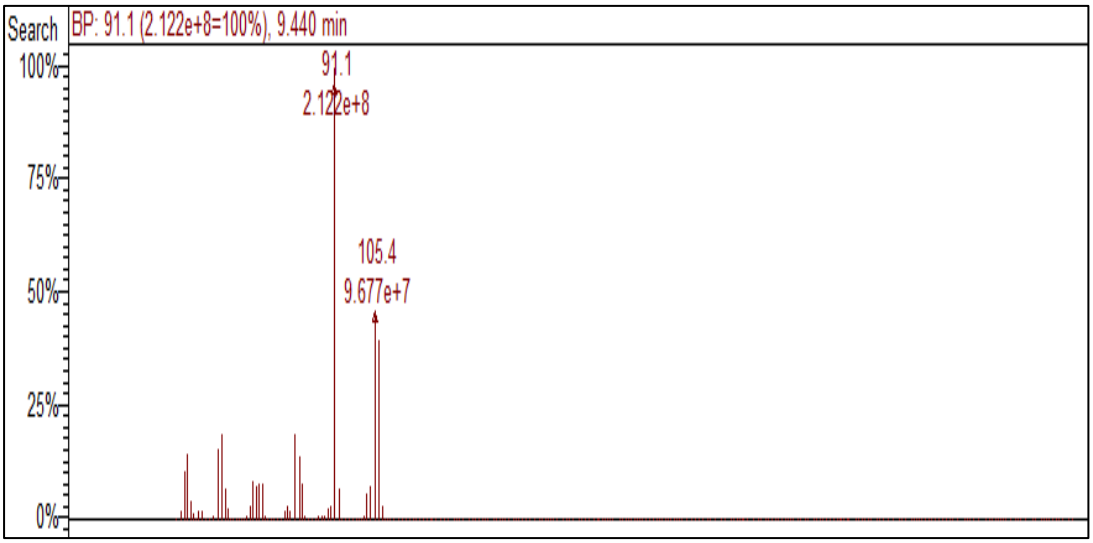
10. 3-aminopyrrolidine (Major ion- 43, Retention time 3.2 ± 0.1, Relative match factor-692).
A) Sample mass spectra, **B)** NIST library mass spectra



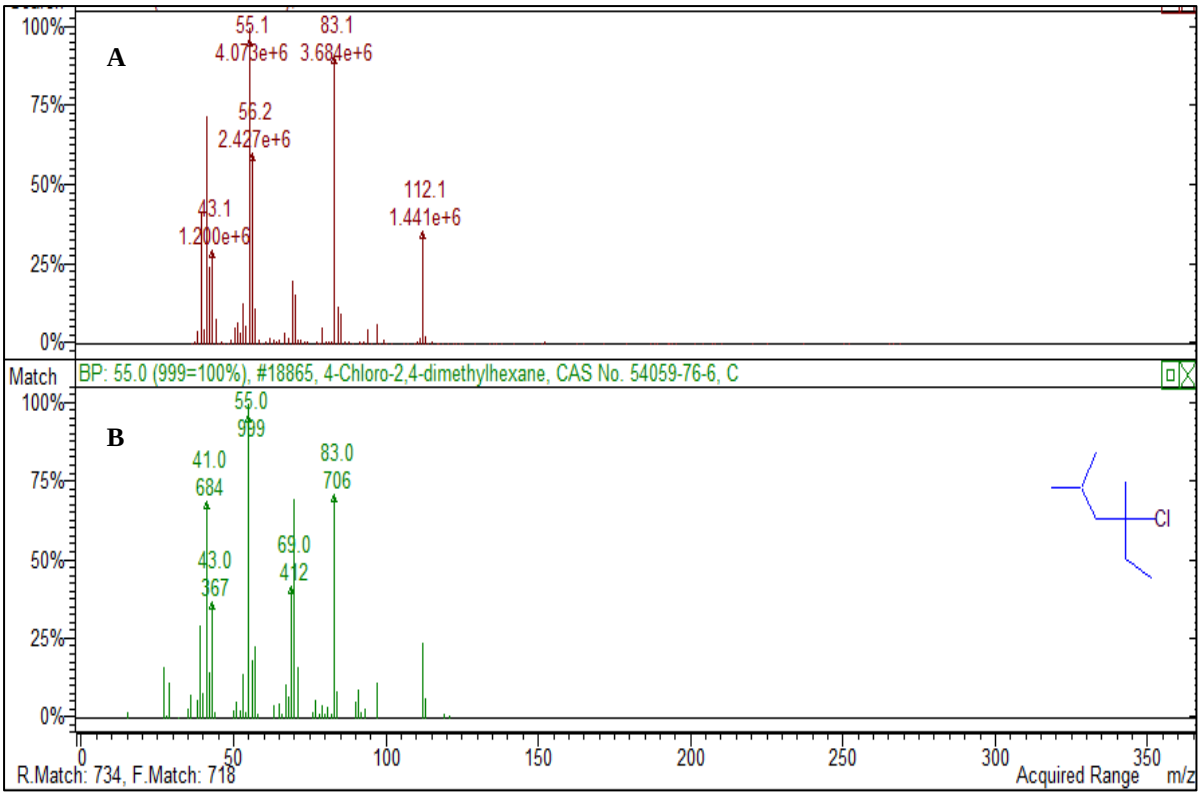
11. 6-ethyl-2-methyl octane (Major ion- 43, Retention time 20.5 ± 0.1, Relative match factor- 790). **A)** Sample mass spectra, **B)** NIST library mass spectra



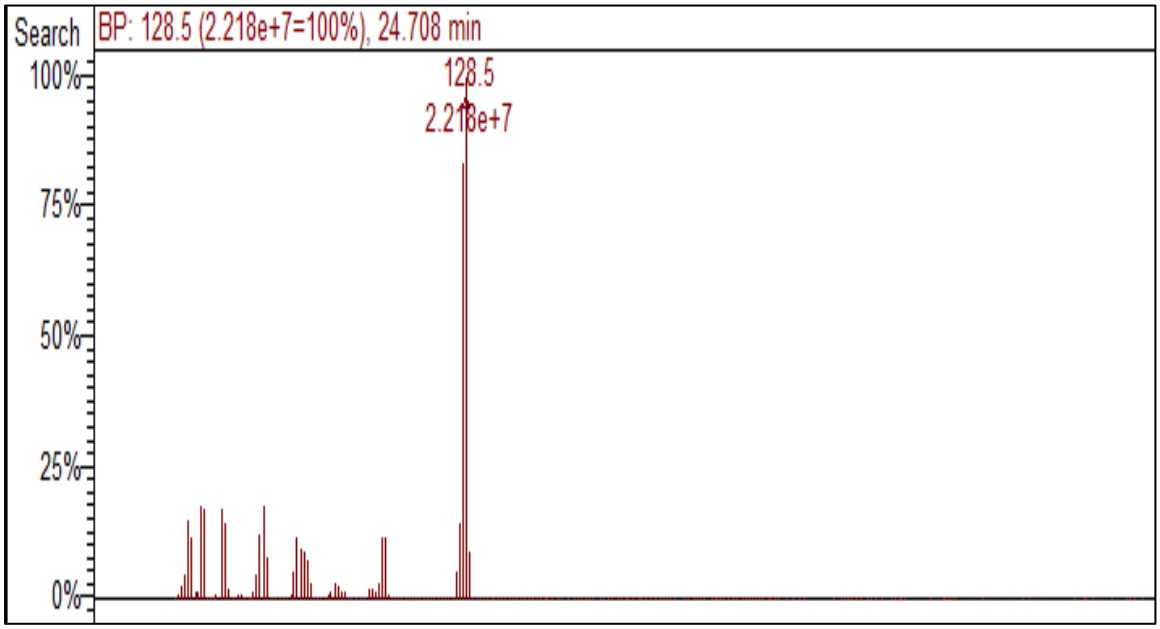
12. P-xylene authentic standard (Major ion 91, Retention time 9.4 ± 0.6)



