Preliminary investigation of the natural contamination of agricultural crops with selected mycotoxins in northern rural South Africa (Limpopo and Mpumalanga Provinces)

by

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Faculty of Science
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Dr. D.R Katerere, Tshwane University of Technology

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Preliminary investigation of the natural contamination of agricultural crops with selected mycotoxins in northern rural South Africa (Limpopo and Mpumalanga Provinces)

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KEYWORDS

Subsistence farmers

Storage practices

Vhembe District Municipality (VDM) (Limpopo Province)

Gert Sibande District Municipality (GSDM) (Mpumalanga Province)

Aspergillus spp.

Fusarium spp.

Aflatoxins

Fumonisins

High-performance liquid chromatography (HPLC)

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

ABSTRACT

Subsistence farmers may contribute significantly to food production, food security, and employment in South Africa. However poor storage practices and contamination with mycotoxins, particularly fumonisins and aflatoxins impacts adversely on production, food safety and food security. Mycotoxins are toxic natural food-borne compounds which frequently contaminate agricultural produce worldwide. They are hazardous to humans and animals and result in significant production losses for farmers.

This study focused on former Bantustans in Northern South Africa, namely Vhembe District Municipality (Limpopo) and Gert Sibande District Municipality (Mpumalanga). The aim was to assess mycological and mycotoxin contamination of crops grown by subsistence farmers. A semi-structured questionnaire was administered to randomly thirty-nine households. Data on demographics, storage practices and production during period of 2011 and 2012 cropping seasons were collected. One hundred and fifteen (115) crop samples (maize, beans and peanuts) were collected for analysis. Standard mycological methods and validated mycotoxin analysis methods (HPLC and LC- MS/MS) were used.

It was found that maize was the staple food in both provinces, with a significant difference ($p = 0.0184$) in its production between the two districts; Vhembe produced 0.6 tonnes compared to 2.4 tonnes in Gert Sibande. The majority of the farmers for storage used traditional open wooden cribs (15/20) and steel tanks (5/20) while VDM farmers used sealed store houses 5/19 and 15/19 used polystyrene sacks. Aflatoxin occurrence was low with <1% of GSDM samples contaminated compared to 11% of VDM samples. No significant difference ($p > 0.05$) was observed in the aflatoxin contamination in VDM samples between the year 2011 and 2012. Samples from VDM households had higher *Aspergillus* fungal infection (maximum incidence 69%) compared to GSDM (27%) over both seasons. The most frequently isolated *Fusarium* species in VDM samples was *F. verticillioides* (92%; 93%), and *F. subglutinans* (97%; 80%) in GSDM samples

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over seasons 2011 and 2012, respectively.

Highest levels of fumonisins ($FB_1 + FB_2$) ranged between 1010 µg/kg and 12168 µg/kg with less than 30% extremely contaminated above the regulated limit in 91% of samples from Limpopo over both seasons (2011 and 2012). Fumonisin levels between the two seasons in VDM showed no significant difference ($p > 0.05$). Only three (less than 5%) from 68% GSDM contaminated maize samples were above the FB_1 and FB_2 limit. In 2011, there were two highly contaminated maize samples (1762 µg/kg and 4598 µg/kg) with the other samples less than 600 µg/kg, whereas in season two (2012) all samples were below 200 µg/kg, except one highly contaminated sample (26115 µg/kg). None of the beans and peanuts from Mpumalanga was contaminated with mycotoxins above the recommended limit, but from Limpopo 1/5 peanuts was found contaminated with aflatoxin G_1 (41 µg/kg).

Natural occurrence and contamination of both fumonisin and aflatoxin in stored home-grown maize from VDM was significantly ($p < 0.0001$) higher than GSDM over both seasons. In general, Limpopo farmers' experience lower harvests and greater mycotoxin contamination of agricultural produce. This may be attributed in part to poor storage practices and environmental and climatic conditions in that agro-ecological zone.

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September 2013

DECLARATION

I declare that '*Preliminary investigation of the natural contamination of agricultural crops with selected mycotoxins in northern rural South Africa (Limpopo and Mpumalanga Provinces)*' is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Pamella Mngqawa 12 September 2013

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DEDICATION

To my grandfather and father, the late Mr Zikhali Mngqawa and Mr Phangalele Mngqawa for all the best memories you have left us with, you were the best male role models I could have ever asked for, we love you and may your souls rest in peace.

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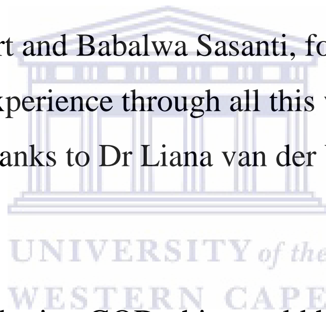

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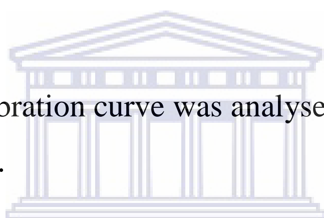

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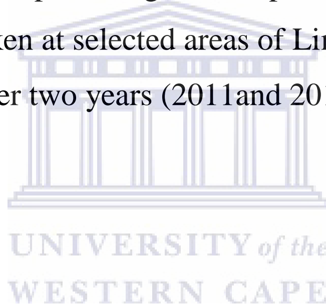
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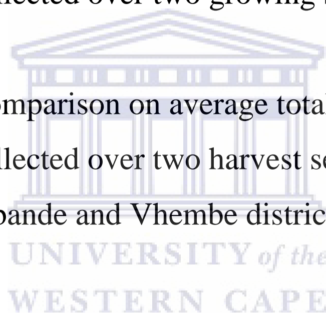
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LIST OF ABBREVIATIONS

VDM	Vhembe District Municipality in Limpopo province.
GSDM	Gert Sibande District Municipality in Mpumalanga Province.
AF	Aflatoxin
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
AFT	Total aflatoxin
FB	Fumonisin
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
AFPA	*Aspergillus flavus* and *A. parasiticus* agar
MEA	Malt extract agar
spp.	Species
HPLC	High-Performance Liquid Chromatography
RP-HPLC-FD	Reversed-phase High-Performance Liquid Chromatography fluorescence detector.
OPA	*o*-phthaldialdehyde
PPE	porcine pulmonary edema
ELEM	equine leukoencephalomalacia
GAP	Good Agricultural Practice
FAO	United Nations Food and Agriculture Organization.
FDA	Food and Drug Administration, United States of America.
EU	European Union
WHO	World Health Organization
PMTDI	Provisional Maximum Tolerable Daily Intake

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JECFA	Joint FAO/WHO Expert Committee on Food Additives
CAC	Codex Alimentarius Commission
IARC	International Agency for Research on Cancer
DAFF	Department of Agriculture, Forestry and Fisheries
MDGs	One of S.A's Millennium Development Goals)
ASGISA	Accelerated and Shared Growth Initiative South Africa
IDP	Integrated Develop Plan
MTL	maximum tolerated limits
IFSS	Integrated Food Security Strategy

DEFINITION OF TERMS

i.	Mycotoxins	toxins produced by fungus.
ii.	Aflatoxins	a group of mycotoxins with similar chemical structures produced by some *Aspergillus* species.
iii.	Fumonisins	a group of mycotoxins with similar chemical structures produced mainly by some *Fusarium* species.
iv.	Fungicide	chemical compounds or biological organisms used to kill or inhibit fungi or fungal spores.
v.	Carcinogenesis	process by which normal cells are transformed into cancer cells.
vi.	Mycotoxicoses-	disease resulting from toxic effect of mycotoxins on animal and human health.
vii.	Aflatoxicoses	diseases caused by aflatoxin consumption.
viii.	Aflatoxicol	is a reductive metabolite of aflatoxin B_1.
ix.	Mouldy	kernels that are visibly infected by fungi and characterized by black, blue, green, yellow, or white fungi growth anywhere on the kernel.
x.	Insect	insects (i.e. weevils) found in maize, living insect that damage stored grain.

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CONTRIBUTIONS OF THE RESEARCH

Parts of data from this thesis have already been integrated into the following publication and presentations at conferences:

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National conferences:

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   (University of Venda in Limpopo, South Africa)

Oral: P. Mngqawa, D.R. Katerere, S. Ngubeni.

Title: Grain storage practices and their effect on mycotoxin contamination among rural subsistence farmers in South Africa.

2. **SANPAD symposium - 19 April 2013.**
   (Tshwane University of Technology, Pretoria, South Africa)

Oral: P. Mngqawa, S. Teffo, SH. Ngubeni, JR Rheeder, GS. Shephard, IR. Green, DR. Katerere.

Title: Natural occurrence of fumonisin and aflatoxin in crops from selected districts of Limpopo & Mpumalanga provinces of South Africa.

International conferences:

1. **Mycored International Conference 4 to 6 April 2011.**
   (CTICC in Cape Town, South Africa)

Poster: P. Mngqawa, GS. Shephard, L. van der Westhuizen and HF. Vismer.
Title: Fumonisin production by *Fusarium proliferatum* grown on maize, millet and sorghum culture patties.

2. **JICSTDA: Joint International Conference on Science and Technology for Development in Africa 26 to 28 June 2012.**
   (Cape Peninsula University of Technology in Cape Town, South Africa)

Oral: P. Mngqawa, S. Ngobeni, S. Teffo and D.R. Katerere.
Title: Comparative study of grain production and mycotoxin ccurrence in the subsistence farming sector in Limpopo and Mpumalanga provinces of South Africa.

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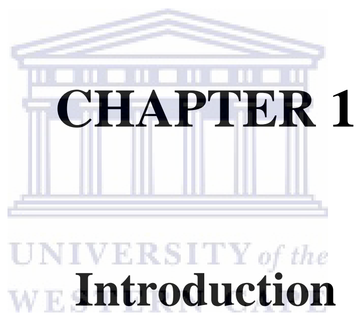
3. **WMF meets IUPAC: 7th Conference of The World Mycotoxin Forum® and the XIIIth IUPAC International Symposium on Mycotoxins and Phycotoxins 5–9 November 2012.**
   (World Trade Centre in Rotterdam, the Netherlands, Europe)

a) **Poster:** P. Mngqawa, JP. Rheeder, GS. Shephard, IR. Green and DR. Katerere.
Title: Investigation of mycotoxin and mycological contamination of maize crops in two rural South African Provinces Limpopo and Mpumalanga).

b) **Poster:** M. Hove, P. Mngqawa, S.H. Ngobeni, D.R. Katerere, and T.C. de Rijk.
Title: Multi-Mycotoxin Analysis of Food Crop Samples from Rural Northern South Africa.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CHAPTER 1

Introduction

1.1 Introduction

Mycotoxins are toxic chemical compounds naturally produced by some fungal species (usually *Aspergillus, Penicillium* and *Fusarium*) which may contaminate human food and animal feed (Proctor, 1994). These fungi are known to be the most important plant pathogens and are commonly detrimental to both human and animals (Bennett and Klich., 2003).

Aflatoxins, deoxynivalenol (DON), fumonisins, ochratoxin, and ergot alkaloids are some of the mycotoxins that adversely affect human and animal health (Bhat and Vasanthi, 2003). High temperatures, drought stress, high moisture content in storage, unseasonal rains during harvest, improper storage and floods may lead to fungal growth (Bhat and Vasanthi, 2003). Some mycotoxins such as fumonisins, DON and ergot alkaloids are produced before harvest, whereas aflatoxin production occurs both in the field and during storage (Bhat and Vasanthi, 2003). Aflatoxins and fumonisins are agriculturally the most important mycotoxins produced by major food–borne fungi that occur largely in sub-tropical and tropical climates throughout the world (Shephard, 2004, Shephard, 2008). These mycotoxins have received much attention in Africa because they are responsible for production loss in maize, peanuts and other grains (Shephard, 2008). Some indigenous agricultural practices used in processing and preserving the crops may also be responsible for fungal growth and mycotoxin production (Moss, 1996). In some other cases the lack of knowledge by the local farmers about fungi often leads to huge losses in crops. Since action is usually taken only when there is discoloration, insect manifestation and rotting signs in the grain and of course by that time it is too late to salvage the crops (Marasas and Vismer, 2003).

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Developing countries are faced by several challenges such as, food insecurity and socio-economic problems associated with poverty. In most African countries issues of food security frequently override issues of food safety due to poverty (Shephard, 2003). It is well-known that agriculture is one of the most important sources of livelihood of the people in the rural areas. However there is a huge lack of information in rural subsistence farming with regard to mycotoxin

contamination and consequently it is imperative to investigate methods to improve crops that are grown and stored by rural subsistence farmers (Abbas, 2005).

The current study investigated the extent of aflatoxin and fumonisin contamination in stored home-grown food crops in the Vhembe District Municipality (VDM) of Limpopo province, and the Gert Sibande District Municipality (GSDM) of Mpumalanga province of South Africa. Limpopo province is close to Zimbabwe and Mozambique while the Mpumalanga province is a neighbour of Swaziland which itself has been reported to have a high prevalence of aflatoxins and fumonisins in food which has been attributed to climatic conditions (Katerere et al., 2008, Sibanda et al., 1997).

1.2 Research Objectives

1.2.1 Aim

This study aims to investigate agricultural productivity and the natural occurrence of aflatoxins and fumonisins in home-grown maize and peanuts in northern rural South Africa, Vhembe District Municipality in the Limpopo province, and Gert Sibande District Municipality in Mpumalanga province. The study's outcome will inform rural subsistence farmers, health authorities and the general public in the areas described about the extent of mycotoxin contamination and possibly assist with intervention strategies.

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1.2.2 Objectives

In the present study we set out to assess and compare the grain and secondary crop production output from selected subsistence farmers in the rural areas of two provinces in South Africa as a measure of household food security status. Maize production, storage and sanitation hygiene are crucial to food security and food safety in the country because maize is the staple food for the majority of the South African population (Mudhara, 2010). We focused on collecting data on maize

production in the 2011–2012 seasons from a total of 39 households in Limpopo and Mpumalanga provinces. The main objectives of this study were to:

(a) Investigate the types of crops and establish baseline production output in selected rural subsistence farmers of Mpumalanga (GSDM) and Limpopo (VDM) provinces.

(b) In addition, we sought to understand the production constraints that subsistence farmers may be experiencing and hence to identify strategic interventions required from policy-makers, government and possibly other stakeholders.

(c) Investigate the natural occurrence of aflatoxins and fumonisins in stored home-grown maize and peanuts over two growing seasons using HPLC and LC-MS/MS.

(d) Compare the extent of mycotoxin and mycological contamination in stored home-grown crops between the selected villages in both Limpopo and Mpumalanga, and explain the possible causes of differences, if any.

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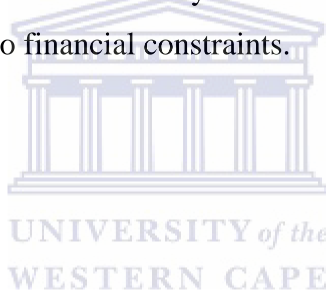
1.3 Significance of the study

In most of South Africa, data on the natural contamination of food commodities by fumonisins has been generated (Gelderblom et al., 1988; Shephard et al., 1996; Marasas, 2001). However, there is lack of information with regard to aflatoxin contamination, production output and crop diversity in the subsistence farming sector. Both fumonisins and aflatoxins are the two most common natural contaminants of food and feed. They are stable to different processing conditions, and they can additionally be present in significant levels in finished products as well (Senyuva et al., 2008). There have been no recent published studies done using the HPLC analytical methods to investigate the natural occurrence of aflatoxins in these two specific areas mentioned above and thus it is of importance

to have these data available for a more comprehensive overview of the national extent of contamination.

1.4 Research limitations

Determination of the quantitative presence of aflatoxins and fumonisins will be done on a limited number of samples which were kept in storage for six weeks over two growing seasons. The study will not include an evaluation on the effects of long term storage, e.g. over several seasons. Samples will be sourced from only two rural areas because of financial constraints. The extent of mycotoxin contamination in the samples collected for this study will probably not represent the entire rural areas which were selected. The number of households from which the samples were collected was relatively small in comparison to the size of each rural area. This was due to financial constraints.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1.5 Research outline

Chapter 1: ***Introduction:***

Presents an overview on mycotoxins (fumonisins and aflatoxins) including the introduction on the current research objectives, justifications, limitations and the outline of the study. The summary of the study chapters will be as follows:

Chapter 2: ***Literature Review:***

Gives background on health risks and economic impact of mycotoxins, aflatoxins and fumonisins to humans and animals worldwide. Describes factors which may contribute to the agricultural production loss in selected rural areas of Limpopo and Mpumalanga. This section also includes strategies to control these toxins in food and feed.

Chapter 3: ***Data assessment on demographics, effects on home-grown storage practices and productivity of subsistence farmers in two rural areas (Limpopo and Mpumalanga):***

Ethics approval for the study was granted and the confidentiality clause of the participants was signed. A detailed survey for the study areas, sampling procedure and farming practices in Mpumalanga and Limpopo are included. Subsistence farming practices revealed the contributing factors to the agricultural production yield which in turn informs the farmers of strategies to control and manage their produce.

Chapter 4: ***Mycoflora isolation of selected, Fusarium and Aspergillus fungal strains in stored homegrown maize, peanuts and beans from selected rural areas of Limpopo and Mpumalanga:***

Isolation of the fungi associated with the production of mycotoxins in stored food crops was achieved. Severity and identity of the incidence of the *Fusarium* and *Aspergillus* fungal species growth from homegrown food stored for six weeks after harvest in Limpopo (VDM) and Mpumalanga (GSDM). A mycological examination for both fungal species was performed by using methods as described by Leslie and Summerell, 2006 and Pitt et al., 1983.

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Chapter 5: ***HPLC validation and analysis of crops (homegrown maize, peanuts and beans) samples for aflatoxins and fumonisins:***

Levels of mycotoxin were investigated in agricultural crops surveyed from Limpopo and Mpumalanga using two in-house validated methods. Fumonisins were determined by an internationally validated method by Sydenham et al., 1996 and an in-house method developed by Shephard et al., 1990 with minor modifications. Aflatoxin was determined using a method by Gnonlonfin et al., 2010 with slight modifications. Standard preparations and sample extraction for maize, peanuts and bean analysis as well as the clean-up and derivatization procedures are clarified.

Chapter 6: ***Multi-toxin quantification of homegrown agricultural produce in selected rural areas of Mpumalanga and Limpopo:***

Mycotoxins studied were analysed using a selective and reliable multi-mycotoxin LC-MS/MS method for detection and quantification of several mycotoxins (Mol et al. 2008; van Asselt et al., 2012). Mycotoxins were extracted following a generic extraction protocol and analysed simultaneously in two chromatographic runs, and detected in both positive (ESI+) and negative (ESI-) ionization electrospray mode. The objectives of using the modern LC-MS/MS multi-analyte method are to achieve simplicity and high throughput by directly injecting sample extracts avoiding any further clean-up (Suloyk et al 2006; Mol et al. 2008; Frenich et al. 2009; Martos et al. 2010). Although other mycotoxins were detected, the main focus was on two commonly occurring and chemically different mycotoxins (aflatoxins and fumonisins) which are produced by the fungal genera *Aspergillus* and *Fusarium,* as contaminants in agricultural commodities.

Chapter 7: ***General Discussion, Recommendations and Conclusions:***

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Results covering seasons 2011 and 2012 for this study will be summarised and the comparison between Limpopo and Mpumalanga subsistence producers will be presented and clarified. Comparison on the occurrence and levels of aflatoxins and fumonisins found in maize, peanut and beans will be stated. Recommendations and conclusions of the study will be clarified.