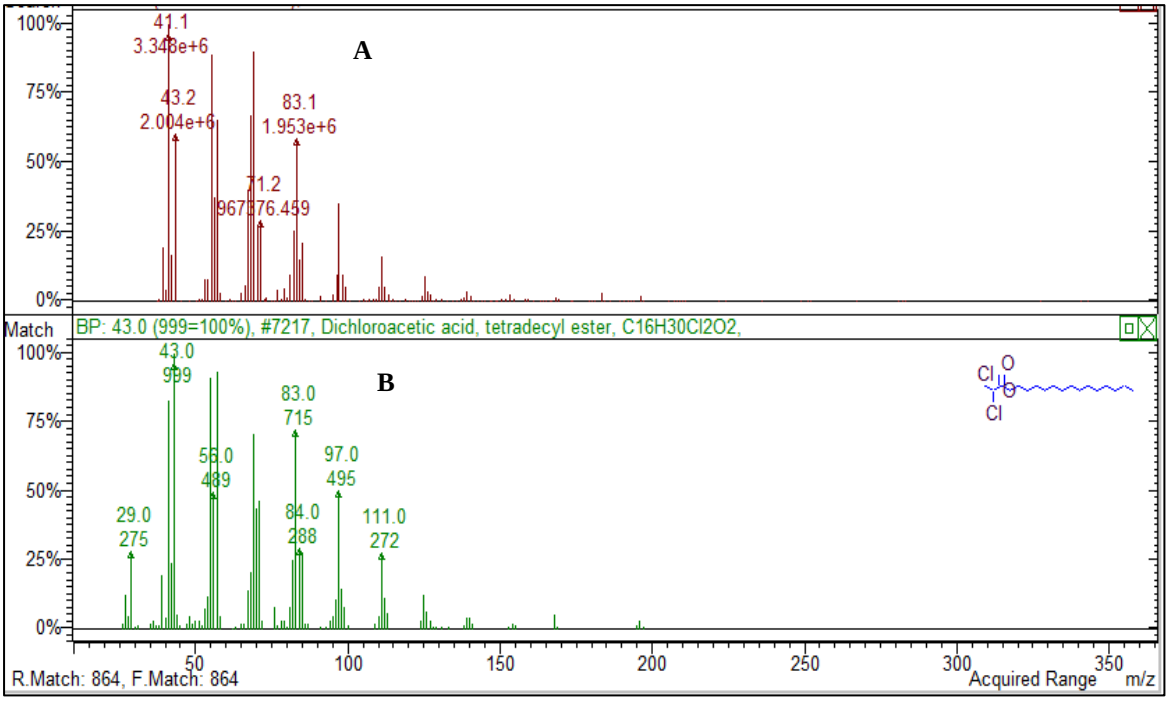
13. 4- chloro, 2, 4 dimethyl hexane (Major ion- 55, Retention time 12.6 ± 0.1, Relative match factor-734). **A)** Sample mass spectra, **B)** NIST library mass spectra.



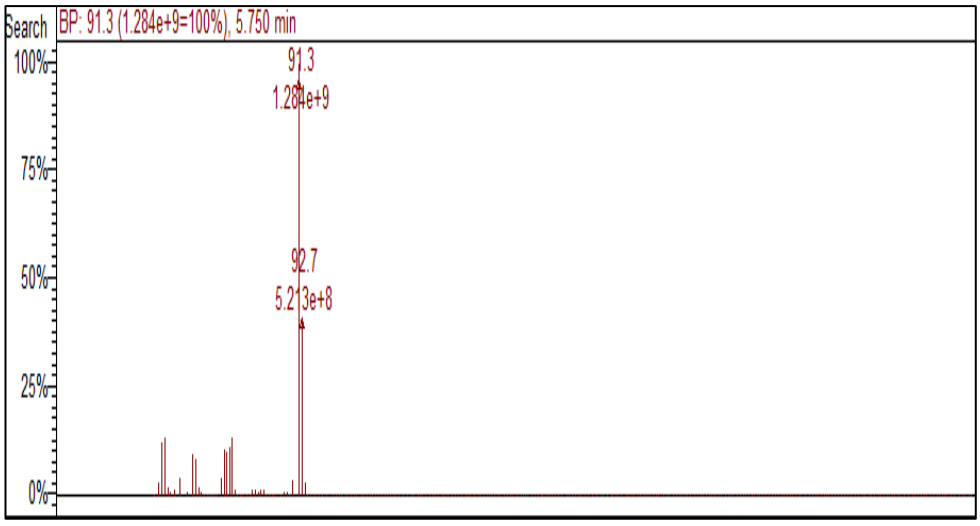
14. Naphthalene authentic standard (Major ion –128, Retention time 24.7 ± 0.1)



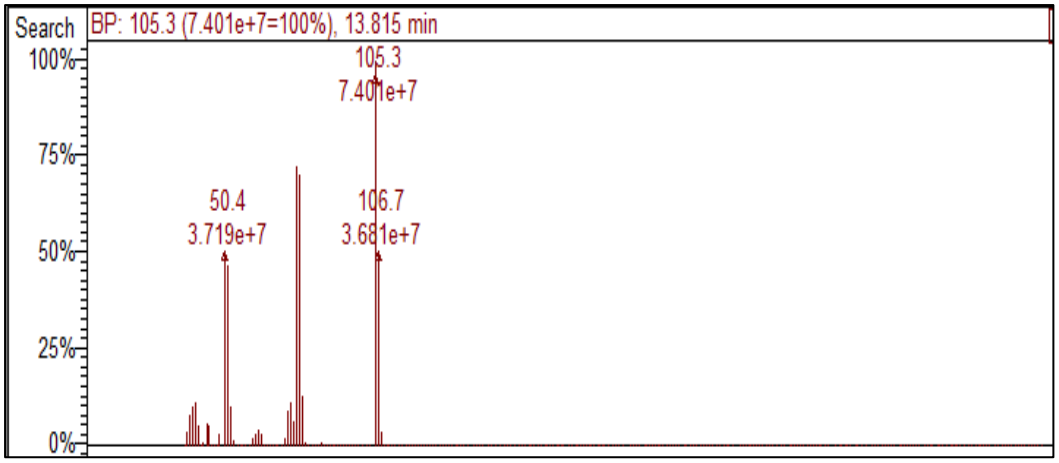
15. Dichloroacetic acid, tetradecyl ester (Major ion-41, Retention time 32.9 ± 0.1, Relative match factor- 864). **A)** Sample mass spectra, **B)** NIST library mass spectra.



16.Toluene authentic standard (Major ion-91, Retention time 5.8 ± 0.1)



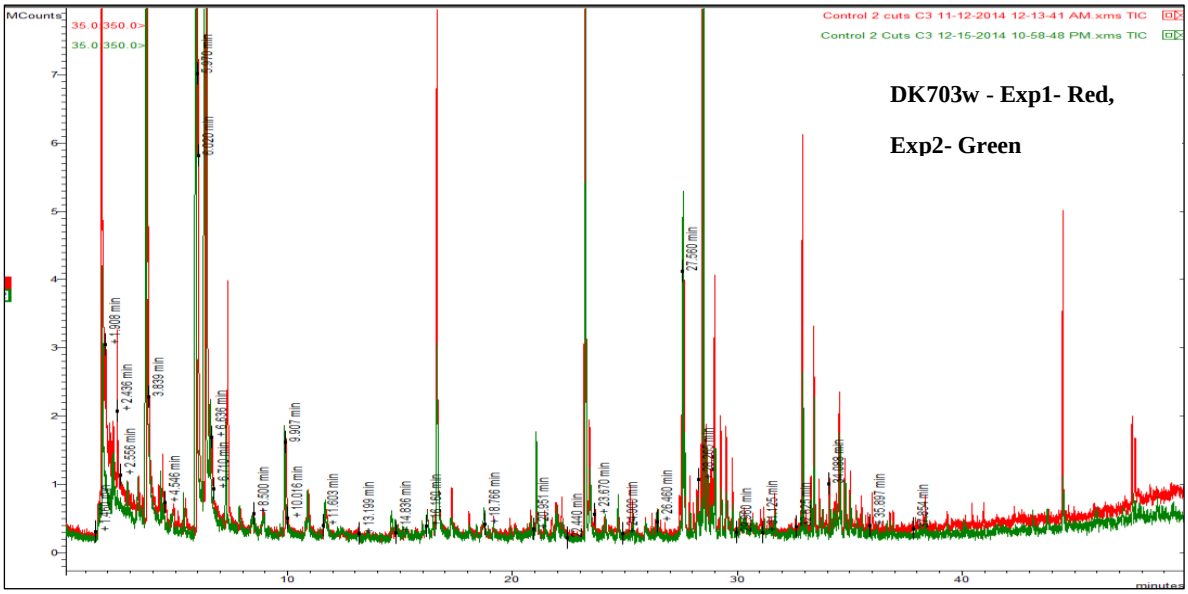
15. Benzaldehyde authentic standard (Major ion- 105, Retention time 13.8 ± 0.2)



Appendix 7: Comparison of GC-MS chromatograms for maize flour samples for variety DK703w, Duma 43 and Pioneer inoculated with 2 % Tween 20 as a control, non-aflatoxigenic and aflatoxigenic isolates of *A. flavus* analysed with Varian 3800 GC and 1200 MS, Agilent 7890A GC and 240 MS and Bruker 451 GC and Scion Single Quad MS respectively.