Appendices:

This section contains an ethics approval letter from the MRC Ethics committee and a semi-structured questionnaire used during the study. Also data on consumption, incidence and concentrations found in samples are presented together with a comprehensive list of multi-analyte spiking components and calibration standards.

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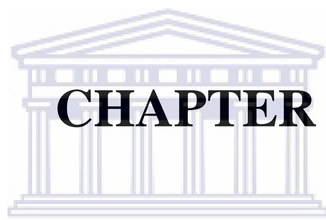
CHAPTER 2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Literature review

2.1 Introduction

2.1.1 Overview of mycotoxins

Mycotoxins are toxic chemical compounds produced by certain fungi (FAO, 1994). They are secondary metabolites produced by a range of filamentous fungi which naturally occur in food commodities (Shephard, 2008). These toxins exert deleterious effects on animals and humans called mycotoxicoses, mainly through exposure from agricultural food and feed (Shephard, 2008; Peraica et al., 1999). There are about 300 mycotoxins known world-wide, but only five groups are considered to be important: trichothecenes such as deoxynivalenol/nivalenol, zearalenone,ochratoxin,fumonisins, and aflatoxins (Akande et al., 2006). They are frequently found in food and animal feed and are the most important mycotoxins relevant for public health and trade (Zain, 2011).

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In most parts of South Africa, maize and peanuts are two of the major crops consumed and thus serve as important nutritional diet components (DAFF, 2013). These crops are also among the most vulnerable to mycotoxins (Schmaile and Munkvold, 2009; Williams et al., 2004). Aflatoxin contamination affects all foods and animal feed types differently depending on the level of carbohydrates (Makun et al., 2012; Muthomi et al., 2009). Mycotoxin contamination occurs throughout the food chain. The interaction between the fungus, its plant host and the environment, determines the type and level of mycotoxin produced and the type of food-crops affected (Pitt, 2000). These crops may be infected by several *Fusarium* and *Aspergillus* species which produce secondary metabolites responsible for severe plant diseases and are the most frequently isolated from grains (Shephard, 2008). The severity of mycotoxicosis depends on the potency of the toxin, the duration and amount of exposure, age and dietary status of the individual and possible synergistic effects with other chemicals (Peraica et al., 1999).

The present study focused on aflatoxins and fumonisins because they are the most important mycotoxins in Africa (Moss, 1996; Pitt et al., 2000). They have been

implicated in both acute and chronic mycotoxicoses which can cause serious and sometimes deadly diseases in humans and animals (Bhat and Vasanthi, 2003; Peraica et al., 1999). The fungi producing these toxins are known to directly develop or continue to develop on grain crops during food storage, processing, production and transport (Aljicevic et al., 2008).

2.2 Effects of mycotoxins on health

Mycotoxins are produced in almost all major food commodities during handling and in storage, and exposure commonly occurs through ingestion and inhalation (Peraica et al., 1999). In most cases mycotoxins which occur in the host species in the soil surface are transferred to the plants by wind and insects (Richard et al., 1993). The most important genera of mycotoxigenic fungi in food and feed are *Aspergillus, Alternaria, Claviceps, Fusarium, Penicillium* and *Stachybotrys* (Peraica et al., 1999).

In humans the organs that may be affected by mycotoxin contamination include the liver, kidney, stomach, lung, brain, womb and the skin (Peraica et al., 1999). In both animals and humans the liver is the most affected organ by mycotoxins. Commonly all animals are vulnerable to mycotoxins depending on their breed, sex, age, nutrition and physiological standing and organs affected among others are, liver and kidney (Zain, 2011; Voss et al., 2007). Deaths of swine and horses in the United States increased interest in mycotoxin contamination significantly (Marasas, 1996).

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2.3 Aflatoxin occurrence

Aflatoxins are secondary metabolites naturally produced by toxigenic species of *Aspergillus,* mainly *A. flavus* and *A.parasiticus* which infect agricultural crops (Castells et al., 2008). *A.flavus* is the main *Aspergillus* species largely produced in agricultural produce (Zain, 2011). They cause disease in economically important crops and therefore have to be fully studied because of their high toxicity and

natural occurrence (Henry et al., 1999, Otsuki et al., 2001). *A. flavus* produces only B aflatoxins, whereas *A.parasiticus* produces both B and G aflatoxin analogues (Hedayati et al., 2007, Peraica et al., 1999). Aflatoxins are fluorescent compounds which were first isolated and identified in 1960 when there was an outbreak of Turkey 'X' disease. It caused the death of 100,000 birds in England which had been fed contaminated groundnuts. To date approximately 20 aflatoxins have been identified (Eaton and Groopman, 1994, Moss, 1996).

There are four basic structures for the aflatoxins (Figure 2.1): aflatoxin B_1 (AFB_1 which represents between 60-80% of the total aflatoxin), G_1 (AFG_1), B_2 (AFB_2), and G_2 (AFG_2). Interestingly, AFB_1 may occur on its own while the others are always found in combination with one another (Weidenborner, 2001). Aflatoxins M_1 and M_2 are hydroxylated metabolic products of aflatoxins B_1 and B_2 and occur in the milk of mammals following aflatoxin B_1 ingestion (Fink-Gremmels, 2008; EFSA, 2004a).

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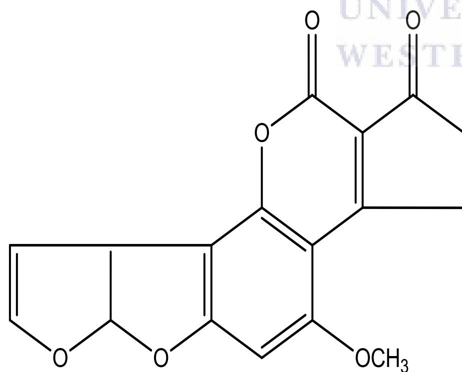
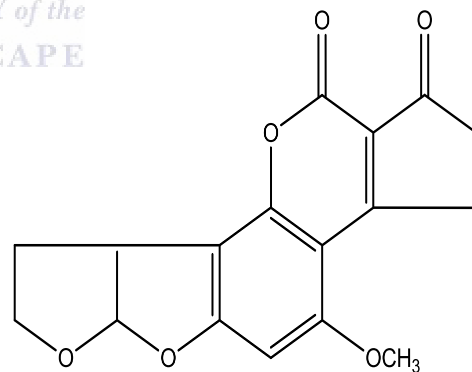
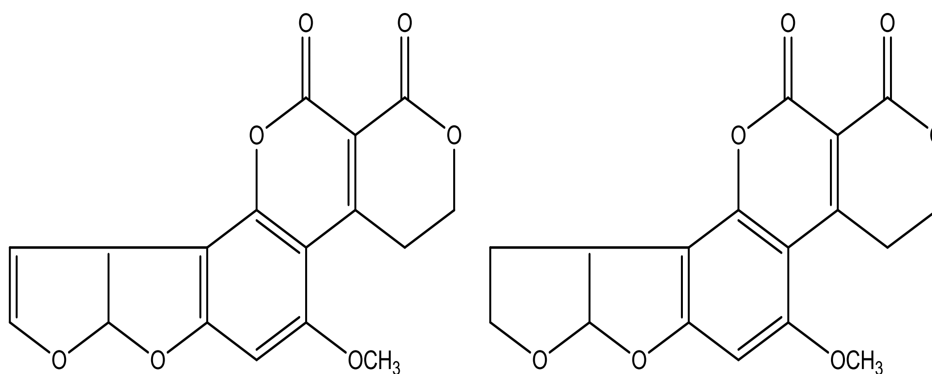


Aflatoxin B_1



Aflatoxin B_2



Aflatoxin G_1 **Aflatoxin G_2**

Figure 2.1: Chemical structures of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2.

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Chemically, aflatoxins are classified as difurocoumarolactones (difuranocoumarin derivatives) (Richard et al., 1993), and comprise of a furan moiety bonded to a coumarin nucleus which is substituted with either a six-membered lactone ring (AFG) or a pentenone ring (AFB and AFM) (Agag, 2004). Peanuts, maize and cotton seed are highly at risk for aflatoxin contamination and this risk maybe exacerbated by inadequate drying or improper storage methods (Castells et al., 2008).

Aflatoxin B_1 has been the most frequently found potent analogue produced by toxigenic strains (Wogan, 1966; Squire, 1981; Mclean & Dutton, 1995). It has been proven to be *hepatoxic, hepatocarcinogenic* and *mutagenic* to humans and animals (Dilkin et al., 2003). It has been classified as a highly potent (group 1) human carcinogen by the International Agency for Research on Cancer (IARC) (WHO-IARC, 2002). Conversion of AFB_1 into the metabolite AFM_1, also a hepatocarcinogen, poses a high risk in children and young animals, and has been classified as a group 2B possible human carcinogen (van Egmond & Dragacci, 2001; IARC, 2002).

Aflatoxins occur mainly in sub-tropical and tropical climates where temperature and humidity levels encourage the growth of aflatoxigenic species (Castells et al., 2008). They are found both before and after harvest (figures 2.2 and 2.3), on practically any food or feed which supports fungal growth, including cereals, oilseeds and edible nuts (Zain, 2011).

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Figure 2.2: *Aspergillus flavus* on maize in the field (Sweets and Wrather, 2009).

Figure 2.3: Stored maize infected by *Aspergillus flavus* (Sweets and Wrather, 2009).

Pre-harvest mould growth and aflatoxin production is intensified by insect damage, mechanical damage, drought stress and excessive rainfall (Miller, 1991). Mould can grow between the maize kernels and develop on the ears of insect damaged kernels (Jouany, 2007). In several African countries, staple foods (such as; maize, wheat, sorghum and ground nuts) have been found to be contaminated with aflatoxins (Makun et al., 2012; EMANc).

There have been reported cases of aflatoxicosis outbreaks in Kenya and South Africa which have claimed the lives of humans and animals, respectively (Probst et al., 2007; Arnot et al., 2012). In South Africa there were various media reports of extremely high levels of aflatoxins (272 µg/kg total AF and 165 µg/kg AFB_1) in peanut butter consumed by Primary school children in the Eastern Cape as part the Primary Schools Nutrition Programme (PSNP) (MRC, 2006). The results 165 µg/kg AFB_1 were more than thirty times higher than South Africa's aflatoxin legal limit of 5 µg/kg AFB_1 (Rheeder et al., 2009). Also extreme cases of groundnut aflatoxin contamination recurred over the years in Nigeria, which in some instance resulted in the deaths of young school children and the produce was subsequently declared unsafe for consumption (Makunet al., 2012). Kenya in particular experienced recurring aflatoxicosis outbreaks (1978, 1981, 2001, 2004, 2005, 2006, 2007, and 2008) causing illness, death, and food shortages (Muthomi et al., 2009). The worst outbreak in Kenya was from January to June 2004 with some maize samples shown to contain as much as 4400 ng/g AFB_1 which iswell above the accepted maximum tolerated levels (MTL) of 10 ng/g (Lewis et al., 2005). In many countries across Africa aflatoxicosis caused by contaminated staple crops has exacerbated food security and compromised food safety in affected communities (Kellerman et al., 1996; Probst et al., 2007).

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2.3.1 Toxicological effects in humans

Aflatoxin contamination represents a serious health concern and aflatoxins are known to be immunosuppressive, teratogenic, mutagenic and carcinogenic to humans (Bhat and Vasanthi, 2003). The liver is the main target for toxicity and

carcinogenicity, and it has been found that exposure to high doses may lead to fatal liver failure (Peraica et al., 1999). The result of exposure to large doses of aflatoxins leads to acute toxicity and death whereas low doses lead to chronic toxicity which has been associated with the development of hepatocellular carcinoma (HCC) as it increases the risk fourfold (Ming et al., 2002; Turner et al., 2002). Early symptoms of aflatoxicosis are diminished appetite, malaise, low fever, other symptoms include vomiting, abdominal pain, and hepatitis (Barrett, 2005, Groopman et al., 1988).

Aflatoxins have been shown to have a negative effect on the immune status and micronutrient absorption of the exposed individuals and stunted growth in infants and young children (Gong, 2002, Maxwell et al., 1989).
Aflatoxin ingestion adversely affects protein energy metabolism, haemoglobin levels and effectiveness of vaccines (Miller, 1996). Naturally occurring aflatoxins are Group 1 human carcinogens that have shown carcinogenicity in animal species (IARC, 2002). It is also an important factor in infant mortality in the developing world (Katerere et al., 2008). Liver cancer incidence has been found to be high in areas of sub-Saharan Africa, Southeast Asia, and China where aflatoxins and hepatitis B virus (HBV) infections were prevalent (Peraica et al., 1999; Liu and Wu, 2010). Aflatoxin exposure with HBV infection have a synergistic impact in inducing a 60-fold increase in the risk of HCC (Liu and Wu, 2010; Cao and Fan, 2011).

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HCC has been found to be one of the important causes of cancer deaths in the world (Henry et al., 2002; Wu and Khlangwiset, 2010). It is estimated that 250,000 deaths are caused by hepatocellular carcinomas yearly in China and Sub-Saharan Africa because of high daily exposure to aflatoxins (Zain, 2011). Chronic HBV, aflatoxin exposure, alcoholism, tobacco smoking, diabetes and obesity are the main risk factors of HCC (Wu and Khlangwiset, 2010). South Africa’s climate in certain areas as well as socio-economic factors may favour the growth of aflatoxin producing fungi and co-infection with hepatitis B virus (Katerere, 2008, Pitt, 2000).

2.3.2 Toxicological effects in animals

Sometimes mycotoxin contaminated crops are used as animal feed, in which case the animals will produce meat and milk products that may contain levels of toxic residues. Aflatoxin in feed can be absorbed by animals and found in their metabolized form in milk or milk products as aflatoxin M_1 and M_2 (Anfossi et al., 2011). When AFB_1 contaminated feed is consumed by mammals, it is converted into hydroxylated form e.g. AFM_1 by the hepatic microsomal cytochrome P_{450} (catalyst) and secreted in dairy milk by the mammary gland (Figure 2.4) (Veldman et al., 1992; Dutton et al., 2012).

Consumption of contaminated feed by animals can in general cause reduced growth rates, illness, and death (Bruns, 2003). No animal species is tolerant to the acute toxic effects of aflatoxins. More than 50% of animal feed is made up of maize products according to the SA Feedlot Association (NDA, 2004). By act 1947 (Amended R227 No. 31958, 2009) maximum levels for dairy feed are set at 5 µg/kg AFB_1 and cattle feed at MTL of 50 µg/kg (S.A Fertilizers, 2009).

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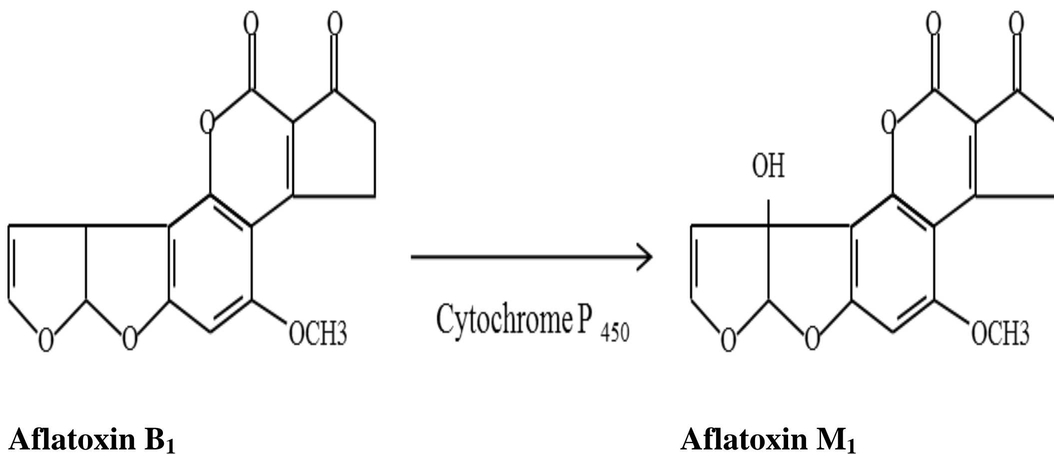

Figure 2.4: Aflatoxin M_1 as a hydroxylated metabolite of Aflatoxin B_1.

During an outbreak of aflatoxicosis in South Africa, approximately over 220 dogs died after consuming toxic pet food with concentrations of up to 4946 µg/kg (Arnot et al., 2012). Acute toxicity is common in livestock and causes severe liver damage and immuno-suppression leading to death, particularly in pigs (Thamaga-Chitja et al., 2004). Chronic toxicity leads to poor food intake, vomiting, stunted growth and weight loss in animals (Peraica et al., 1999). Aflatoxin ingestion has severe toxic effects on the internal organs, tissues and compromises the reproductive capabilities of animals (Dilkin et al., 2003).

2.4 Fumonisins occurrence

Fumonisins are often produced in high levels by *Fusarium verticillioides* and *F. proliferatum* which often occur on different kinds of foods and feeds (da Silva et al., 2004). However, maize and maize-based products are the most vulnerable to the genus *Fusarium* (Marasas, 2001). They have also been shown to be produced by *Aspergillus*, in particular FB2 can also be produced by *A. niger* (Frisvad et al., 2007). *A. niger* has even been granted the GRAS (Generally Regarded As Safe) status in certain industrial production processes by the Food and Drug Administration of the US government (Perrone et al. 2007). *Fusarium spp*. are commonly found in different environmental conditions. In maize they are linked with ear rot, cob rot, stalk rot and some diseases associated with insect infestation (Leslie and Summerell, 2006). Figure 2.5 shows rather dramatically what the effects are of maize infected with ear-rot.

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Figure 2.5: Maize ear-rot infected by *Fusarium spp.*

More than 28 fumonisin analogues have been identified and isolated since they were first discovered in 1988 at the South African Medical Research Council (SA MRC), from *F. verticillioides* strain MRC 826 (Bezuidenhout et al., 1988, Gelderblom et al., 1988, Rheeder et al., 2002). Fumonisins are long-chain polar compounds with a chemical structure based on an eicosane hydroxylated hydrocarbon chain substituted with methyl and amino groups (Marasas, 2001). *Fusarium* species are generally isolated in more than 50% of maize in tropical and subtropical regions and cause grain discoloration and reduction in nutritional value (Shephard et al., 1996, Fandohan et al., 2003). However, *Fusarium spp* can also be produced during storage (Marasas, 1995, Ross et al., 1992). Figure 2.6 gives a representation of the chemical structures of FB_1, FB_2, and FB_3. FB_1 is a polyhydroxy alkyl amine, esterified at C14 and C15 with 2 molecules of tricarballylic acid (Bezuidenhout et al., 1988, ApSimon et al., 1994).