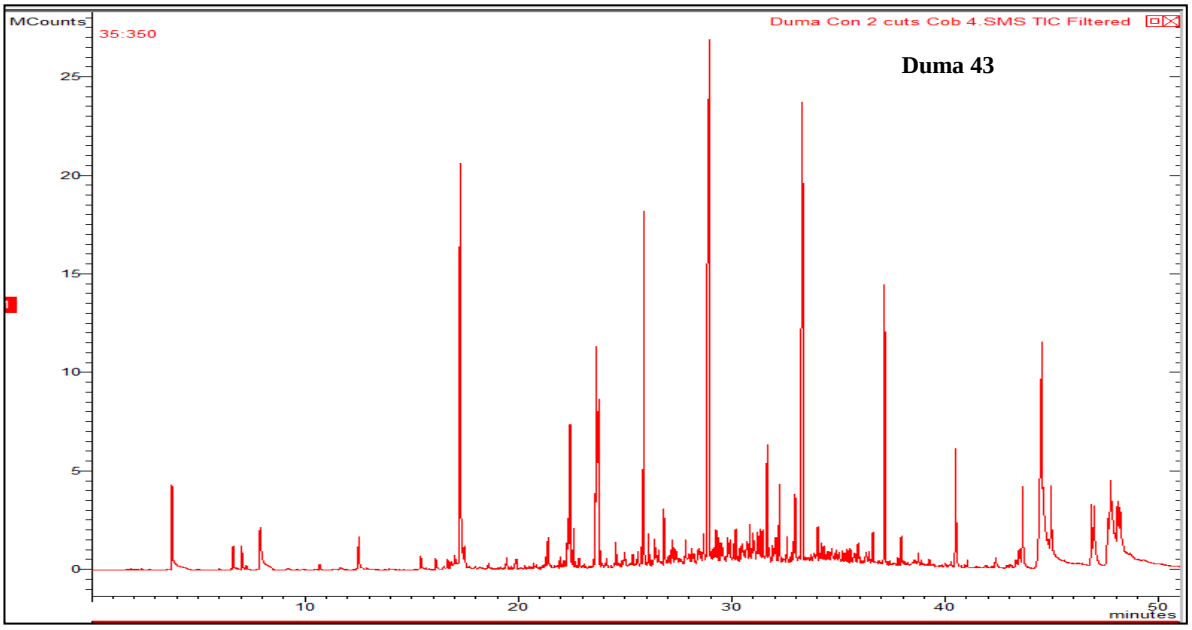
1) Control samples

A) Varian 3800 GC and 1200 MS

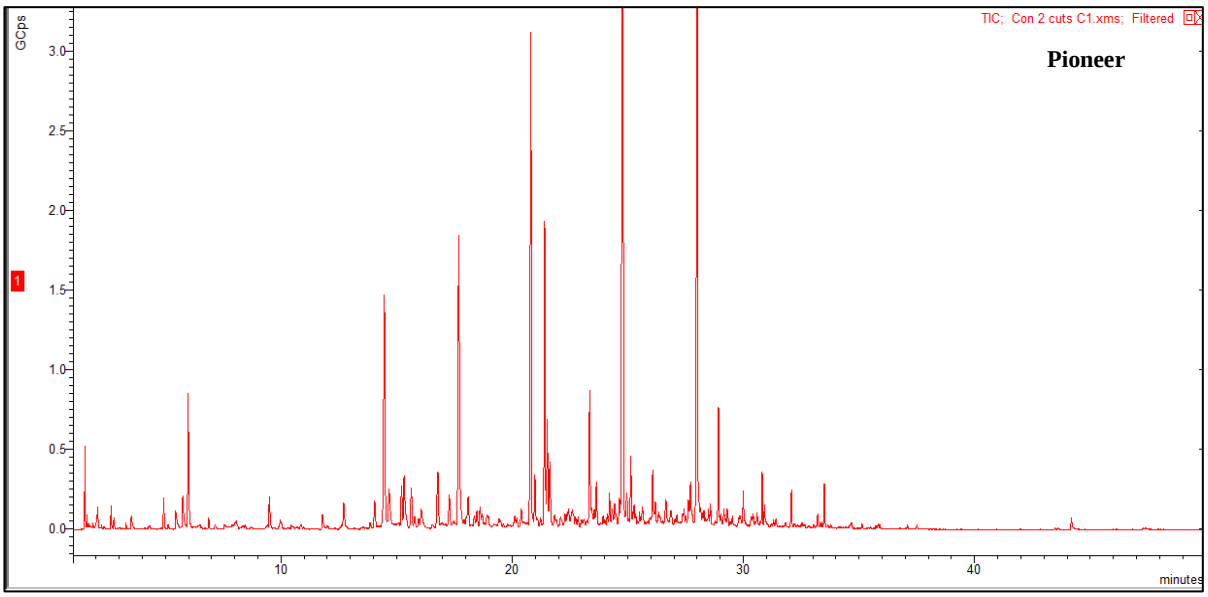


*Exp- Experiment

B) Agilent 7890A GC and 240 MS

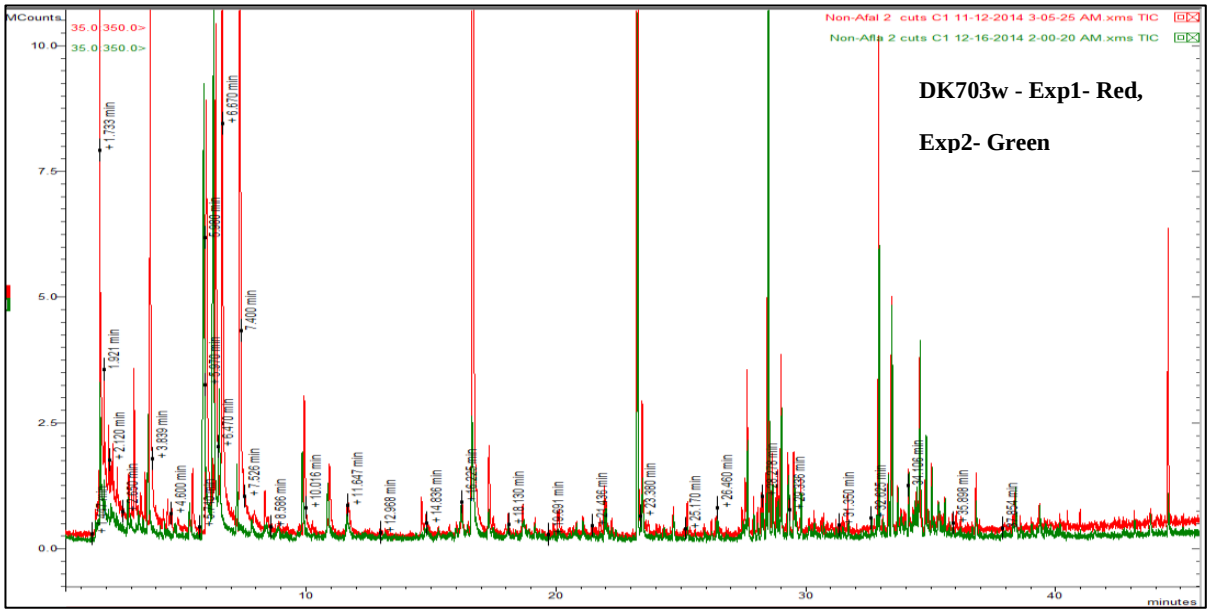


C) Bruker 451 GC and Scion Single Quad MS



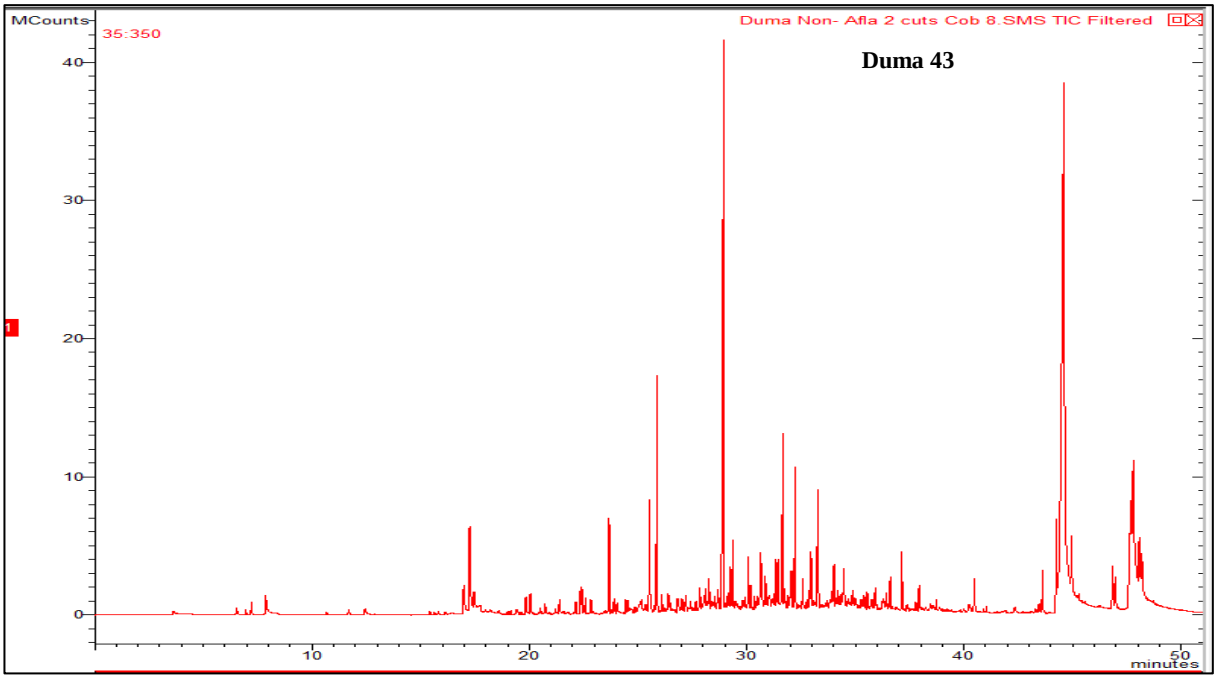
2) Non-Aflatoxigenic samples

A) Varian 3800 GC and 1200 MS

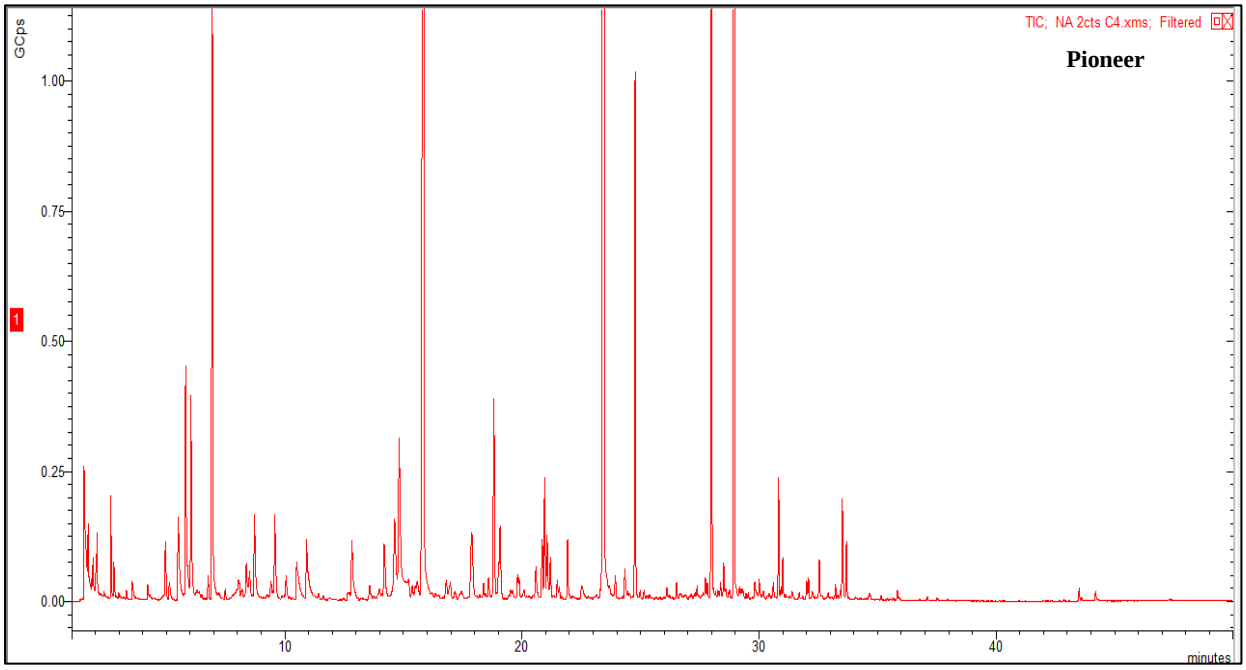


*Exp- Experiment

B) Agilent 7890A GC and 240 MS

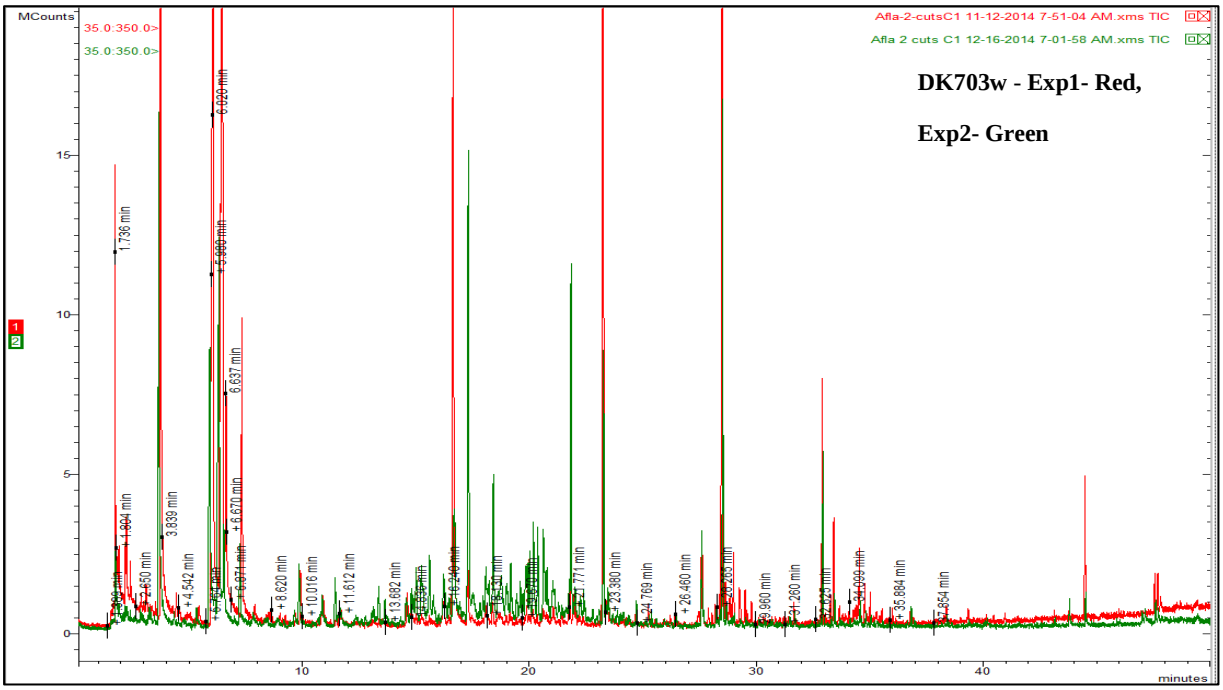


C) Bruker 451 GC and Scion Single Quad MS



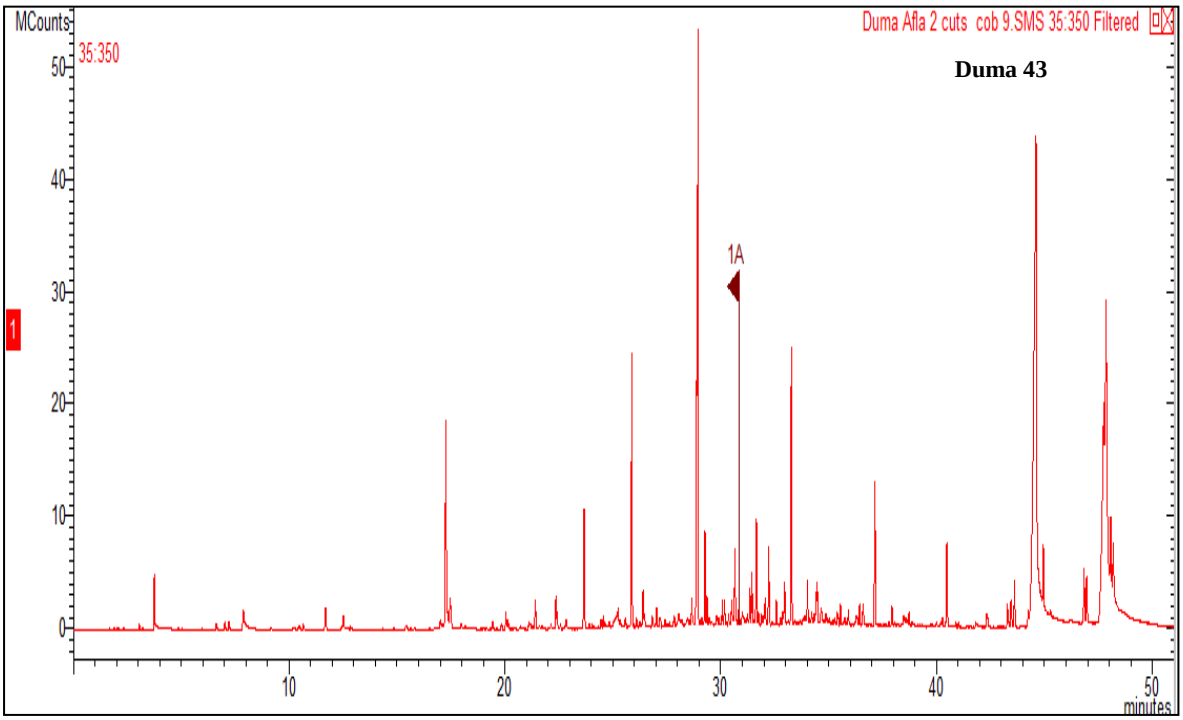
3) Aflatoxigenic samples

A) Varian 3800 GC and 1200 MS

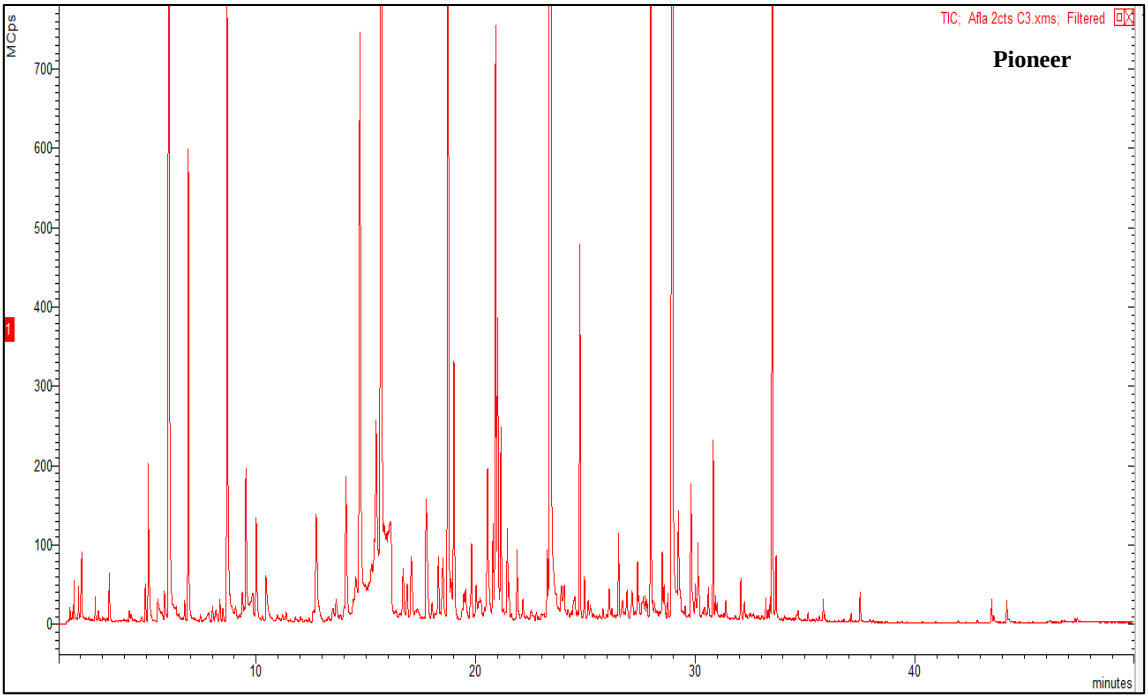


*Exp- Experiment

B) Agilent 7890A GC and 240 MS



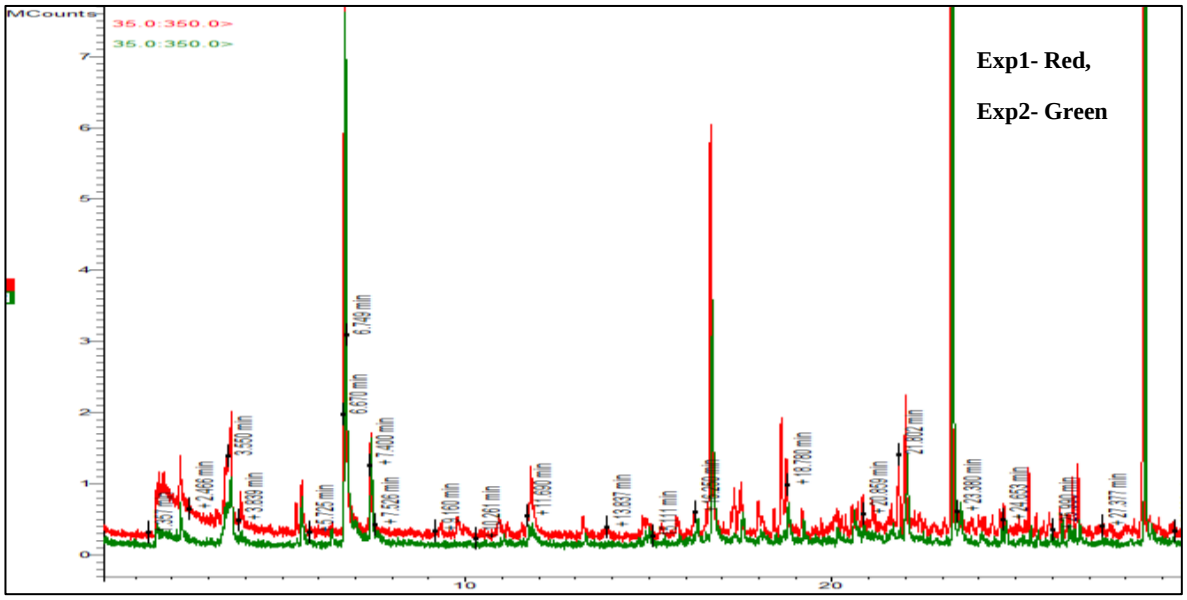
C) Bruker 451 GC and Scion Single Quad MS



Appendix 8: Comparison of GC-MS chromatograms for maize flour samples and blank 10 mL glass vial (Supelco, Bellefonte, PA- sealed with silicon/teflon magnetic autosampler vial caps (Agilent Technologies, Australia)) analysed with Varian 3800 GC and 1200 MS, Agilent 7890A GC and 240 MS and Bruker 451 GC and Scion Single Quad MS.