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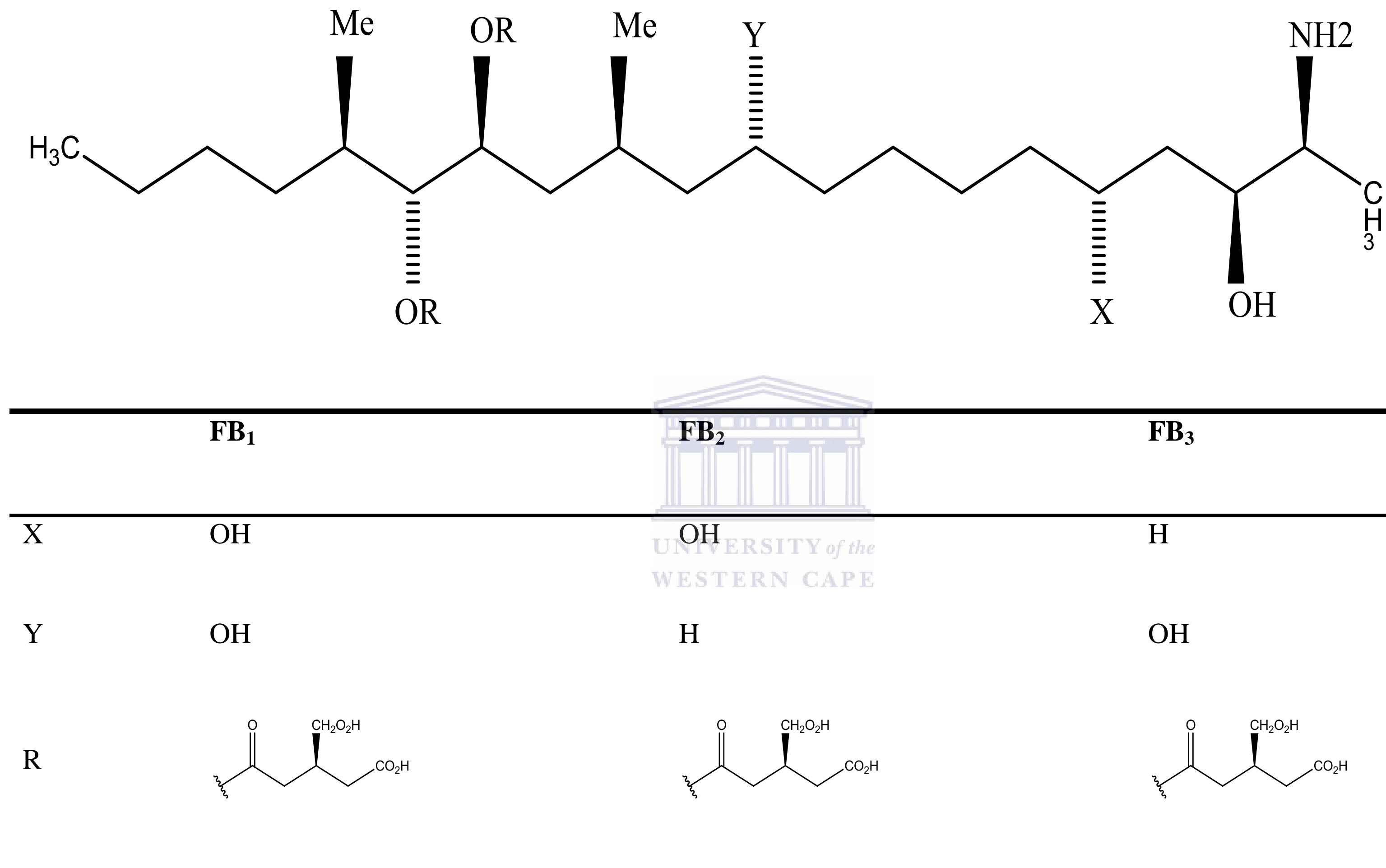

	FB$_1$	FB$_2$	FB$_3$
X	OH	OH	H
Y	OH	H	OH
R	O, CH_2O_2H, CO_2H	O, CH_2O_2H, CO_2H	O, CH_2O_2H, CO_2H

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Figure 2.6: Chemical structures of fumonisin B_1, fumonisin B_2, and fumonisin B_3.

There are five groups of fumonisins viz., A, B, C and P which are chemically stable (Rheeder et al., 2002; Lawley et al., 2008). Fumonisins, A, C and P occur naturally at less than 5% levels of the total fumonisins detected (Rheeder et al., 2002). The B-type represents agriculturally the most important and widespread of the mycotoxins (Marasas, 1996). Fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) are produced by *F.verticillioides* with FB_1 predominant (Shephard et al., 1996) and accounts for 70 to 80% while FB_2 accounts for 15-25% of the total content. Other fumonisins may be produced at relatively low levels (Rheeder et al., 2002). Higher FB_1 production in comparison to other analogues (FB_2 and FB_3) has been reported worldwide in both naturally contaminated and cultured maize (Chu and Li, 1994; Fotso et al., 2002; Rheeder et al., 2002).

FB_1 is by far the most abundant and toxic metabolite in terms of its occurrence and toxicity and found in naturally contaminated commodities (Krska et al., 2007, Rheeder et al., 2002). Although FB_1 was found to be neither mutagenic nor genotoxic, indications are that it is a cancer promoter (rather than initiator) (Gelderblom et al., 1991; Pitt, 2000). Fumonisins are widespread in maize growing regions and maize-based foods and feeds in the world (Shephard et al., 1996). In some regions of Africa, China and Italy, FB_1 was frequently found in much higher levels in maize crops where there was higher incidence of oesophageal cancer than other regions where FB_1 levels were lower (Chu and Li, 1994, Jaskiewicz et al., 1987, Marasas, 1981; Somdyala et al., 2010). This led to the conclusion that fumonisin exposure may be one of the risk factors for the development of oesophageal cancer (Marasas, 2001).

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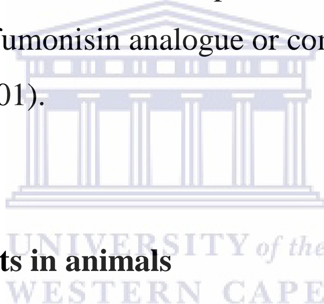
2.4.1 Toxicological effects in humans

There has not been confirmation of human health effects caused by fumonisins. However, it is considered an important risk factor in human oesophageal cancer in the former Transkei regions of South Africa and Santa Catarina State, Brazil (Rheeder et al., 1992; van der Westhuizen et al., 2003). It has been associated in

the development of neural tube defects in the womb which subsequently affects babies especially in certain regions of South Africa, China, and Italy (Bhat and Vasanthi, 2003; Chu and Li, 1994; Marasas et al., 2004; Shephard, 2001). In the Eastern Cape Province, in the area formally known as Transkei in Southern Africa, fumonisins were found to be endemic in maize which is the major staple food (Shephard, 2001).

FB_1 has been categorized as a group 2B carcinogen (probable carcinogenic to humans) by the IARC (WHO-IARC, 2002). Main symptoms of acute exposure are severe abdominal pain and continuous bloody diarrhea (IARC, 2002; Peraica et al., 1999). A provisional maximum tolerable daily intake (PMTDI) guidance value of 2µg/kg body weight per day for fumonisin in contaminated food has been recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for either each fumonisin analogue or combination of fumonisins B_1, B_2 and B_3) (Bolger et al., 2001).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2.4.2 Toxicological effects in animals

Fumonisins have low acute oral toxicity in several animal species and are hepatotoxic in some animals (Fako et al., 2004). In Syrian hamsters, embryo toxicity is only observed simultaneously with maternal toxicity (Marasas et al., 2000). Contaminated maize obtained from the areas with high oesophageal cancer in the Eastern Cape Province was fed to rats for a long period, and found to cause toxic and preneoplastic lesions in the liver of male BD IX rats (Gelderblom et al., 2004). It has been established that low concentrations of FB_1 are taken up by the liver and kidney and can be excreted through the urine (Marasas et al., 2000). In experimental work, fumonisin B_1 was found to cause hepato- and nephrotoxicity in rats, mice and rabbits (Gelderblom et al. 1988; Gumprecht et al. 1995; Sharma et al. 1997).

The most sensitive species to fumonisin toxicity are horses and pigs in particular. In horses it causes equine leukoencephalomalacia (ELEM) or *"hole-in-the-head-*

disease" syndrome which affects the central nervous system followed by lesions in the brain (Marasas, 1996; Marasas et al., 1988). In pigs porcine pulmonary edema syndrome (PPE) occurs within 4 – 7 days of ingesting contaminated feed (Voss et al., 2007). The first symptoms noted were a decrease in feed intake, sluggishness followed by convulsions and eventually leading to death (Voss et al., 2007).

2.5 Other mycotoxins

There are various other mycotoxins, which may contaminate food stuffs in different concentrations and affect humans and domestic animal health (Amadi and Adeniyi, 2009; EMANa). Most of the other mycotoxins found in grain are produced by *Fusarium*, *Aspergillus* and *Penicillium* fungi (Hainzte al., 2007; Roigé et al., 2009). These several important types of mycotoxins may cause health problems and include deoxynivalenol and other trichothecenes, ochratoxins, ergot alkaloids and zearalenone (Driehuiset al., 2008; Schmaile and Munkvold, 2009). Some of these mycotoxins may cause gastrointestinal problems, kidney lesions, and may interrupt protein and DNA synthesis (Pestka, 2007; Zeljezicet al., 2006). They frequently co-contaminate maize and maize products worldwide (Khayoon et al., 2010; Dorn et al., 2009). Absorption of deoxynivalenol, ochratoxins , zearalenone , fumonisins in milk; have been detected in lower levels compared to aflatoxins (EFSA, 2006; 2004b; 2004c; 2004d; Gazzotti et al. 2009)

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2.6 Economic impact of mycotoxins

Climatic conditions and dependence on maize diets by many countries in Africa exacerbates the problem of mycotoxin occurrence. Mycotoxin contamination of foods and feeds can cause serious economic hardships to producers, processors, and the consumer (Cary et al., 2009). This is due to losses in crop yields, livestock production, and research and regulatory costs to monitor and minimize contamination and exposure (Schmaile and Munkvold, 2009; Wu, 2004). Massive economic losses have been estimated to be experienced by developing countries

in Asia and sub-Saharan Africa, due to high levels of mycotoxin contamination in food and feed which in most of these countries is unavoidable (Swaans et al., 2009). The Food and Agriculture Organisation (FAO) has estimated that mycotoxins account for 25% of trade losses (WHO, 1991). International trade in agricultural commodities such as groundnuts, maize and other grain products amounts to hundreds of millions of tonnes of loss due to rejected imports each year (JECFA, 1999).

2.7 Legislation/ Regulations

Risk assessments for natural toxins have been set by the food safety agencies such as JECFA for both import and export of all food commodities for the protection of consumers. Since mycotoxins in food cannot be totally eliminated, they have to be regulated. These regulations vary throughout the world. Where well developed countries usually set lower maximum limits than emerging economic countries and most of the regulations in Africa apply for aflatoxins only (Wagacha and Muthomi, 2008). More than 100 countries regulate mycotoxins in commodities (FAO, 2004; van Egmond et al., 2007). Most include maximum tolerated or recommended levels for specific commodities and included in this list are only 15 African countries (Wagacha and Muthomi, 2008) which are mainly for AFB_1 rather than total aflatoxins.

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Different specific limits which apply to some international countries range from 4-30 µg/kg in food suitable for humans. The low limit of 4 µg/kg total aflatoxins applies to countries in the European Union (EU) where aflatoxin B_1 is set at 2 µg/kg where the limits for AFB_1 and the total aflatoxins are enforced for various products (Espinosa-Calderón et al., 2011). In Latin American countries and the United States, the limit is set at 20 µg/kg sum of aflatoxins. The United States is also one of the first countries to establish aflatoxin regulatory limits (Abbas, 2005). Young children and infants' food is controlled and limits are set at very low levels (0.10µg/kg AFB_1, 0.025 µg/kg AFM_1 and 200 µg/kg FB_1 & FB_2) (E.C., 2006). Food for children and children is essential in their nourishment and their

consumption per unit body weight is greater and they are more sensitive with under developed immune and gastro systems. Mycotoxins have been regulated to try and reduce the exposure in maize and maize products for human and animal consumption. Maximum levels regulated for the the sum of FB_1 and FB_2 which apply to natural maize products, milled grains and maize based foodstuffs for human consumption are 2000 µg/kg, 1000 µg/kg and 400 µg/kg (E.C., 2006). Table 2.1 shows a list of the mycotoxins in food that are regulated in Africa. The only African country which has detailed mycotoxin regulations is Morocco. It has different regulations for specific foods and even has regulations for childrens' food.

Table 2.1: List of regulated mycotoxins in African commodities (Abbas, 2005; E.C., 2006).

Regulated mycotoxins in Africa	
AFB_1	Aflatoxin B_1
AFT	Total aflatoxins
AFM_1	Aflatoxin M_1
OTA	Ochratoxin A
PAT	Patulin
AFG_1	Aflatoxin G_1
ZEN	Zearalenone

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Countries worldwide have a maximum tolerated AFB_1 limit in food and ranges from 1-20 µg/kg. A 5 µg/kg limit can be found in twenty one countries, in Africa, Asia/Oceania, Latin America and Europe (Abbas, 2005). Five countries have set limits of 10 µg/kg for total AF which includes South Africa, three countries have regulations for total AF of 15 µg/kg, and only two countries have regulations of 20 µg/kg.

Table 2.2 shows regulations in place for aflatoxin in the commodities from South Africa and its neighbouring countries. The maximum limit for AFM_1 in milk is 0.05 µg/L in South Africa, and in other international countries 0.05 µg/kg (EU) and 0.5 µg/kg by the U.S. Food and Drug Administration (Dutton et al., 2012; EC, 2003; FDA, 2011).

Table 2.2: Southern African countries with aflatoxin regulations (EC, 2010; FAO, 2004).

Aflatoxins: Maximum Tolerated Levels		
African countries	aflatoxin (µg/kg)	food and feed
South Africa	5 AFB_1; 10 total	All Foods
	0.05 AFM_1	Dairy milk
Zimbabwe	5 AFB_1; 4 AFG_1	maize, groundnut & sorghum
	10 AFB_1 & AFG_1	Poultry feed
Mozambique	10 total	Peanuts, peanut milk
	10 total	Feed
Malawi	5 AFB_1	Peanuts export

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Mycotoxin contamination in Africa is a significant health risk mostly in informal trading channels. Subsistence farmers grow agricultural crops mostly for their own consumption, and hence most of the existing regulations will have no effect on them. Regulation of mycotoxins in foods and feeds creates an excellent and sustainable opportunity for international trade. It can act as a non-tariff trade barrier as well. FAO and WHO do not consider the regulatory standards for aflatoxins as an option for subsistence farmers as they have no proper infrastructure in place (Bhat and Vasanthi, 2003).

To have regulations and policies is very important in controlling mycotoxins, however, it is not always appropriate, especially in Africa where most food is traded through informal channels. Acute toxicity is rare in developed countries,

where mycotoxin contamination levels are monitored, controlled and diverse diets are consumed. Modern agricultural practices, regulated food processing and marketing systems have greatly reduced mycotoxin exposure in the developed countries (Shephard, 2008).

2.8 Reduction strategies

Most rural communities in Africa cannot afford bio-control or pesticides for improving their commodity productivity. Mycotoxin production on maize whilst in storage can be minimized by properly drying crops, sorting and disposing of visibly moldy or damaged kernels before storage (Fandohan et al., 2005a). A study done on simple preventative measures to reduce aflatoxin levels in groundnut before storage in West Africa, resulted in 50% reduction (Turner et al., 2005). It was further found that storage structures used, the quantity, extent of time and the form of maize stored (i.e., cob, or loose grain) impacts on the mycotoxin production level (Hell et al., 2000). There have been efforts to control fumonisins and aflatoxins in Benin by processing maize into traditional products (Fandohan et al., 2005b). Effective control of mycotoxin contamination of grains is best achieved at the pre-harvest stage by harvesting early and general good agricultural practices (GAP) such as crop waste removal (sorting), fertiliser application, prevention of drought stress by irrigation, and harvesting at the correct moisture level (Cary et al., 2009). There are other options which are less costly and less effective but can minimize the contamination, like cleaning of contaminated crops and the use of plants as preservatives (Makun et al., 2012).

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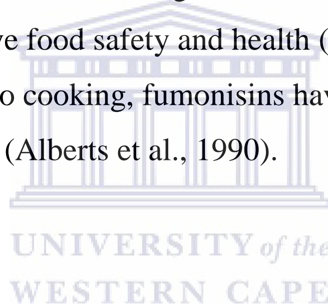
Early planting and rotation between crops that are prone to different mycotoxigenic fungi are very important for balancing soil fertility in order to reduce plant and mycotoxin infection (Jouany, 2007) but may not be practical due to erratic rainfall patterns and limited arable land. Improper storage methods together with insect and animal pest damage can result in fungal growth in grains within days of harvesting and even during transportation (Ncube et al., 2011). It was found that fast post-harvest effective drying can reduce or prevent production

of mycotoxins (FAO, 2004). Controlled moisture content and temperature in storage need to be monitored so that variation of both is minimized. Maintaining moisture levels of stored commodities below 0.7 a_w between a temperature range of 10 - 40°C prevents fungal and mycotoxin production (Whitlow et al., 2010; Paterson and Lima, 2010; EMANb). The U.S. Food and Drug Administration (FDA) made some recommendations in the year 2000 as means for safe moisture level before storage the maize moisture content should be in the region of 255 to 200 mg/g and dried out to 155 mg/g (Bruns, 2003; Ncube et al., 2011). For storage of maize and groundnuts the moisture levels have to be in the region of 14% and 7% at 20°C respectively (Lawley et al., 2008).

Fumonisin exposure in rural subsistence farming may be controlled and reduced by simple methods such as hand sorting and washing of contaminated grains before cooking to improve food safety and health (van der Westhuizen et al., 2010). But with regards to cooking, fumonisins have been found to be fairly stable during cooking (heating) (Alberts et al., 1990).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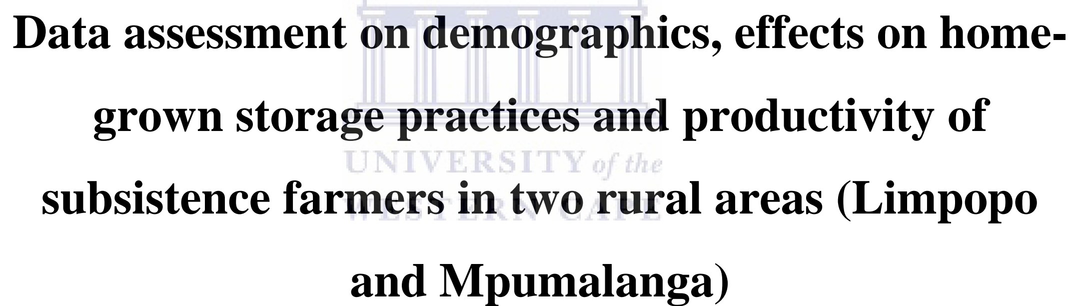
CHAPTER 3



Data assessment on demographics, effects on home-grown storage practices and productivity of subsistence farmers in two rural areas (Limpopo and Mpumalanga)

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3.1 Introduction

The agricultural sector (livestock, field crops and horticulture) plays an important role in economic growth and poverty alleviation and way of life for most people in Africa (Mlambo-Ngcuka, 2006; DFID, 2005). It ensures food security and increases employment opportunities in rural economies (FAO, 2008). Subsistence agricultural farming is when most of the farmers grow food mainly to be consumed by themselves and family, with little, if any, for trade (Baiphethi & Jacobs, 2009). Most research in Africa has shown that each subsistence farm has less than 0.5 hectares of rain fed farm land with agriculture being their primary source of food (Ellis et al., 2003; Ellis & Freeman, 2004). Agricultural contributions of subsistence farmers in Africa is around 80% (generally producing staple foods) and thus empowering subsistence farmers will increase productivity which will in turn reduce hunger (FAO, 2008; Moyo, 2010).