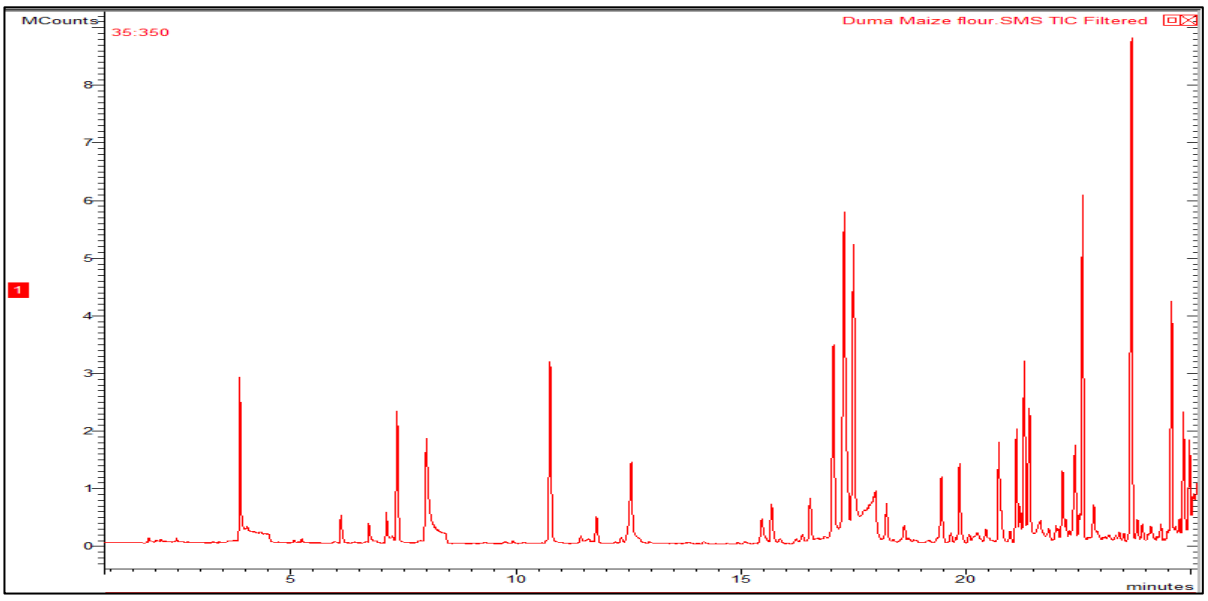
1) Maize flour sample

A) Varian 3800 GC and 1200 MS

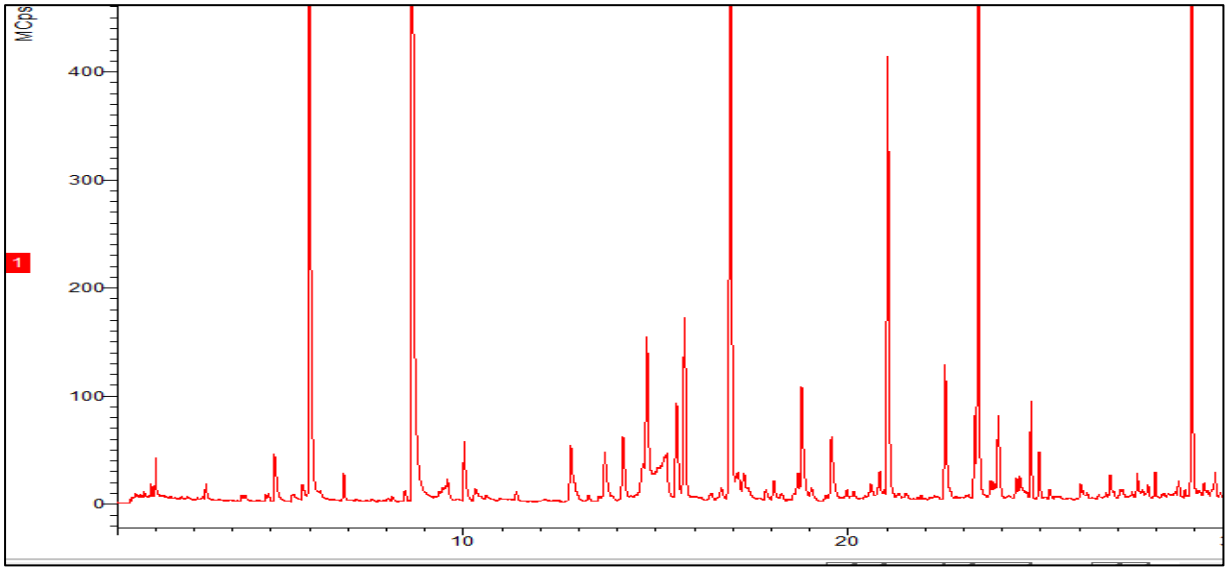


*Exp- Experiment

B) Agilent 7890A GC and 240 MS

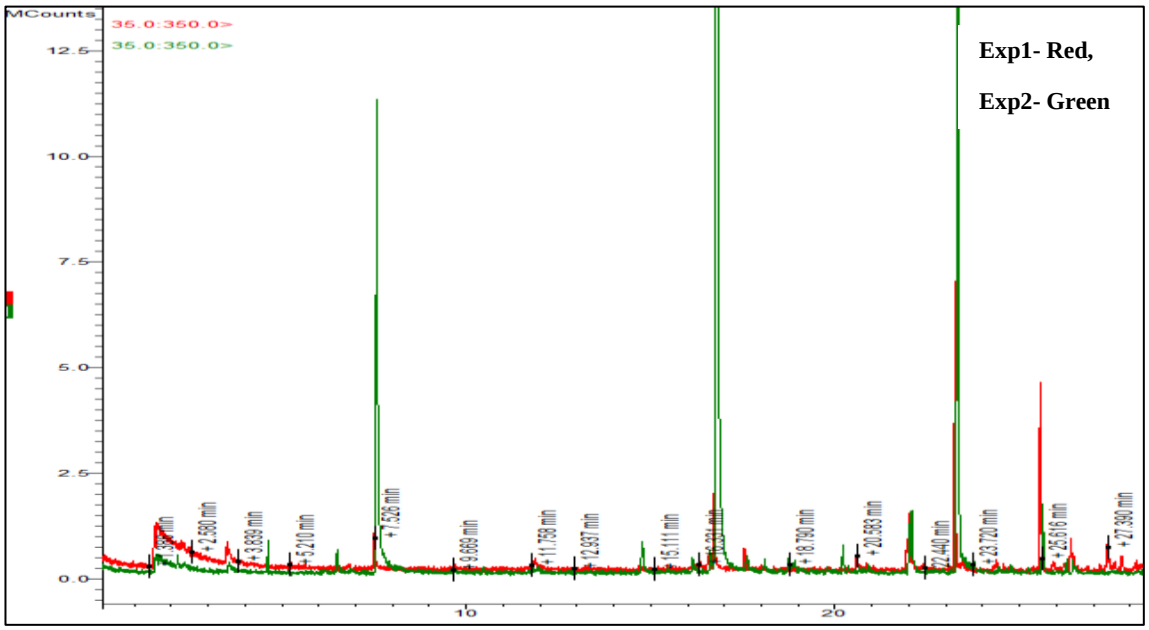


C) Bruker 451 GC and Scion Single Quad MS



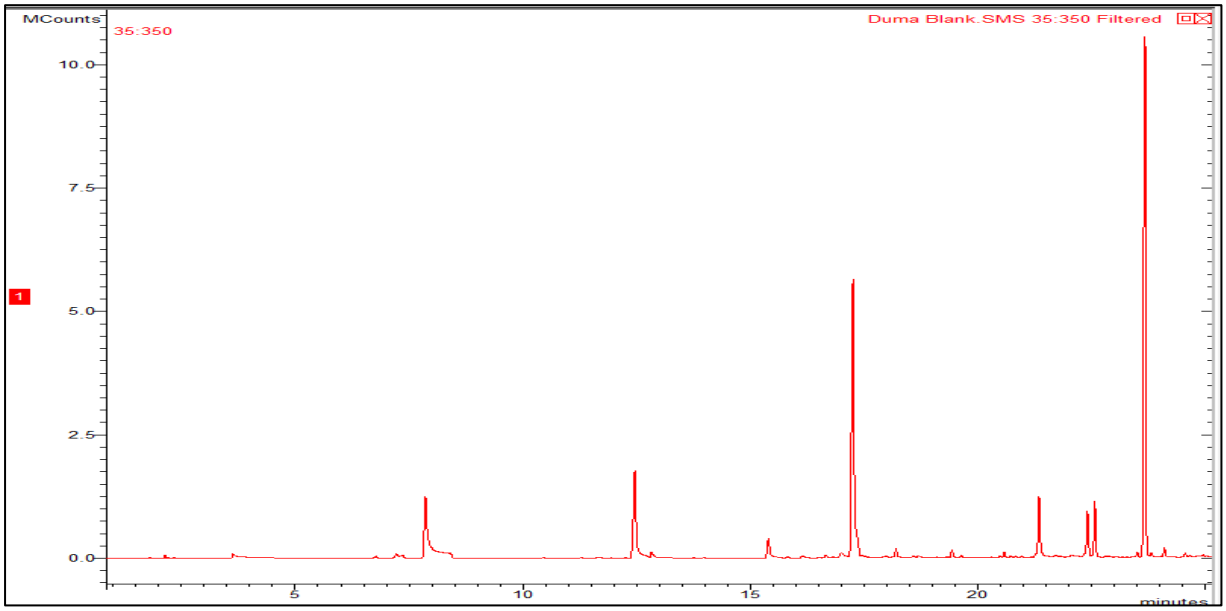
2) Blank vial sample

A) Varian 3800 GC and 1200 MS

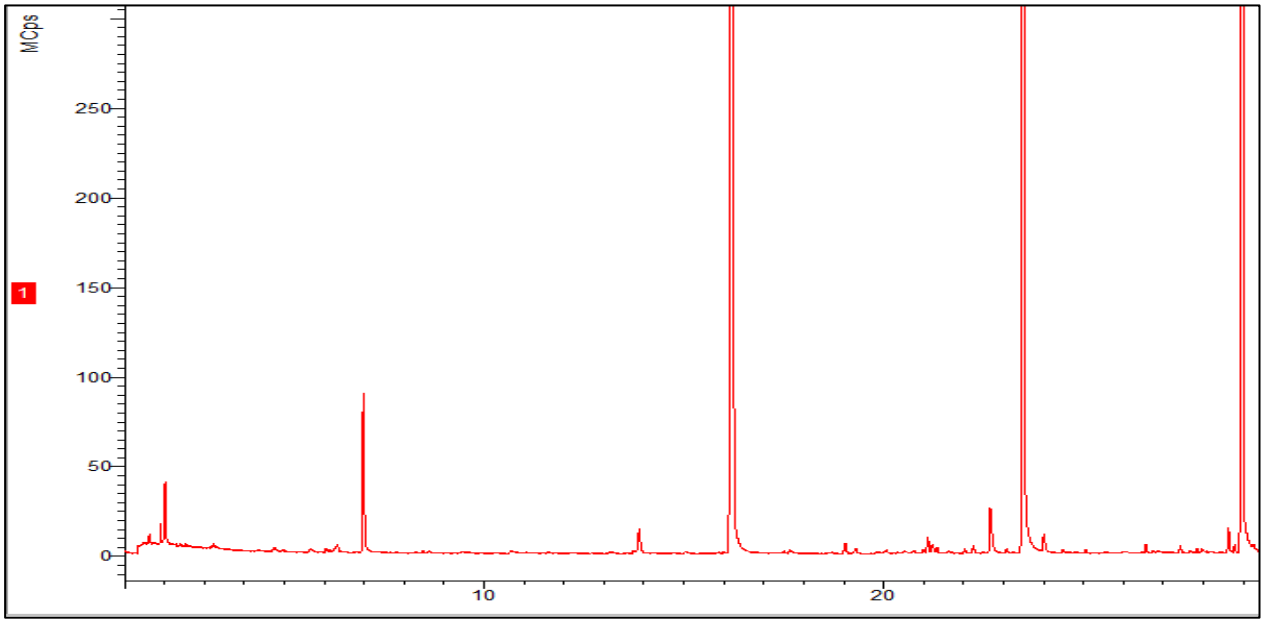


*Exp- Experiment

B) Agilent 7890A GC and 240 MS



C) Bruker 451 GC and Scion Single Quad MS



Appendix 9: Difference in sensor response intensities between experiment 1 and experiment 2 for DiagNose analysis of Australian maize variety DK703w artificially inoculated with 2 % Tween 20 as a control and non-aflatoxigenic and aflatoxigenic isolates of *A. flavus*.