South Africa (S.A) has two agricultural farming sectors: well-developed commercial and subsistence farms (most produce consumed by the farmer with little to sell or trade) situated in the former homelands (May & Carter, 2009; Scotcher, 2010). About 46.3% of the population in S.A resides in the rural areas with many people being subsistence farmers and depending on agricultural activities as a way to alleviate poverty (Mathivha, 2012). South African government strategy through Integrated Food Security Strategy (IFSS) is to improve household food security and trade (DOA, 2002).

It’s evident that food insecurity is an important problem, which needs to be alleviated. There is a need to identify factors which hamper the agricultural growth in subsistence farms. Such factors are infections and improper storage practices of grains from two former Bantustan areas located in Limpopo and Mpumalanga.

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3.2 Food Security

The World Food Summit in 1996 linked food security to health on three main accounts i.e. food availability (access to sufficient quantities of food regularly), food access (adequate resources to obtain food), and food safety (safe and healthy food) (WHO, 2011). Sub-Saharan Africa continues to struggle with the inability of people to gain access to food due to poverty, which is the root cause of food insecurity in developing countries (InterAcademy Council, 2004). Poor productivity not only impacts on the economic status of rural subsistence farmers but it also exacerbates food insecurity and compromises food safety. While S.A is generally regarded to be a food-secure country, it ranks very high for income inequality (in terms of the GINI index) among other middle-income countries; up to 30% of the population experiences food insecurity (Altman et al., 2009). This commonly occurs in the rural areas and informal urban settlements (Bonti-Ankomah, 2001). Children are the most severely affected by food insecurity which impacts on their learning ability and physiological development and function (Bonti-Ankomah, 2001).

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When food is in short supply, the available food may not be safe enough for consumption due to spoilage (e.g. mycotoxins, bacteria and other contamination), which is a cause of concern (WHO, 2005). However, many people living in emerging economic countries have little choice but to consume spoiled food which is why mycotoxicoses are more frequent in these regions (Shephard, 2005; Katerere et al., 2008). Even though there are maximum tolerated limits (MTL) in place for certain mycotoxins, consumption level plays a big role in food safety (Shephard, 2008). MTL's are poorly enforced in developing countries and have no impact on subsistence agriculture where crops grown in the farm are consumed by the farmer.

One of S.A's Millennium Development Goals (MDGs) incorporated in the Accelerated and Shared Growth Initiative South Africa (ASGISA) was to reduce poverty and unemployment by 2014. It was done in the recognition that the agricultural sector was the crucial economic industry (SSA and UNDP, 2010;

Mlambo-Ngcuka, 2006). A further goal was to decrease hunger by the year 2015 and sustain rural areas by them contributing to food security (DAFF, 2011; Mudhara, 2010).

3.3 Subsistence farming in South Africa

S.A agriculture contributes nearly 3% to the gross domestic product (GDP) in which about 1.3-million hectares are irrigated with half (50%) of S.A's water supply (Mathivha, 2012; Dube et al., 2013). Agriculture remains an essential sector for food security and provides employment for over 4 million people mostly residing in the former homelands (Aliber, 2009; DAFF, 2011). The constitution states that every South African citizen has the right to have access to sufficient food irrespective of rural and urban poverty which are a reality (DAFF, 2012). To the subsistence sector, agriculture is an essential resource which they can benefit from if operated efficiently to boost productivity. Subsistence sectors are viewed as farms with degraded soils, approximately 1.5 hectare per farmer and typified by a low production (Runge et al., 2004; LDA, 2008). Good farming land location is important as it determines the household food productivity and safety which has been stated by many studies (Oldewage-Theron et al., 2006; NAMC 2009; Jacobs 2009).

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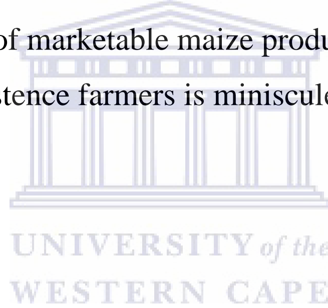
For most subsistence farmers, agriculture is the primary source of food and income (Runge et al., 2004). They have limited access to arable land and have historically held only 0.5–1.5 ha. of land per household (Lahiff and Cousins, 2005). As a result of this as well as resource constraints and historical and current challenges, production among subsistence farmers is generally much less than the land's potential (Walker and Schulze, 2006). In general, the agricultural subsistence farming sector (particularly communal land in former homeland) has been identified as being underutilized, poorly capitalized and economically unsustainable (Aliber, 2009; DAFF, 2010). Instead a number of households participate in subsistence farming as an extra food source not as a main food source (Aliber, 2005; 2009).

Recognizing the importance of agriculture in rural economic development and the role that subsistence farmers can play in poverty reduction and sustainable development, the South African government has put in place support interventions under the Comprehensive Agricultural Support Programme (CASP), and the *Illima / Letsema* Programme. One of the aims of these programmes is to transform subsistence farmers currently subsisting in the former subsistence farms into viable market farmers (NDA, 2006). Each farmer gets seeds and fertilizer as well as being ploughed and planted one hectare. Improving the productivity of subsistence farmers and facilitating market access will be important factors in the government's strategy. Currently, most of these farmers are mainly engaged in maize monocropping and produce mainly for household consumption. This is largely due to limited hectarage and resources, poor infrastructure, inadequate storage facilities and lack of access to formal markets (Altman et al., 2009). Thus of the 12 million tonnes of marketable maize produced annually in South Africa, the contribution of subsistence farmers is miniscule and insignificant (DAFF, 2010).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3.4 Ethical approval

A formal approval, protocol **ID no. EC11-002** (see Appendix 1.1) for the study by the Medical Research Council of South Africa was obtained from the MRC Ethics Committee. A meeting with community leaders and villagers was completed, and relevant preliminary information collected. Households were then randomly selected. Information obtained during research, storage and access to data about research participants will be kept confidential. The personal data about research participants will be anonymous information. No personal data will be published.

3.5 Study areas

This study focused on two rural district areas, i.e., Vhembe District Municipality (VDM) in Limpopo Province and Gert Sibande District Municipality (GSDM) in Mpumalanga Province. The sampled rural subsistence areas chosen are located in

provinces formally known as Bantustans. They were selected because they are situated on marginal lands with high concentration of subsistence farmers and the fact that these areas lie in the sub-tropical climatic zone. Two villages were selected in VDM, Tshidimbini which falls under Thulamela local municipalityand Matshavhawe found under Makhado local municipality of the Limpopo Province (Figure 3.1). Nine villages in GSDM, (i.e. Waverley, Fernie, Oshoek, Ntababomvu, Hereford, Bellvedia, Swaluwsnest, Ndonga and Mayflower) in Albert Luthulilocal municipality (Figure 3.2) in Mpumalanga Province were additionally selected for the study areas.

Limited current data is available on the prevalence of aflatoxin contaminated food in home grown maize and groundnut in South Africa (Steyn et al., 2009; Ncube et al., 2010). This was also mentioned in a review study by Katerere et al. (2008), in which most of the data was two decades old (Ramjee et al., 1992; Dutton & Kinsey, 1995; van Halderen et al., 1989; van Rensburg et al., 1990). Although there have been recent reports on aflatoxin contaminating feed and food (Otto, 2011; Chilaka et al., 2012) no surveillance of mycotoxins has been done in these areas.

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VDM is located in the Northern corner of South Africa, bordering Zimbabwe, Botswana and Mozambique. These countries neighbouring South Africa have been reported to have high aflatoxin contamination (VDM) (Siwela & Caley, 1989; Mphande et al., 2004; Wyket al., 1999). This municipality is situated in the lowveld and has a subtropical climate with mild, moist winters and wet, warm summers (Durand, 2006; VDM, 2008/9). VDM receives an annual average rainfall of approximately 500 mm mostly from October to March (growing season) with temperatures throughout the year from 10ºC minimum in winter to 40ºC maximum in summer (Durand, 2006; VDM, 2008/9). It is largely rural, rich in natural resources (fruit and vegetables) such as citrus, avocado, mango and banana, and exports nuts (VDM, 2010/11). This province has land which is very fertile and contributes over 4.4% of South Africa's total agricultural output, most of which is attributed to fruit and vegetable production (Poto & Mashela, 2008).

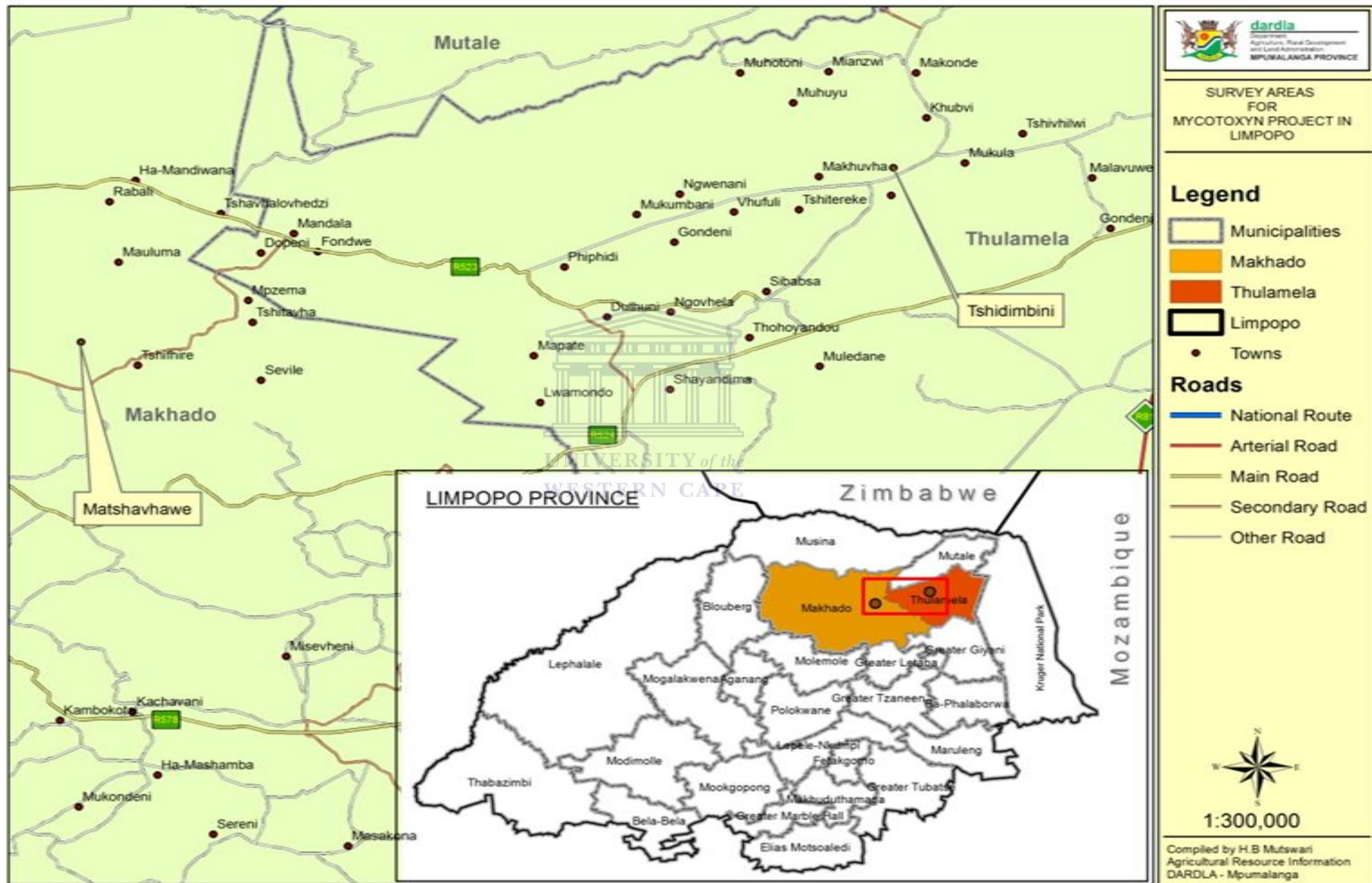
Mutale
Thulamela
Makhado
Tshidimbini
Matshavhawe
SURVEY AREAS
FOR
MYCOTOXYN PROJECT IN
LIMPOPO
Legend
Municipalities
Makhado
Thulamela
Limpopo
Towns
Roads
National Route
Arterial Road
Main Road
Secondary Road
Other Road
1:300,000
Compiled by H.B Mutswari
Agricultural Resource Information
DARDLA - Mpumalanga
LIMPOPO PROVINCE
Zimbabwe
Mozambique

Figure 3.1: Map showing villages in Vhembe District Municipality of Limpopo Province, Tshidimbini in Thulamela local municipality and Matshavhawe in Makhodo local municipality (source: Mpumalanga Provincial Department of Agriculture, Rural Development and Land Administration (DARDLA).

GSDM is situated in the lowveld, east of Mpumalanga close to Mozambique and Swaziland which has been reported to have levels of aflatoxin in its maize products (Peers et al., 1987; van Wyk et al., 1999). It has a climate which is subtropical with an annual rainfall of approximately 800 mm to 1000 mm in summer and occasional maximum temperatures above 25°C and mostly below zero minimum temperatures (GSDM, 2011/12). In Mpumalanga Province the Gert Sibande District is the biggest district that has the largest agricultural land as well as mostly rural (DAFF, 2009). In the district, agriculture (crops and livestock) contributes less than 10% to the economy and 33% of the province's GDP. The province is dominated by mining, manufacturing and electricity generation industries (GSDM, 2011/12).

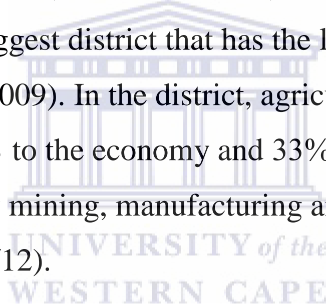

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Figure 3.2: Map showing villages in Gert Sibande District Municipality of Mpumalanga Province in the Albert Luthuli local municipality (source: Mpumalanga provincial Department of Agriculture, Rural Development and Land Administration (DARDLA).

3.5.1 Sampling and data collection

Twenty subsistence farmers from villages in Mpumalanga Province and nineteen subsistence farmers from the two selected villages in Limpopo Province were recruited. Households were randomly selected for recruitment into the study and interviewed using a semi-structured questionnaire translated into the vernacular (Tshivenda and siSwati). The questionnaire (Appendix 2.1) also captured information on household size, farm size, types of agricultural crops grown, storage practices and several food security indicators, including agronomic data. Based on the financial constraints of the project only twenty households from Gert Sibande District (Mpumalanga) and nineteen from Vhembe district areas were recruited. They were surveyed just after harvest time; samples were collected approximately within six weeks after storage of the harvest for the period of July 2011 and 2012. Some of the selected households had various storage forms (e.g. steel tanks, polypropylene sacks and wooden cribs) and samples were obtained from each of the random forms. Some matrices were sometimes sampled as course or finely ground. For quantitative analysis of multi-analyte contamination by LC-MS/MS, a representative (~100 g) of each sample collected was send to the Netherlands.

The numbers of samples collected as well as sample types were uneven; these samples were sampled based on availability and different storage forms in each household. A total of 136 matrices, maize (approximately 500g, each), peanuts and beans (handful ~100g, each) were collected using a standard collection protocol and stored in cloth bags to prevent moisture. A total no of maize (n=115; comprising of white, yellow and mixed maize), peanuts (n=6) and fifteen bean samples were milled and homogenized and subsampled prior to analysis.

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In November 2011, another collection was made and only maize samples were collected (if farmers still had any in store). The total maize also includes crops collected in November 2011; eight yellow maize (YM) samples from Vhembe and three maize samples collected from the silo (termed as (R) found inside the silo, and two samples (milled - M) and shelled - K) from store room next to the silo) in Gert Sibande district.

The second collection was done to evaluate if there is a difference in mycotoxin contamination level in samples stored over a longer period. Before the sample collection was done, a field demonstration was done as part of the study to ensure compliance to the sample collection protocol. All collected samples were finely ground (the grinder was thoroughly cleaned in-between samples with a commercial sample to avoid cross-contamination) and stored in the refrigerator at 5°C at the MRC at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) unit.

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3.6 Statistical data analyses

Data obtained for household variables in this study was subjected to the mixed procedure in SAS statistical package v. 9.3, (SAS® Institute Inc., 2011) which was used for regression modelling. The kg (tonne) variables had a skewed distribution, so subsequently for this log transformed variable kg (i.e. kg (tonne) log) a mixed effects regression model was used with year and province as fixed effects. A year by province interaction term was also included to investigate whether weights differed across the two years (2011/2012) and two provinces (Limpopo and Mpumalanga). Least Squares Means (LS Means) were used to estimate the fixed effects, and their comparisons as well as 95% confidence intervals for these effects (household size) and differences were obtained.

3.7 Results and discussion

Demographic and production output data were collected using the questionnaire and administered through individual interviews. It is well-known that maize in South Africa and other African countries is the staple crop for rural communities and a cash crop for commercial farmers around the world (Manyong et al., 2000; Chilaka et al., 2012). Maize was chosen as the main commodity to be studied as it is generally produced in both provinces as well as other grain crops which may be produced in the selected areas as secondary crops. All these crops from the subsistence farms have the potential to boost the agricultural economy of these provinces and to generate employment in rural areas so as to prevent people from migrating to the urban areas.

Samples which were collected after both harvest seasons viz., 2011 and 2012 were as follows; maize (3; 8), in GSDM and VDM, respectively. The eight samples collect in November from Limpopo were maize samples reserved as seeds for the 2012 seasons. The rest of the households did not have any harvest left. For the second production season, a number of farmers purchased seeds from other subsistence farmers or from commercial suppliers. Only maize from the silo was sampled, participants from GSDM did not have much harvested maize left and the seeds which were available were bought from the shop or supplied by the government.

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3.7.1 Demographic profile and Productivity of respondents.

The ages of farmers in VDM were on average 11 years older (average 62 years; 49–81 years) than those in GSDM (average 51 years; 37–75 years) (Table 3.1). The formal education background of Limpopo farmers varied from none to secondary. However all farmers in Mpumalanga reported having had only primary education (Table 3.1). This corresponds to the findings by Oni et al. (2003), who reported that most subsistence farmers in Mpumalanga Province are ageing men and women with poor formal education. This might imply that they will find it difficult to learn new farming techniques to improve production (Oni et al., 2003).

Table 3.1: Demographic indicators for subsistence farmers in both districts (VDM and GSDM).

Components of Indicators	VDM	GSDM
Age of household head (years) [1]	61.5 (49-81)	51.1 (37-75)
Amount of land used (ha) [2]	1 (0.5-4)	1 (1-4)
Average number of family size /household [3]	6.3 (1-23)	8.7 (3-15)
Literacy level [4]	2.0 (1-3)	2.0 (2)

[1]Primary data. An average age of household head, cultivated hectare.

[2]Land cultivated per hectare (ha) on average.

[3]Average numbers of occupants (adults & children) in each household

[4]Literacy level refers to the education level of the household head. 1-no education, 2-primary education, 3-secondary education.

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In general, farmers are known to have limited arable land with VDM farming on average 1 ha, ranging from 0.5 to 4 ha. One correspondent (the headman) had 4 hectares and the rest had 0.5 or 1 ha. GSDM farmers had hectares ranging from 1 to 4 ha. each (Table 3.1). Most of the subsistence farmers were women (13/19; 68%) in VDM whereas only 10% (2/20) of women were involve in farming in GSDM. Williams (1994) has reported that in general 70% of subsistence farmers in South Africa were women similar to other African countries which accounts for 60-80% of farmers (Baiphethi & Jacobs, 2009) as in VDM, and most men reside in urban areas in order to earn an income. In contrast to this 90% of subsistence farmers in GSDM were men. In the Nguni ethnic group, farming is culturally regarded as an activity reserved for men which is why more men are farmers (EGSA).

On average, household size in GSDM was large (8.7 occupants) with more adults which could be the reason why there is more maize production than in VDM with fewer adults (6.3) in each homestead. Household size between the two districts showed a statistically significant difference of Least Square Means (LS Means) between Limpopo and Mpumalanga for both 2011 (-2.4460;-1.3796) and 2012 (-

1.8931;-0.7879) seasons, respectively. But, when comparing 2011/2012 for each province, Limpopo respondents were not significantly different (-0.4892; 0.4011) and Mpumalanga residents were significantly different (0.1034; 0.9530).

This is the first attempt to compare grain production among subsistence farmers in South Africa. This study shows that maize is the major crop while groundnuts and beans were produced by a minority of farmers as secondary crops in both areas (Table 3.2). While all farmers in the selected areas cultivated maize there were distinct differences in the maize varieties grown and the subsistence productivity in both districts (Table 3.2).

Table 3.2: Varieties of crops and productivity output for subsistence farmers in Vhembe (Limpopo) and Gert Sibande (Mpumalanga) District Municipalities.

Districts	Year	Average maize harvested (tonnes)[1]	WM[2]	YM[3]	Peanuts[4]	Beans[4]
VDM	**2011**	0.4 (0.08-2.4)	1/19	18/19	2/19	3/19
	2012	0.7 (0.1-2.5)	n/a	17/17	3/17	2/17
GSDM	**2011**	3.0 (0.3-10)	17/20	12/20	1/20	7/20
	2012	1.8 (0.4-5.0)	19/19	9/19	n/a	3/19

[1]Average of maize harvested – average maize produced per hectare presented as tonnes and the range of the harvested maize (range of harvested maize).
[2]WM-White maize
[3]YM- yellow maize
[4]Number of beans and peanuts cultivated as secondary crops.
N/A- not applicable

Farmers from the two districts consume maize daily at an average of approximately 400g per person per day. A study conducted by Shephard et al. (2007) in rural areas of the Eastern Cape (formerly known as Transkei) indicated comparable mean maize consumption of up to 456 g per person daily. In addition,

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van der Westhuizen, 2011 reported an exposure level of 13.8 μg/kg body weight/day total fumonisin upon daily consumption of FB contaminated maize.

In 2011 and 2012 seasons, respectively 9/20 and 7/19 of GSDM household farmers produced both yellow (60 & 47%) and white (85 & 100%) maize but farmers in VDM mostly only cultivated yellow maize (95 & 100%). However, only one white and mixed maize samples were collected. There were households which appeared to cultivate both landrace and hybrid maize varieties in Mpumalanga as illustrated in Figure 3.3. All farmers planted landrace maize which they indicated has a high germination rate, tasting comparable to the hybrid seed variety.

In contrast, in a study by Mabhaudhi and Modi, (2010) on seed performance between the two variations (landrace and hybrids), landrace was significantly different ($p < 0.001$) from the hybrid seed and found to germinate at a slower rate than the hybrids. However, in the same study, landraces were more tolerant to stressful conditions than hybrids (Mabhaudhi and Modi, 2010). Landraces is an indigenous variety mostly grown by subsistence farmers because of cultural preferences (Bellon and Hellin, 2011; Sibiya et al., 2013). The majority of residents from three villages of KwaZulu-Natal Province in South Africa preferred growing local landrace because of its taste and tolerance to stress (Sibiya et al., 2013). A few farmers planted hybrid maize in small quantities as a secondary crop, stating that it is much more filling.

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Figure 3.3: Maize varieties cultivated by some GSDM households, hybrid maize has big kernels with few rows and land race has short smaller kernels, more rows (Picture taken by D.R. Katerere, 2011)

In the GSDM rural areas, white maize was grown for human consumption only, whereas yellow maize was used specifically as feed. In South Africa white maize (approximately 60%) has been reported as mainly for human consumption and yellow (approximately 40%) for animal feed (Durand, 2006; DAFF, 2011/2012). Selling livestock in GSDM serves as another source of income. Animal husbandry was found to be an important part of the livelihoods and culture of these farmers who are of Nguni ethnicity. Nguni people (Xhosa, Swati and Zulu) are historically pastoralists and cattles in particular play a significant part in providing food, bridewealth and savings (Lahiff and Cousins, 2005). Nationally, Lahiff and Cousins (2005) have previously reported that 25 - 50% of rural households own cattle in South Africa. Widespread stock theft, frequent droughts, lack of water supply and grazing pastures are reported to limit the growth of the rural herd (Pender, 1999; Poto and Mashela, 2008). Apart from large stock farming, the GSDM farmers also reported keeping sheep, goats, pigs and chickens.

In contrast, in the VDM, yellow maize is the culturally accepted variety for human consumption. The farmers reported no ownership of large stock because of lack of pasture land and water. Only chickens and goats were observed in some of the households as reported in the Vhembe district municipality in 2011 and 2012. Integrated Develop Plan (IDP) review that goats mostly are kept for socio-economic and cultural reasons (VDM, 2011/12). Some farmers in VDM indicated that they send their large livestock to the nearby areas in the district where there is better grazing.

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GSDM farmers reported an average annual maize yield of 3.0 and 1.8 tonnes which was 2.6 and 1.1 tonnes more than that reported by VDM farmers in the first and second season, respectively (Table 3.2). The least (0.08 tonnes) in 2011 and (0.1 tonnes) in 2012 productive farmers were both found in VDM (Table 3.2). This is partly reflected by the average age of the farmers, most of whom are elderly, poorly educated and past their most economically productive phase of life.

Odhiambo (2011) reported that most of the land in Limpopo has degraded and the current unproductive soil has resulted from factors which include overcrowding and poor land management. This was evident in Matshavhawe village, where maize was mostly planted in backyards in which the yields were low. In the second season, all GSDM farmers indicated that their fields were planted late due to problems within the agricultural sector compared to the previous year. As a result of late planting, it was too late for one farmer to cultivate. However, maize production in VDM has shown an average increase of 0.3 tonnes from 2011 to 2012. GSDM maize production has decreased in 2012 by 1.2 tonnes as a result of late crop planting. Consequently, this difference in productivity between the two provinces presented an analysis variable with a statistically significant difference ($p < 0.05$; 0.0184) for both seasons. Good nutrition is important for plant health and delayed planting has been known to influence production and exposes the crop to higher temperatures, inducing fungal infection and consequently mycotoxin contamination (Kendra, 2009; Soonthompoct et al., 2001). Two participants from VDM were not available for collection, and were thought to have migrated to the urban areas for employment.

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In these two districts a number of respondents indicated that due to limited resources, very little or no fertilizer was applied to the crops and this may partly account for poor harvest. Two farmers in Matshavhawe indicated that they sold all their maize green/fresh at the local market and thus no storage facility was required. The sale of green maize is mostly practised by farmers in the Matshavhawe village in order to purchase much needed additional food supplies, where they plant maize under irrigation. In VDM area maize can be planted up to two to three times a year. Although farmers in GSDM also have limited hectarage they appear to be more productive with an average yield of 2.4 tonnes (0.4 – 7.5) in two seasons. The main reason appears to be the support they get from the provincial government which provides access to basic production equipment and agricultural extension services. Such support was lacking in VDM and Baloyi (2010) had previously pointed out that access to resources such as land, water, infrastructure, capital and good resource management is necessary for subsistence

farmers to increase their productivity. Such support could improve productivity substantially (World Bank, 2007; CAADP, 2009).

In general maize production in both areas is characterised by low yields and in some households the average yield was inadequate to meet their food needs. This was evident especially in VDM where 53% of household crops are used up within five months. It further appears that very few farmers produce surplus quantities of the maize to allow for trade. Although VDM might not be food sufficient as they only produce one type of maize and low grain productivity unlike in GSDM where they plant white and yellow and the production is much higher, there are various other reasons such as storage practices that influence productivity and are discussed in detail below.

Comparing the two seasons (2011 and 2012) on maize harvest duration, VDM (10/19 and 9/17), showed no difference in maize which lasts less than six months. But, a decrease in number of households with maize that lasts for 12 months from 9/19 to 5/17 in the first and second season respectively was observed as represented in appendix 3.1. However, even though in GDSM there was no harvest which lasted for less than six months, yield lasting for a year declined from 15/20 to 10/19. The rest persisted for just less than a year in 2011 and 2012. (Appendix 3.1). Selling of maize to hawkers or other subsistence farmers had considerably decreased in 2012 as there was not enough food harvested in the second year. Only 1/17 and 3/19 household maize was sold compared to the year 2011, where maize was sold by approximately half of the households in both districts.

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3.7.2 Storage Practices

In both study areas, the practice was to leave maize in the field for a period of one week to three months to dry naturally prior to harvesting. This generally happens from April to May and harvesting begins in June. Harvesting at the correct time is important to minimize mycotoxin contamination. Lengthy drying in the field for

more than three weeks has been shown by Kaaya et al. (2006) to increase the levels of aflatoxin contamination by approximately 4 times. Drying delays in the field and less than 20% moisture levels may encourage *Fusarium* and mycotoxin growth (Kendra, 2009). Instead early harvesting followed by drying to safe moisture levels to prevent the fungal growth was advised. Before storage, the farmers indicated that physically damaged grains are sorted and given to livestock (i.e. chickens, pigs and cows) as feed.

It was observed that storage structures used for maize by the farmers differed from district to district. A study by Hell et al. (2000) in Benin, West Africa found that maize kept in different storage structures vary in fungal and aflatoxin contamination. Some farmers had their storage facilities for a range of 2 to 30 years while some could not recall exactly when their storages were erected. For season one and two respectively, 21 and 24% of the farmers in Limpopo used enclosed cement or mud storehouses called Duru for storage which were sealed. The respondents (79 and 76%) were frequently observed using sacks, with a few storing their sacks directly on the floor inside the houses where they live (Figure 3.4). Kankolongo et al. (2009) observed the same process in Zambia, where households store maize harvest in sacks inside their houses.

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(a) (b) (c)

Figure 3.4: Common maize storage methods used in VDM (a-c) (a) enclosed mud storage house (Duru) roofed with iron sheets containing maize on cob; (b) maize stored on cement floor and (c) polypropylene sacks kept in-house on the cement floor against cement bricks with either loose or milled maize (Pictures taken by P. Mngqawa, 2011).

However in VDM there were no community silo facilities found, the majority (78%) of farmers shelled or milled maize directly from the field, and then stored in sacks in the house as loose grain and on cement or mud floors. There was no temporary drying storage facilities observed. Storing maize in sacks or plastic drums (which is common in VDM) presents obvious problems not only with weevils (*Sitophilus zeamais*) infestation, rats and stalk borers but also predisposes the crop to heat, humidity and moisture. Signs of spoilage were obvious on visual inspection in most of the bags. Stored maize from Zambia and West Africa has been found to be commonly contaminated with insects, specifically *Sitophilus zeamais* and *Prostephanus truncatus* which have an impact in fungal and mycotoxin infection (Kankolongo et al., 2009; Meikle et al., 2002). The most common insects found in storage maize facilities from the Republic of Benin were *S. zeamais* infested at 85% and *P. truncatus* at 54% (Meikle et al., 2002). Most of the maize samples collected from VDM were contaminated with weevils, this was also commonly found in other part of Africa such as Zambia as reported by Kankolongo et al. (2009). Insect damage increases the risk of mycotoxin contamination in maize, and disperses *Fusarium spp.,* particularly *F. verticillioides* (Schulthess et al., 2002; Munkvold, 2003). *Sitophilus zeamais* has been shown to carry and encourage the development of *A. flavus* and aflatoxin contamination in stored maize (Beti et al., 1995; Bankole and Adebanjo, 2003).

These storage structures were similar to granaries used in Zimbabwe except that those in Zimbabwe are usually mud huts and raised on boulders from the ground (Marchand, 1989). This is meant to reduce dampness and increase aeration. In Ibadan, Nigeria, storage structures are made from grilles of wood and bamboo (FAO, 1994). Zambian small-scale farmers use different types of agricultural storage forms, some of them similar to Limpopo and Mpumalanga from open cribs, bags and steel drums (Kankolongo et al., 2009). Steel drums tend to create a cool well-ventilated atmosphere which reduces spoilage.

The sacks were usually kept on the cement kitchen floor or in an empty separate room in the house with a corrugated iron roof. Inside the Duru, on-cob maize was

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stockpiled while in sacks, maize was stored on-cob, loose or milled. Some households' who kept crops in the kitchen, cooked with fire wood. Most other farmers in the area do not pre-store their harvest due to the small size of the yield. Open wooden cribs were used by the majority of farmers from GSDM for further drying and 75 & 84% farmers made use of the cribs as dual methods (both for continuous drying and as an on-cob crop storage), while 25 & 26% used the community silo for storage, respectively. The community silo is a temperature and humidity controlled storage system with ventilation (Figure 3.5). Ventilation system with cooling and drying in the silo are important in order to avoid unnecessary deterioration of agricultural commodities caused by fungal growth and activity during storage (Jouany, 2007).

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Figure 3.5: Community silo in Dundonald provided by the government for bulk storage of maize for the rural areas in the Albert Luthuli local municipality (Picture taken by P. Mngqawa, 2011).

Wooden or bamboo cribs are commonly used in Africa as traditional storage methods positioned on raised platforms to prevent moisture, insect and rodent

damage to agricultural crops (Hell et al., 2010). They may be covered with thatch or corrugated iron sheet or not covered. In GSDM only a few wooden cribs were covered with corrugated iron roofing, allowing access to environmental conditions. Farmers (15 & 32%), used the wooden cribs solely as storage and 5 &11% of farmers s stored maize in sacks inside the house during season one and two, respectively (Table 3.3).

Table 3.3: Different commodities storage and pre-storage forms used by the respondents.

Storage methods	VDM number of households %		GSDM number of households %		GSDM pre-storages %	
	2011	**2012**	**2011**	**2012**	**2011**	**2012**
Sacks	79	76	0	0	0	n/a
Steel tanks	0	0	25	26	25	26
Wooden cribs	0	0	15	32	75	84
Storehouses	21	24	n/a	n/a	n/a	n/a
Silo	n/a	n/a	13	5	n/a	5

*In Vhembe District Municipality, pre-storage of crops was not done.

*Most households have used more than one storage method.

*N/A- not applicable

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Even though further drying of crops on the wooden cribs is encouraged, they are also exposed to extreme environmental conditions such as the sun, rain and rodent invasion. *Fusarium graminearum*, continually grows in maize cobs stored in cribs to drying, particularly in tropical areas (EMAN). On that score the cement and mud houses used in Limpopo seemed to be superior but humidity and growth of mould was possible due to poor aeration. Proper ventilation is one of the important factors throughout drying and storage in order to eliminate possible fungal growth (Wagacha & Muthomi, 2008). We found that in the past most GSDM farmers used to store shelled maize in steel tanks in the homestead, but

they now have access to a community silo which not only stores the maize for them but also mills it for a small charge. Some residents stored and pre-stored in houses, steel tanks, open wooden cribs, roof covered wooden cribs as shown on Figure 3.6 and roof tops as means of storage and pre-storage instead of adequately ventilated silos.

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(a) (b) (c)

Figure 3.6: Different maize storage methods used in GSDM (a-c), (a) wooden crib with corrugated iron roofing above ground containing maize on cobs; (b) Open wooden crib raised up off the ground; (c) maize grains in a sealed steel tank on a raised stand (Pictures taken by D.R. Katerere, 2011 & 2012).

One individual from Matshavhawe village in VDM during the second season revealed that they had milled their maize produce comprising of weevils, because they have not produced enough food and that after maize has been milled, weevils cannot be visually seen. This shows that people would rather eat contaminated food than go hungry. Further, weevil, rats and stalk borer damage can only be prevented by the use of synthetic pesticides which most of the farmers reported buying and utilizing for preserving agricultural crop.

Majority of households from Vhembe district villages (16/19) used synthetic fungicides which can be costly pesticides while half 10/20 from Gert Sibande district villages indicated not using any form of pesticide to protect stored commodities.Types of pesticides used to treat stored crops (specifically maize) in Vhembe were Shumba super and Actellic Chirindamatura Dust. Both pesticides are manufactured in Zimbabwe as a method of protecting and controlling infested stored grain (Gadzirayi et al., 2006; Dube, 2008). In Gert Sibande a variety of commercial chemicals was applied in agricultural products; Cutworm & Cricket bait, Alphathrin Protek, Debello glue trapping, Phostoxin® tablets and pellets for fumigation, Cyperin and Roundup Herbicideas as weed controller. These types of chemicals were used to control weevils, stock borers and rodents which negatively affect maize produce. Some synthetic chemicals (fungicides, insecticides and pesticides) can probably be toxic and harmful if ingested or inhaled. If not applied without protective measures, they can cause unintended harm, such as skin and neurological problems (Ngowi et al., 2007; Anjorin et al., 2013).

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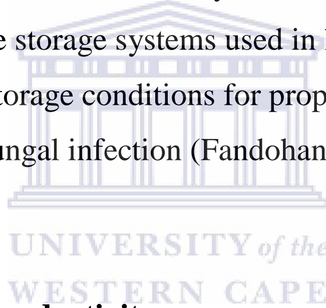
But, as stated before, factors such as financial constraints, poor resources and incorrect or no application of fertilizers contributes to the poor level of production. There were participants who make use of indigenous plants (natural pesticides) which are possibly environmentally safer to human health (Varma and Dubey, 1999). *Lippia Javanica*, commonly known as: lemon bush (English), msudzungwane (Tshivenda), mutswane (Swati) (Thembo, 2012) was used in place of synthetic pesticides. *Lippia Javanica* is a common perennial plant found all year round in both areas and was observed being used in Limpopo household

maize storage. It has been reported to have protected grain from pests (Omolo et al., 2005). Subsistence farmers in GSDM mentioned using cow manure and ash to prevent pest and fungal infection during the storage period, as well as to add to the soil in the attempt to improve soil fertility.

Other nutritional plants used to protect grain from different mycotoxigenic fungi are as follows: *Tagetes minuta* L, particulary *Vigna unguiculata* L, and *Amaranthus spinosus* L, which have been found by Thembo et al. (2010) with potential chemical components to inhibit fungal infections. Both rural districts areas indicated that the storages were cleaned before filling in the new harvest before every season. It has been reported that fumonisin in maize may increases as a result of the age of maize in storage (Warfield and Gilchrist, 1999; Chulze et al 1996). This has been regarded as a sanitary measure of reducing mycotoxins (Hell et al., 2000a). Most of the storage systems used in humid and semi-humid zones generates unfavourable storage conditions for proper drying of maize which subsequently promotes fungal infection (Fandohan et al., 2006).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3.7.3 Factors affecting productivity

As noted earlier, these farmers are located on semi-arid degraded soil areas and marginal agricultural areas which are drought prone. Only 7% of South Africa's farmland is irrigated (CEEPA, 2006; Scotcher, 2010). Climate change is one of the many challenges that the farmers encounter, resulting in increase in temperature and changes in humidity. Climate change may affect the development of agricultural crop production. The climate is expected to worsen in the future with forecasts of further 3 - 6% reduction of agricultural productivity in South Africa (World Bank, 2003; Mudhara, 2010). Some farmers in GSDM were additionally affected by floods and were thus not able to produce their expected quota of maize. The consequent expected impact of climate change will discourage farmers in their activities. Soil degradation caused by overcrowding, limited access to agricultural technical assistance and lack of modern farming

knowledge have been previously cited as impacting on productivity (Mwaniki, 2006).

These above factors or conditions were observed in both areas, particularly in VDM. In GSDM, the farmers had on average larger plots whereas in VDM all farmers reported farming on no more than 1 ha of land. Apart from these considerations, there appears to be a general lack of farming equipment, poor storage infrastructure, lack of access to credit and inadequate extension support services. The farmers in GSDM were in a slightly better position as they received some government assistance under the "*Asibuyele emasimini*" (back to farming) programme which provides tillage for 1 hectare and starter seed packs and fertilizers.

Thirty-one percent of farmers in GSDM and almost 50% in VDM reported using the previous year's harvest as seed, because of the higher costs of seed. The rest of the farmers purchased commercial seed. In VDM about 16% of households planted the previous season's harvest as well as seed bought from commercial retailers. This practice has previously been reported elsewhere in rural South Africa and in other countries (Gouse et al., 2006; Ncube et al., 2011; Longley et al., 2001). Previous year's seeds have a distinct possibility of low germination and cross-pollination (Ajeigbe et al., 2009). High quality seed is essential for overall plant health, good crop germination and agricultural productivity. Poor post-harvested seeds are susceptible to fungal infections and may yield low productivity as they are commonly kept under unfavourable conditions (Gouse et al., 2006). Most mycotoxigenic fungi are entophytic; re-using harvested crop for seed only serves to re-infect the new crop. An illustration of the disease cycle of *F. verticillioides* and fumonisin production in maize is illustrated in Figure 3.7. However this increases the risk of common mycological infection (Wilke et al., 2007) and impacts on crop health and productivity output.

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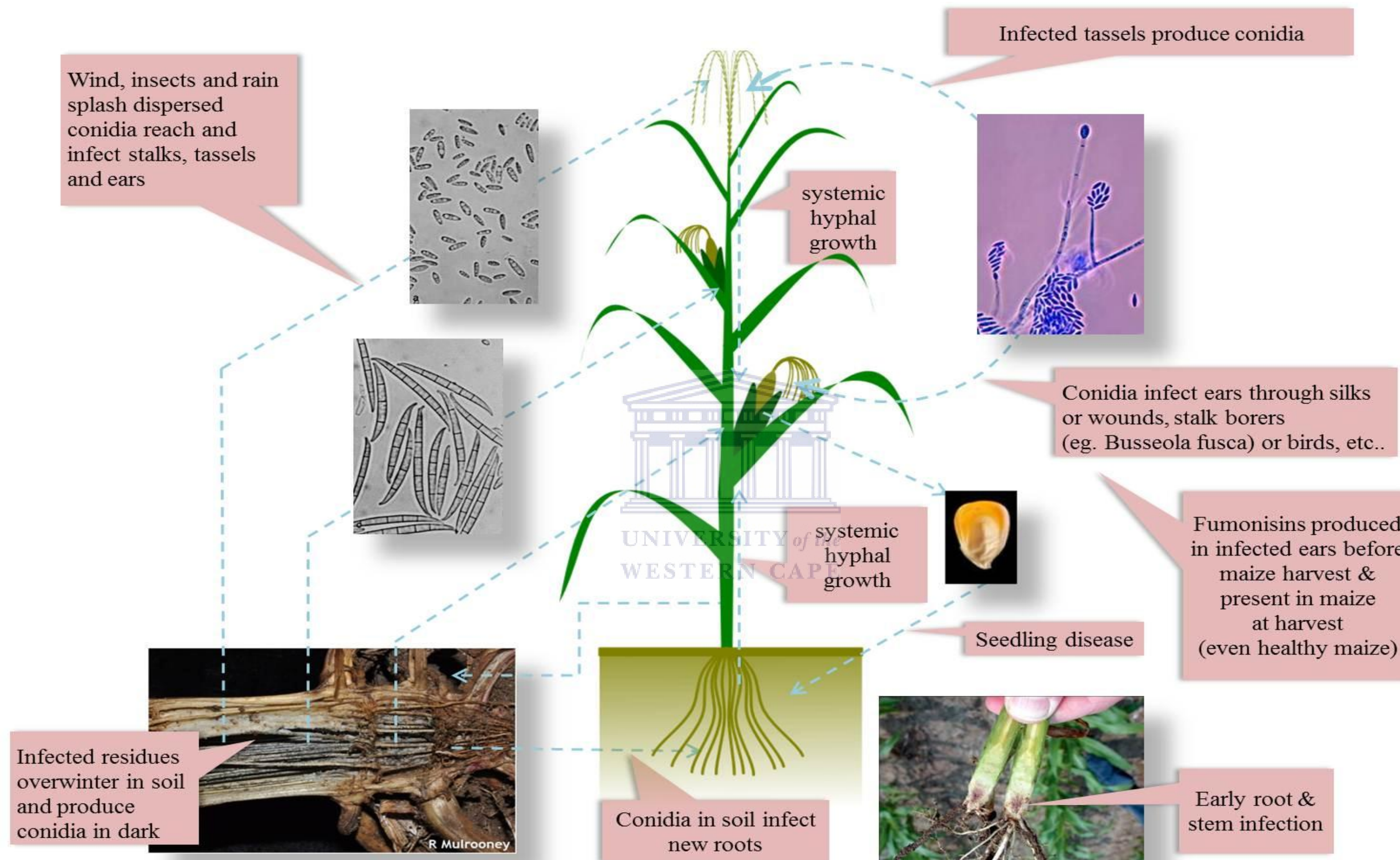

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Figure 3.7: *Fusarium verticillioides* pathogen and fumonisin production in maize (pre-harvest) (by Dr.H. Vismer, 2013).

3.7.4 Climatic data of Mpumalanga and Limpopo Provinces

The magnitude of mycotoxin and fungi infection in agriculture commodities depend on the climatic parameters of the area and the storage practices of each household. Variations and distribution in temperature and rainfall have a negative effect on agricultural production in South Africa (Durand, 2006; Dube et al., 2013). These sampled areas are vulnerable to poor and unreliable rainfall, participants from both districts had stated that they had experienced insufficient late rain with high temperatures over the years.

South African maize has been known to grow well in temperatures of 12 - 24°C minimum and maximum of 26 - 29°C for better growth and is highly sensitive to humid parameters (Durand, 2006). Mpumalanga normally receives rain during summer and Limpopo Province receives enough rainfall but has high temperatures (Durand, 2006). Seven year climatic data was obtained to check if there was any climatic changes from year to year (Table 3.4). Climate data from October to March growing season in South Africa, was as follows; VDM in 2011 and 2012, respectively ranged from 18 - 30°C and 17 - 30°C with relative humidity (33 - 93% & 32 - 87%) and maximum rainfall of 434.3 mm and 202.18 mm. Mpumalanga had a temperature from 15 - 33°C & 14 -32°C, a relative humidity of 31 -92% & 29 – 89% and a maximum rainfall of 221 and 206 mm during 2011 and 2012 respectively. High temperatures in combination with high humidity elevate mycotoxin production (EMAN).

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Some parts of Africa have the most important climatic factors which highly favour the growth of toxigenic fungi and mycotoxin production (high moisture content of 17 to 25%, high relative humidity (≤ 70%) and 2 to 55°C temperatures) (Bankole and Adebanjo, 2003; Negedu et al., 2011). Phillice (2007), in his study has reported that fungus has an optimal temperature and relative humidity growth of close to 30°C and 80% respectively.

Table 3.4: Shows annual averages of climatic data conditions (temperature (°C) and relative humidity (%) and rainfall (mm) patterns) for Mpumalanga (Mp) and Limpopo (Lim) Provinces of South Africa.

	Minimum temperatures (°C)		**Maximum temperatures (°C)**		**Minimum Relative Humidity (%)**		**Maximum Relative Humidity (%)**		**Rainfall (mm)**	
Year	**Mp**	**Lim**	**Mp**	**Lim**	**Mp**	**Lim**	**Mp**	**Lim**	**Mp**	**Lim**
2006	12.72	17.3	26.53	28.73	28.21	33.12	76.84	79.88	63.48	55.22
2007	12.67	16.99	27.28	27.22	32.22	37.41	84.89	79.27	42.9	85.03
2008	13.04	16.96	26.91	27.22	35.41	38.99	86.78	80.23	101.1	68.08
2009	13.07	16.95	26.73	26.77	36.66	40.51	87.65	80.52	63.8	79.14
2010	13.35	17.22	26.87	26.84	38	43.69	88.27	83.41	64.83	75.44
2011	10.07	16.51	25.34	26.37	31.07	40.99	85.98	80.55	59.69	105.5
2012	12.98	16.57	27.84	27.04	30.53	35.16	83.31	77.8	49.98	47.7

*Weather data provided in this study was supplied by the Institute for Soil Climate and Water of the Agricultural Research Council in Pretoria, South Africa. The reported weather data are from the closest weather stations to the study areas.

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3.8 Conclusion

This study has identified the fact that food safety and security in rural South Africa remains a challenge which needs to be addressed urgently. It has revealed an average of 75 % and 47% of farmers in GSDM and VDM, respectively reported producing maize crops that would last to the next season's harvest. The farmers stated that they purchased maize either from other subsistence farmers or retail outlets in order to last them until the next harvest. A similar situation was previously reported in the Northern KwaZulu-Natal by Thamaga-Chitja et al. (2004). When household size was taken into consideration, most families were prone to food insecurity if they were to rely solely on crop farming. In order for the subsistence farmers in rural South Africa to improve food supply for themselves and the country, they need to increase and diversify crop production.

However at the moment they are faced with many challenges which impact on this i.e. access to arable land and knowledge on mycotoxin effects. Access to more arable land than VDM and agricultural extension services were the major reason why the farmers in GSDM were more productive. Some methods for reducing mycotoxins such as drying, physical separation, and early harvesting are to some extent used but not consistently. Kaaya et al. (2006) has reported common practices such as sorting of damaged grains before storage, storage of loose grain and the use of different synthetic pesticides as being useful in reducing fungal contamination and mycotoxin occurrence in maize (Kaaya et al., 2006). In a study by van der Westhuizen et al. (2010) where the process of sorting and washing of maize contaminated with fumonisin was followed, fumonisin contamination was reduced.

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At the macro-economic level, policymakers should focus on these two elements (land reform and extension support services) while at the farm level, there appears to be a great need to create awareness about Good Agricultural Practices (GAP) which protect the land, reduce production losses and ensure improved food quality. These practices should include training on proper post-harvest handling of

crops. The findings show the inconsistency in farmer support programme roll-out among provinces, which exacerbates inequalities in farming communities.

Increasing productivity of the subsistence agriculture sector by encouraging farmers to improve their management skills of their harvest from the field to storage would increase food security. This would consequently reduce the high dependence of having to buy food from the shops and improve the food quality. Food quality and quantity are vitally important and the prevalence of mycotoxins which are a problem all over the world certainly negatively affects food security and food productivity. There needs to be a full understanding of the mycotoxin-producing strains in contaminated in home-grown harvests in order to appreciate the intervention methods needed for their eradication.

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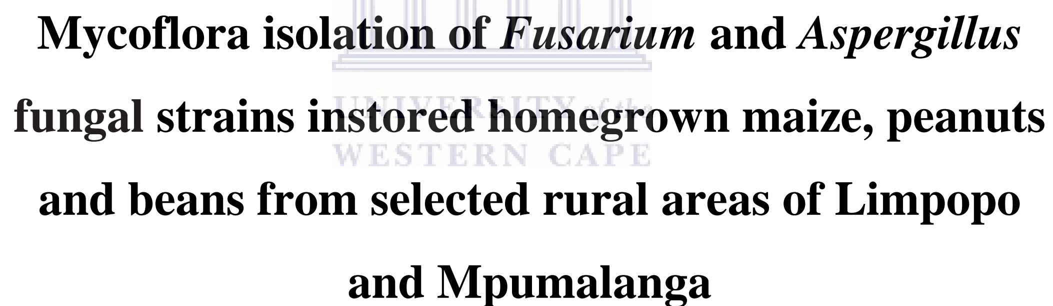
CHAPTER 4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Mycoflora isolation of *Fusarium* and *Aspergillus* fungal strains instored homegrown maize, peanuts and beans from selected rural areas of Limpopo and Mpumalanga

4.1 Introduction

Natural contamination of food with fungi and mycotoxins is an important consideration which affects food safety as a global concern. Filamentous fungi (also capable of producing mycotoxins) are environmental microorganisms which produce secondary metabolites, and cause many plant diseases (Peraicaet al., 1999; Adrio and Demain, 2003; Reverberi et al., 2010). Complex interactions between environmental factors and nutritional composition cause fungal growth (Charmley et al., 1994; Hollinger and Ekperigin, 1999).They may occur in the soil surface and be transferred to plants by wind, water and insects (CAST, 2003; Richard et al., 1993). Fungi impact on agricultural productivity and crop quality (Kendra, 2009; Charmley et al., 1994; Korir and Bii, 2012). *Aspergillus*, *Fusarium* and *Penicillium* are some of the most important fungal contaminants in food and feed (Gelderblom et al., 1988; Bryden, 2007; Chilaka et al., 2012).

Food may also be contaminated by other common fungi such as *Penicillium*, *Alternaria* and *Diplodia* spp. which produce toxins (Marasas and van der Westhuizen, 1979; Latterell & Rossi, 1983; Piotrowska et al., 2013). Several toxin-producing *Fusarium spp.* often dominate (by causing seedling diseases, damaging the roots, stalks, and ears of the plants) in agriculture and produce mycotoxins. They include *Fusarium verticillioides* (*Sacc*) Nirenberg =*moniliforme* Sheldon, *F. graminearum* Schwabe, *F. subglutinans* (Wollenw and Reinking) Nelson, Toussoun and Marasas (Shephard, 2006; Summerell et al., 2003; Rheeder et al., 2002; Nelson, et al., 1983). But out of all the *Fusarium spp.*, *F. verticillioides* is the most encountered in agricultural commodities particularly in maize (Marasas, 1996 and 2001). It causes cob and stalk rots in maize and is a main producer of fumonisins (Munkvold and Desjardins, 1997; Leslie, 2005).

Aspergillus fungi can be fatal to humans, depending on the level of exposure and infects a wide variety of food and feed, particularly grains and peanuts, *A. flavus* being the most dominant (CAST. 1979; Cotty, 1990). *Stenocarpella macrospora* (*Diplodia macrospora*) and *S. maydis* (*D. maydis*) have been well documented to infect maize ears and stalks. Marasas et al (1979) and Latterell & Rossi (1983)

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found that *S. maydis* is the most common species in South Africa and the USA. Crops produced by subsistence farmers in the two selected districts of Limpopo and Mpumalanga are prone to pests and fungal contamination due to the tropicaland subtropical climate which exists in these regions. This is probably exacerbated by factors such as poor handling and inadequate storage facilities.This two-year study investigated fungal contamination of homegrown crops which had been stored for about six weeks after harvesting in Vhembe District Municipality (VDM) in Limpopo and Gert Sibande District Municipality (GSDM) in Mpumalanga.

4.2 Materials and methods

Home-grown samples intended for human and animal consumption were collected for mycology testing during the year 2011 and 2012 as shown in Table 4.1.

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Table 4.1: Samples collected from VDM and GSDM during seasons 2011/2012.

	Year	no. of samples	maize kernels	samp (grainy)	maize meal	peanuts	beans
GSDM	2011	33	32	0	1	1	7
VDM		27	26	0	1	0	0
GSDM	2012	30	30	0	0	0	2
VDM		22	15	3	4	1	2

Different *Fusarium spp.* (*F. verticillioides, F. subglutinans*, *F. graminearum* and other *Fusarium spp*), *Diplodia spp. (D. maydis* and *D. macrospora)* combined with other fungal species as well as *Aspergillus spp*., (*A. flavus*) were identified and isolated. Kernels were plated on either MEA or AFPA as described in detail below. Results were expressed either in percentage kernel infection as the isolation frequency of the fungal genera in whole kernels or as colony forming units per gram (cfu/g) in milled samples.

4.3 Chemicals and reagents

AFPA (*Aspergillus flavus* and *A. parasiticus* agar): Yeast extract and Dichloran (2, 6-dichloro-4 nitroaniline) (20g and 0.002g) were purchased from Oxoid-Unipath Ltd, Basangstoke, UK. Peptone (10g) and 15g Agar were acquired from Difco, Kansas, USA. 0.5g Ferric ammonium citrate and 0.1g Chloramphenicol (dissolved in 3-5 ml ethanol before adding to other ingredients) were bought from Sigma-Aldrich, St Louis, Mo., USA. Distilled water (1L) was added.

MEA (*1.5%Malt extract agar*): For mycological enumeration of maize samples. A 15g Malt extract (Oxoid L39 - diastase free) (Oxoid-Unipath Ltd, Basangstoke, UK), 17g Bacto agar (Difco, Kansas, USA) and 0.15g Sodium Novibiocin purchased from Merck, Darmstadt, Germany with 1L distilled water was used. Heating was necessary to dissolve the reagents and sterilize the agar (121°C for 15 minutes). Thereafter the agar was allowed to cool to 55°C and poured into 90mm plates.

4.3.1 Experimental apparatus

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Petri dishes (9cm plastic disposable, sterile petri dishes) obtained from Concorde Plastics Ltd, Longdale Roodepoort, S.A and an incubator controlled at 25°C and 30°C (LEEC Ltd, Nottingham, UK) were used.

4.4 Determination of mycotoxigenic strains associated with maize, peanuts and beans.

Kernels from each sample were subsampled to isolate the contaminated species and to determine the isolation frequency of *Fusarium*, *Aspergillus* and *Diplodia spp*. infections. Samples were plated out for identification and isolation of fungal species as follows:

I. Whole maize kernels were transferred on both MEA and AFPA.

II. Finely ground and coarse maize samples were dilution plated onto AFPA only under sterilized conditions.
III. Whole peanuts and bean samples were only plated on AFPA.

4.4.1 Isolation and identification of fungal infection on maize, beans and peanuts commodities.

***MEA*:** A subsample (approximately 200 g) from each of the well mixed samples was surface-disinfected for 1 min in a 3.5% sodium hypochlorite solution and rinsed twice in sterile water (Rheeder et al., 1992). One hundred kernels per subsample were plated (five kernels per petri dish) onto 1.5% malt extract agar (MEA), containing 150 mg/L Novobiocin to minimize bacterial growth (Rheeder et al., 1992). The MEA plates were incubated in the dark at 25°C for 5-7 days. All the fungal genera which developed from the kernels were identified according to their morphological characteristics (Leslie and Summerell, 2006**).**

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***AFPA*:** The samples were prepared for the isolation and identification of *Aspergilli*, using the selective *Aspergillus flavus* / *A. parasiticus* Agar (AFPA) and isolates were identified by the pigmentation of colony as described by Pitt et al. (1983). This agar medium was used for both whole kernel and dilution plating. Briefly, one hundred kernels (maize, beans and peanuts) per subsample were plated (five kernels per petri dish) onto AFPA and incubated as described below. For the AFPA dilution plates, under aseptic conditions, 1 g from each sample was mixed with sterile distilled water (9 ml) as the first dilution. Then followed by another five serial dilutions; 1 ml of each dilution was transferred to an empty petri dish, mixed with 15 ml of cooled (± 50° C) AFPA and left to solidify. The inoculated plates were then incubated at 30°C for 3 - 4 days. The number of yellow-orange pigmented fungal colonies per gram of sample was calculated and expressed as colony forming units per gram (cfu/g) (Pitt et al., 1983).

The relevant *A. flavus* /*parasiticus* isolates on AFPA were identified according to their unique orange colouration on the reverse side of the plates according to Pitt

et al., 1983. *Fusarium* and *Aspergillus* fungal species were isolated purely because they are the two most important fungal genera in terms of toxicity.

4.5 Results and discussion

The two fungal genera (*Aspergillus* and *Fusarium*) isolated in samples from selected villages in both districts during the 2-year survey were observed.

4.5.1 Incidence of *Fusarium spp.* genera

In season one (2011), *Fusarium* recovered from maize ranged from 0 - 80%; 0 - 56% in GSDM and VDM (Table 4.2), respectively.
Maize samples (31/31) from GSDM and 16/18 from VDM were identified with *Fusarium spp.*

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Table 4.2: Percentage (%) frequency isolation of fungal species in maize samples from GSDM (Mpumalanga) and VDM (Limpopo) during the first season (2011).

Huosehold no's	***F. verticillioides***	***F. subglutinans***	***F. graminearum***	**Other *Fusarium***	***D. maydis***	***D. macrospora***	**Total other fungi**	***A. flavus* (*MEA*)**	***A. flavus* (*AFPA*)**	**Huosehold no's**	***F. verticillioides***	***F. subglutinans***	***F. graminearum***	**Other *Fusarium***	***D. maydis***	***D. macrospora***	**Total other fungi**	***A. flavus* (*MEA*)**	***A. flavus* (*AFPA*)**
	GSDM										**VDM**								
1w[1]	6	8	0	0	0	0	45*	0	0	**1y**	27	1	0	0	0	0	21	0	0
2w	52	6	3	2	0	0	5	0	0	**2y**	12	0	0	1	2	0	36	0	2
3w	1	24	2	0	0	0	25	0	0	**3y**	5	2	0	1	4	5	50	2	12
3y	2	30	5	1	2	2	15	0	0	**4y(1)**	21	0	0	2	0	0	50	4	11
4w	0	27	6	0	1	0	42	0	0	**4y(2)**	7	0	0	0	3	0	48	2	7
4y(1)[2]	0	12	0	0	0	0	50*	0	7	**5y**	7	0	0	0	1	0	41	4	5
4y(2)	0	22	3	0	0	0	9	0	0	**6y**[5]	19	0	0	0	0	0	56*	0	1
5w	0	31	5	2	1	0	14	0	0	**7y**	13	2	0	0	0	0	42	1	0
5y	0	14	16	1	2	0	19	0	0	**8y(1)**	12	0	0	1	7	4	43	0	1
6w	3	18	10	2	14	0	17	0	0	**8y(2)**	56	0	0	0	6	6	20	0	0
6y	0	11	40	9	6	0	28	0	0	**9mix**	0	0	0	0	0	0	40	3	2

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Huosehold no's	***F. verticillioides***	***F. subglutinans***	***F. graminearum***	**Other *Fusarium***	***D. maydis***	***D. macrospora***	**Total other fungi**	***A. flavus* (*MEA*)**	***A. flavus* (*AFPA*)**	**Huosehold no's**	***F. verticillioides***	***F. subglutinans***	***F. graminearum***	**Other *Fusarium***	***D. maydis***	***D. macrospora***	**Total other fungi**	***A. flavus* (*MEA*)**	***A. flavus* (*AFPA*)**
	GSDM										**VDM**								
7w	0	30	43	0	2	0	11	0	0	**10y**	3	0	0	0	1	0	24	0	1
8w	0	7	34	1	2	0	29	0	0	**12y**	10	0	0	1	0	0	27	0	4
8y	0	5	30	4	8	5	22	0	0	**13y**	17	0	0	0	1	17	22	0	0
9y	0	2	0	0	1	0	8	0	0	**14y**	11	0	0	0	1	1	43	0	4
10w	0	7	11	0	0	3	25	0	0	**15y**	12	0	0	0	0	0	45	18	32
11y	0	17	12	0	18	0	14	0	0	**16y**	15	2	0	0	0	0	43	5	8
12y	0	63	0	2	1	0	9	0	0	**17w**	0	0	0	0	0	0	0	0	0
13w	3	26	1	0	0	0	36	0	0	**9(1)**	13	0	0	0	0	0	43	0	4
13y[3]	0	12	0	17*	0	0	4	0	0	**9(2)**	14	0	0	0	3	0	49	0	3
14w	9	42	1	2	0	0	25	0	0	**10**	6	0	0	0	0	0	40	9	1
15w	0	6	17	2	17	0	30	0	0	**13**	17	1	0	0	0	0	48	0	1
16w	0	10	1	1	0	0	17	0	0	**14(1)**	14	0	0	0	0	1	44	0	3
16y	0	4	3	8	2	0	32	0	10	**14(2)**	15	0	0	0	1	0	36	0	5
17w	1	11	9	0	12	0	38	0	0	**15**	15	0	0	0	0	0	42	0	35

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GSDM

Huosehold no's	*F. verticillioides*	*F. subglutinans*	*F. graminearum*	Other *Fusarium*	*D. maydis*	*D. macrospora*	Total other fungi	*A. flavus* (*MEA*)	*A. flavus* (*AFPA*)
17y	0	7	38	5	0	0	4	0	0
18w(1)[4]	0	80*	0	0	20	0	4	0	0
18w(2)	4	3	0	0	0	0	27	0	0
19w	0	2	0	4	0	0	47	0	0
19y	3	4	2	0	0	0	44	0	0
20w	4	7	2	1	1	0	29	0	0
21-K	3	0	1	0	1	0	11	8	10

VDM

Huosehold no's	*F. verticillioides*	*F. subglutinans*	*F. graminearum*	Other *Fusarium*	*D. maydis*	*D. macrospora*	Total other fungi	*A. flavus* (*MEA*)	*A. flavus* (*AFPA*)
20	16	1	0	0	1	0	27	0	12

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W-white maize; Y-yellow maize; Mix-white & yellow maize

Highlighted area represents maize collected in November 2011.

[4]80% - Darkly pigmented *F. subglutinans.*

[1]45% - *Chaetomium spp*. prevalent

[2]50% - *Penicillium spp*. prevalent

[3] 17% - *F. poae* prevalent

[5]56% - *A. niger* prevalent

Only two samples from VDM which were not contaminated with *Fusarium spp.* Figure 4.1 shows the isolation of *Fusarium* species from maize kernels plated on MEA^{+} plates for 5 - 7 days at 25°C incubation. Maximum infection of kernels from Limpopo was observed at 56 % *F. verticillioides*, prevalence in one of the samples.

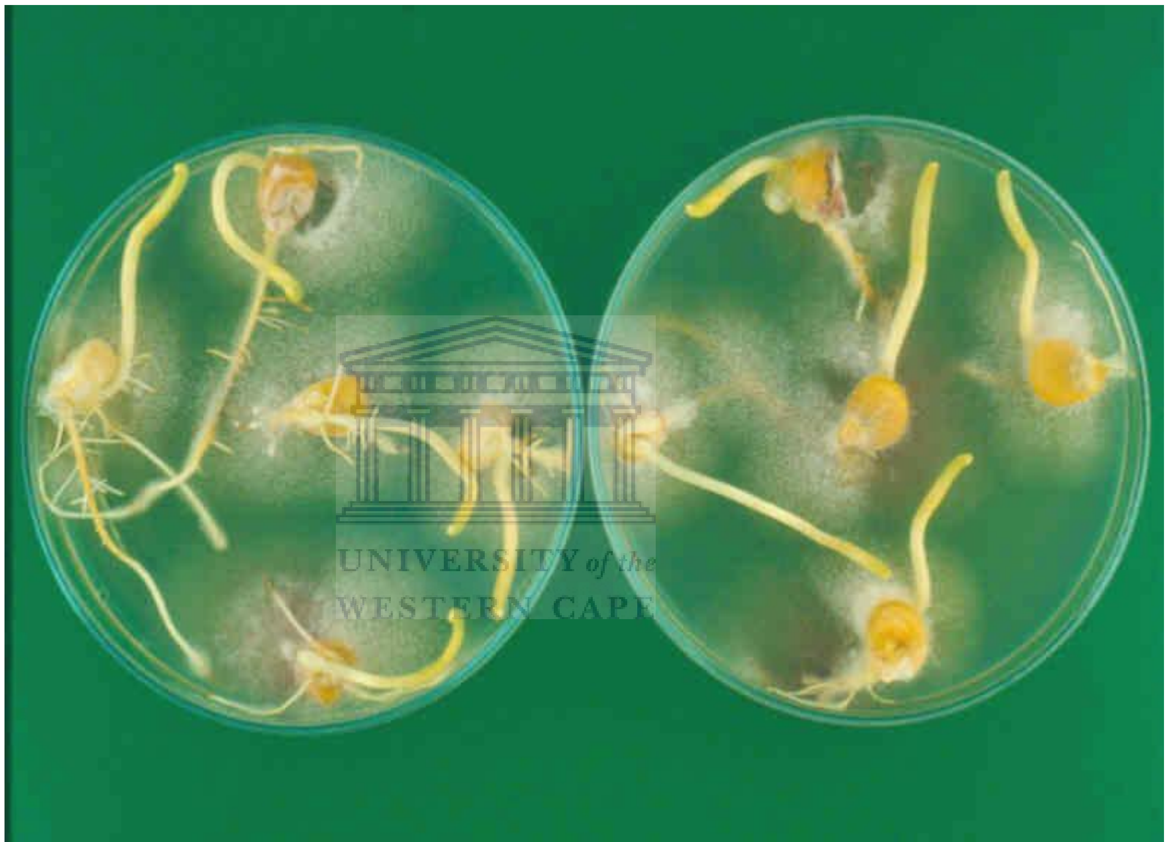
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Figure 4.1: Isolation of surface disinfected stored whole maize kernels on MEA^{+} plates after 5 days of incubation at 25°C (picture taken by J.P. Rheeder).

Out of eight maize seed samples collected from VDM in November 2011, only two *Fusarium* species were observed, 100% *F. verticillioides* and 25% *F. subglutinans*. The seeds were predominantly infected with *F. verticillioides* ranging from 6 - 16%. In Mpumalanga, 80 % *F. subglutinans* in maize kernels and

52% *F. verticillioides* were the highest frequencies observed. From three maize samples collected from GSDM during November 2011, one sample was isolated with 3% *F. verticillioides spp*. and another was isolated using a dilution plate method. Fungi could not be isolated from the third sample as it was badly spoiled.

In the second season (2012), the species recovered from maize ranged from 0 - 23% in VDM and 0 - 90% in GSDM (Table 4.3). In GSDM, 28/30 and from VDM 14/15 samples were identified with *Fusarium* species.
The highest isolated % frequency levels of infected kernels were *F. subglutinans* (90%) and *F. verticillioides* (75%) from GSDM. Highest isolated species in VDM were found to be *F. verticillioides* (23%).

4.5.2 Incidence of *Aspergillus spp.* genera.

Aspergillus spp. ranged from 0 – 10%; in maize samples from GSDM and 0 - 35% in maize from VDM (Table 4.3) during season one. Two out of thirty one maize samples from GSDM, as well as 14 out of 18 from VDM were isolated with *Aspergillus* species. Maize kernels from Limpopo were found with maximum infection of 32% *A. flavus* (AFPA) and 56% *A. Niger* prevalent in one of the samples. *A. flavus* (AFPA) ranging from 0-35% had the highest incidence in maize samples collected from VDM in November 2011.

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Table 4.3: Percentage (%) frequency of fungal isolates in maize samples from GSDM (Mpumalanga) and VDM (Limpopo) over the second season (2012).

GSDM

Huosehold no's	*F. verticillioides*	*F. subglutinans*	*F. graminearum*	Other *Fusarium*	*D. maydis*	*D. macrospora*	Total other fungi	*A. flavus* (*MEA*)	*A. flavus* (*AFPA*)
1w	0	90	7	0	0	0	3	0	0
1w	0	57	1	0	2	0	46	0	0
2y	2	87	3	2	16	0	13	0	0
3w	0	12	0	0	0	0	7	0	0
3y	0	24	8	0	28	0	11	0	0
4w	0	30	4	0	12	0	9	0	0
4 y	0	48	0	0	0	0	5	0	0
5w	0	2	2	1	0	2	15	0	0
5y	0	32	0	0	0	0	4	0	0
7w	0	16	0	0	5	0	8	0	0
8w	0	1	1	1	0	0	7	0	0
8y	1	0	0	0	9	0	11	0	0
9y	75	3	1	0	0	0	9	0	1
9y	4	0	0	0	0	0	5	0	0
11y	6	2	10	0	24	0	18	2	8
12w	0	3	0	2	0	0	18	0	0

VDM

Huosehold no's	*F. verticillioides*	*F. subglutinans*	*F. graminearum*	Other *Fusarium*	*D. maydis*	*D. macrospora*	Total other fungi	*A. flavus* (*MEA*)	*A. flavus* (*AFPA*)
1y	0	0	0	0	0	0	24	2	0
2y	2	0	0	0	0	0	8	0	0
4y	11	1	0	0	0	0	24	4	10
5y	9	0	0	0	0	0	30	11	50
6y	23	0	0	0	5	0	33	12	69
7y	6	0	0	0	0	0	20	5	9
8y	15	0	0	0	13	1	16	16	19
9mix	5	0	0	0	0	0	42	1	0
10y	4	0	0	2	0	0	42	3	4
11y	10	1	0	0	0	0	21	9	2
12y	13	0	0	0	0	0	34	14	21
13y	5	0	0	0	1	0	18	0	0
14y	3	0	0	0	4	0	18	0	0
15y	3	0	0	1	0	0	24	0	3
16y	15	0	0	0	0	0	16	0	1

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Huosehold no's	*F. verticillioides*	*F. subglutinans*	*F. graminearum*	Other *Fusarium*	*D. maydis*	*D. macrospora*	Total other fungi	*A. flavus* (*MEA*)	*A. flavus* (*AFPA*)
					GSDM				
13w	0	0	0	0	0	0	20	4	4
13y	0	4	1	2	0	0	25	2	9
14w	3	0	0	0	0	0	22	0	0
15w	0	0	0	0	0	0	65	27	7
16w	0	4	0	0	0	0	22	0	16
16w	1	0	0	0	2	0	16	0	0
17w	0	11	0	0	19	0	8	0	0
17y	0	7	19	1	9	0	10	0	0
17w	0	3	0	0	90	0	7	0	0
18w	0	88	1	0	17	0	4	0	0
19w	0	26	1	0	0	0	26	0	0
19w	1	4	22	0	0	0	13	0	0
20w	0	4	0	0	0	0	24	0	1
21w	1	32	0	0	2	0	11	0	3

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W-white maize

Y-yellow maize

Mix-white & yellow maize

Mpumalanga had the highest percentage infection of 10% *A. flavus* (AFPA) observed. One (milled) of three November samples was observed with with 600 cfu/g *A. flavus*. None of the peanuts and beans samples collected from both districts in the first season were infected with *Aspergillus* fungi.

Recovery of *Aspergillus* species during season two from maize ranged from 0 – 69% with dilution plated samples ranging from 10 – 700 cfu/g in VDM. However, *A. flavus* in GSDM maize kernels (Table 4.3) ranged from 0 - 27%. Twelve out of fifteen sample kernels from VDM and 8/30 from GSDM were identified with *Aspergillus spp.* Highest isolated % frequency levels found in infected kernels were *A. flavus* (MEA, 27%) and *A. flavus* (AFPA, 16%) in GSDM, whereas, kernels from VDM were isolated at a maximum frequency of 16% MEA, and 69% AFPA. *A. flavus* fungus, isolated from whole maize kernels and whole peanuts plated on AFPA at 30°C in the dark, were observed daily for 3 - 4 days, and are illustrated in Figure 4.2. Only one of each peanut (1/3; 3%) and bean (1/2; 2%) samples from Limpopo were contaminated with low frequencies with *A.flavus*.

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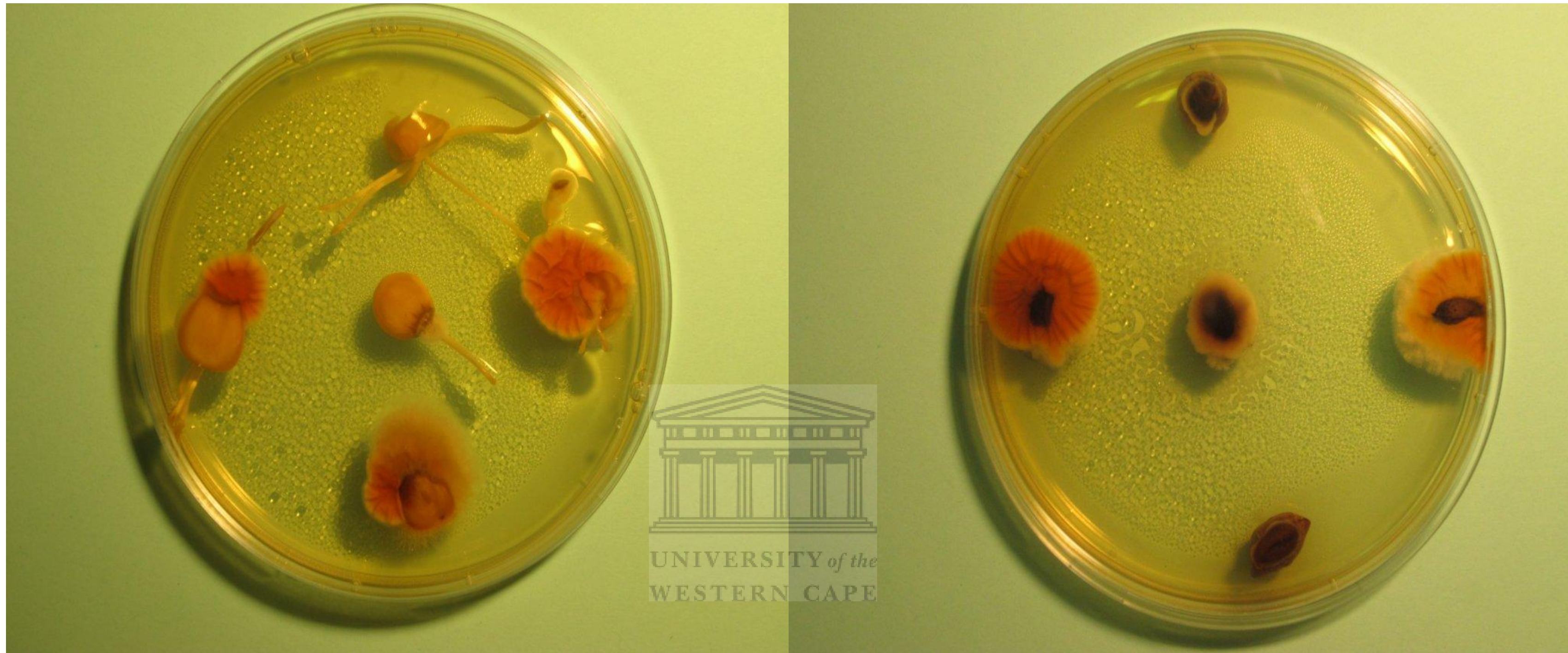
Seven VDM maize samples were measured by a dilution plating procedure. The total number of *A. flavus* species fungal counts recovered varied as illustrated in appendix 4.1. None of the fungal counts were above the acceptable limits of 10^2 to 10^4 cfu/g recommended by the International Commission on Microbiological Specification for Foods (Elliott, 1980; ORSI et al., 2000).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Whole maize kernels **Whole peanuts**

Figure 4.2: *A. flavus*, an aflatoxin producing fungus, isolated from surface disinfected stored maize and peanuts plated on AFPA medium in the dark for 3 - 4 days at 30°C (picture taken by J.P. Rheeder).

4.6 General frequency in isolated and identified commodities.

Mean isolation frequency of *F. subglutinans* (18%; 2011 and 20%; 2012) and *F. verticillioides* (2.8%; 2011 and 3.1%; 2012) fungi from GSDM was found with a minor increase in 2012 (Table 4.4). In contrast, *F. verticillioides* (13.7%; 2011 – 8.3%; 2012) isolated in maize from VDM had slightly decreased. *Diplodia* species varied in both districts for both seasons with *D.maydis* being highly isolated in the first season. *Aspergillus* species had an increased mean incidence range of 0-1% and 2-5 MEA and 0.5–2% and 5.0–13% AFPA compared to the first season for GSDM and VDM, respectively.

Table 4.4: Isolation frequency of fungal species (mean % frequency) from maize kernels of sampled in Limpopo (VDM) and Mpumalanga (GSDM) in 2011 and 2012.

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Fungi isolated	**(%) frequency**			
	Species in VDM		**Species in GSDM**	
	2011	2012	2011	2012
Fusarium				
F. verticillioides	14	8	3	3
F. subglutinans	0.4	0.1	18	20
F. graminearum	0	0	10	3
Other *Fusarium*	0.3	0.2	2	0.3
Diplodia				
D. maydis	1	2	4	8
D. macrospora	2	0.1	0.3	0.1
Aspergillus				
A. flavus (MEA)	2	5	0	1
A. flavus (AFPA)	5	13	0.5	2

There were considerable differences in the number of kernels infected by the genus *Fusarium*, *Diplodia* and *A. flavus* (Appendix 4.2). Differences were observed between the two provinces, when comparing 2011/2012 seasons. Total amount of *Fusarium* (43) as well as *Diplodia* (19) in Limpopo were recovered at higher occurrence levels compared to Mpumalanga (135; 34), respectively. *A. flavus* was the most common with 41 isolates at higher frequencies compared to Mpumalanga with 14 isolates at very low frequencies. Yellow and white maize samples collected form Mpumalanga showed obvious differences in the average incidence rate observed when comparing 2011/2012 of each maize variety (Table 4.5).

Table 4.5: Comparison on average percentage of fungal species between white and yellow maize samples from Mpumalanga (GSDM) during both seasons (2011 and 2012).

Maize samples	***F. verticillioides***	***F. subglutinans***	***F. graminearum***	**Other *Fusarium***	***D. maydis***	***D. macrospora***	***A. flavus* (MEA)**	***A. flavus* (AFPA)**
		Season one (2011)						
WM	4.5	18.2	7.7	0.9	3.7	0.2	0.4	0.5
YM	0.4	15.6	11.5	3.6	3.1	0.5	nd	1.3
		Season two (2012)						
WM	0.3	19.2	2.0	0.2	7.5	0.1	1.6	1.6
YM	8.8	20.7	4.2	0.5	8.6	nd	0.4	1.8

WM-White maize
YM-Yellow maize

But when comparing the average % incidence between yellow and white maize, there were slight differences in *F. verticillioides* species recovered (Figure 4.3). White maize during 2011 was observed with high % frequency of *F.*

verticillioides, however in 2012 the reversed situation occurred. In 2011 to 2012 there was an average increase of *F. Verticillioides* from 0.4 to 9% and *D. maydis* from 3 to 9% in yellow maize with a decrease of *F. graminearum* and other *Fusarium* present by more than 2-fold, respectively. *A. flavus* incidence varied between yellow and white maize with slightly higher incidence in the second season. On the other hand, *A. flavus* (MEA; 0.4 - 1.6%) and (AFPA; 0.5 - 1.6%) incidence in white maize increased in 2011/2012 seasons (Table 4.5).

The combined overall number of kernels infected showed an amount of 93% (38/41) *F. verticillioides* species isolated in maize from Limpopo. *F. subglutinans, Diplodia* and other *Fusarium* were isolated in less than 40% whereas *F.graminearum* was absent (Appendix 4.2). *A. flavus* was isolated in kernels at 46% (19/41) on MEA and AFPA at 76% (31/41), the latter being the most predominant. Mpumalanga had the highest amount of isolates observed at 89% (55/62) *F. subglutinans* followed by 61% (38/62) *F.graminearum*, *F. verticillioides* (34%, 21/62). Infection by *D. maydis* occurred at 50% (31/62), while other *Fusarium* were isolated at 37% and *D. macrospora* at less than 10%. *F. graminearum* has been shown to cause cob rot all over the world together with *F. subglutinans* and it is known to be transferred by seeds (Sutton, 1982; Hussein et al., 2002). It has also been reported as the frequent contaminant of cereal crops (Bennett & Klich, 2003). Strains of *A. flavus* isolated were found in 8% (5/62) of the samples on MEA and 18% (11/62) when plated onto AFPA agar.

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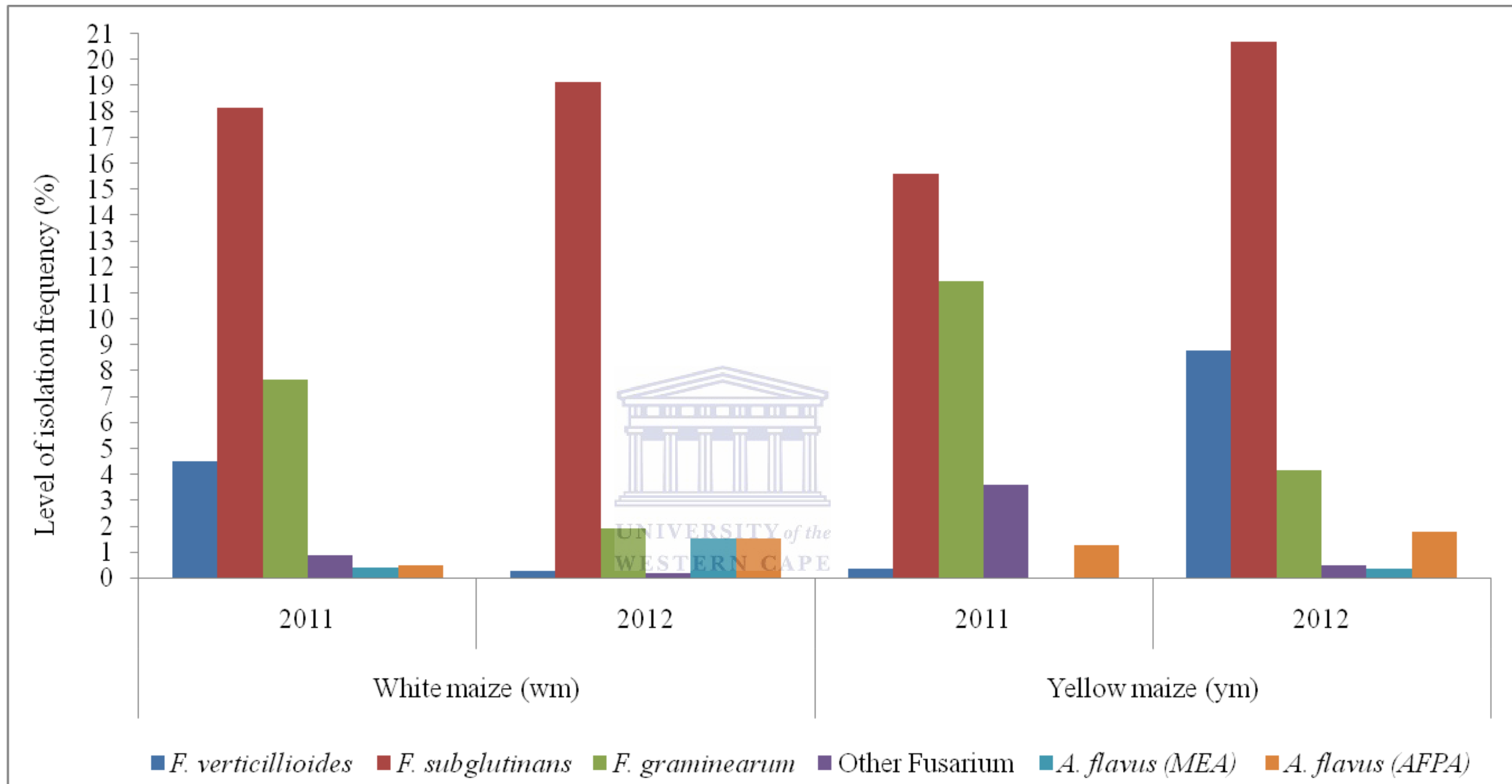

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Figure 4.3: Average frequency (%) of fungal species isolated from yellow (ym) and white (wm) maize samples collected over two growing seasons from GSDM.

The high total number of *F. graminearum* isolates in Mpumalanga is of concern as it has been reported by Marasas et al., 1991 as one of the most toxic *Fusarium* species in animal feed. Most high frequencies of the species are found in YM which is used as animal feed in GSDM. *F. subglutinans spp*. has been reported to be more prevalent as well as comparable in colour to *F. graminearum* species (Shurtleff 1984; Lew et al., 1991). Fungal growth of *F. graminearum* and *F. subglutinans* can be suppressed by *F. verticillioides* (Rheeder et al., 1990; Reid et al., 1999). This explains the absence of *F. graminearum* and the ≤ 2% incidence of *F. subglutinans* in kernels from VDM during seasons 2011/2012, where there was predominance of *F. verticillioides*. Figure 4.4 shows the mean incidence of all species isolated over the two seasons. *A. flavus* was by far the most frequent species detected in Limpopo compared to Mpumalanga.

F. subglutinans was overall the most dominant species in Mpumalanga followed by *F. graminearum*, which correlated with a recent study by Ncube et al. (2011). Temperature in Mpumalanga in growing seasons ranged from 15 - 33°C (2011) and 14 - 32°C (2012), this makes it conducive for the growth of *F. graminearum* in the field before storage. *F. graminearum* has been reported with an optimal growth occurring between 24 - 26°C (EMAN).

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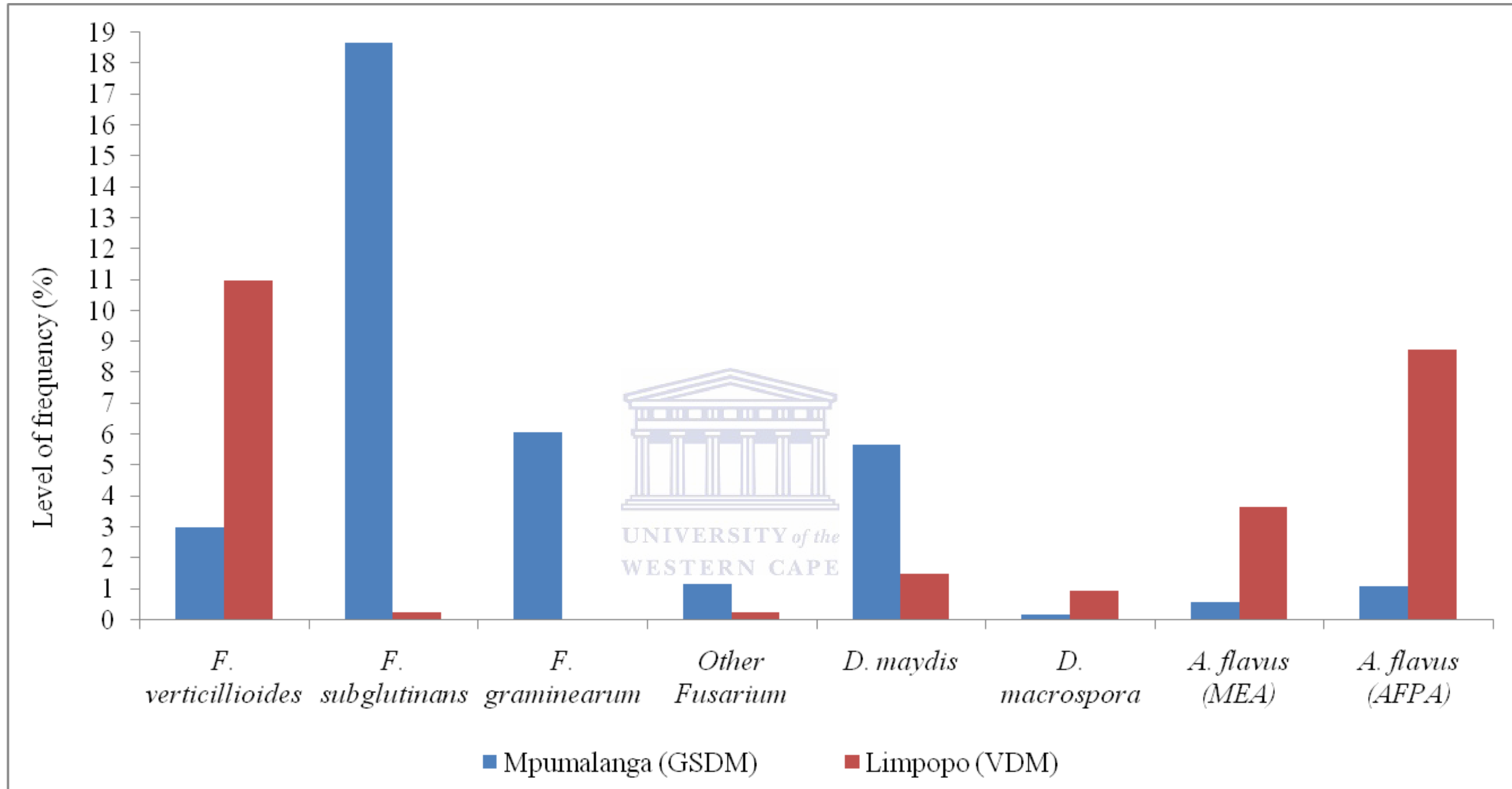

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Figure 4.4: Comparison on average total frequency (%) of maize collected over two harvest seasons between Gert Sibande and Vhembe district.

In Limpopo, maize was mostly infected with *F. verticillioides*. These results correlate with the study by Ncube et al. (2011) and Phoku et al. (2012), who both reported *F. verticillioides* as the most prevalent species in maize from Limpopo province. Minimum and maximum temperature data in Limpopo during the growing seasons one (2011) and two (2012) respectively ranged from 18 - 30°C and 17 - 30°C. Marín et al. (1995) observed that 25 to 30°C was an optimal growth temperature for *F. verticillioides,* and even at temperatures above 30°C growth was occurring. *A. flavus* has been reported as developing in a minimum 10 - 12°C to a maximum of 48°C (EMAN). The two yearly weather conditions from both districts show a great potential for *Fusarium* and *Aspergillus* production in commodities before storage. Phoku et al. (2012) detected mostly *F. verticillioides* (70%) in maize compared to less than 30% found in porridge and faecal samples from Limpopo.

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High incidence of *F. verticillioides* in maize has been reported in other parts of South Africa; Limpopo, Zululand and Transkei region (presently part of the Eastern Cape) by Marasas (2001), and Ncube et al. (2011). This also occurs in other parts of the world such as Brazil, Eastern and Southern Africa, where high prevalence has been observed of *F. verticillioides* in maize by van der Westhuizen et al. (2003); Orsi et al. (2000), and Doko et al. (1996). The development of *F. Subglutinans* (a non-producer of fumonisins) is commonly characterized by moderate temperatures that occur in GSDM and has been reported to be more prevalent under favourable conditions by Ncube et al. (2011) and Vigier et al. (1997). Both *F. graminearum* and *F. subglutinans* which have been found mostly in GSDM kernels were reported by Reid et al. (2002) to favour temperate climates as compared to *F. verticillioides*. A report by Ncube et al. (2011) revealed *F. verticillioides* is South Africa's most important maize fungal pathogen. It has been reported by Summerell and Leslie (2011) as one of the most frequent strains linked to the highest production of fumonisins during harvest and as an endophyte of maize found practically in every individual maize plant. *F. subglutinans* has been reported to cause ear rot diseases in maize (Marasas et al., 1979).

Overall, the highest %incidence rate of *A. flavus* (AFPA) was found in maize (69%) isolated from VDM in the second season. In the first season maize (35%) from VDM collected in November 2011was the most frequently isolated with *A. flavus* (AFPA). The genus *Aspergillus* was recovered at moderate to low levels, less than 27%, in kernels from Mpumalanga whereas *F. verticillioides* was isolated in higher percentages:52% (2011) and 75% (2012) (GSDM). The other samples from GSDM had a frequency of isolation of less than 10% which would result in a low mycotoxin production and less effect on maize quality. *Aspergillus spp*. have been reported as always to be found at lower levels compared to other genera such as *Fusarium* in South Africa maize crops (Dutton and Kinsey, 1995). *F. verticillioides* and *F. subglutinans* which have been found to dominate the current study, have been previously found to commonly occur in maize (Moretti et al., 1996). Fungal incidence in home-grown maize is of concern, particularly *Aspergillus spp* as it is known to cause severe illness and sometimes death. *F. verticillioides* as well has been classified as carcinogenic to animals upon evidence that its cultures and FB_1 are capable of promoting liver cancer in rats (Gelderblom et al., 1996). Another concern is the occasional mixing of homegrown seeds with commercial seeds for planting. Overall, in the two years the occurrence of *Fusarium* species was greater in maize compared to peanuts and beans.

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4.6 Conclusion

Mycology results showed the dominance of *Aspergillus and Fusarium* genera in VDM maize whereas GSDM maize was less contaminated. Most households from the Limpopo province are at risk by mycotoxin exposure caused by an abundance of *F. verticillioides*. The highest incidence of fungal species was isolated in maize from Limpopo compared to Mpumalanga, which is possibly due to a general poor harvest, inadequate storage practices and climatic conditions among other factors. The samples collected in the first season and kept as seeds for the next season had no apparent mould contamination. But, the highest incidence of *A. flavus* was found in one bag of the seeds, which indicated that fungal development does not

only occur on visibly mouldy crops. A considerable variation was observed on levels of incidence with regard to sample types (maize, beans and peanuts).

Households sampled in VDM stored their commodities in sacks without the use of any pesticides. These bags are prone to fungal infection and insect infestation as observed in the maize collected. Most maize crops collected from VDM during this study showed discolouration and severe weevil infestation which have been reported to encourage fungal pathogens. Storing on a cement floor increases the probability of absorbing moisture from the floor or the wall which can cause fungal and insect infection. Both rural populations heavily depend on home-produced maize crops as their primary source of food. They consume *Fusarium* contaminated maize on a daily basis as it cannot be completely eliminated from their harvested crops.

In this survey, infection by *Fusarium* and *Aspergillus spp* were observed more in maize compared to the secondary crops (peanuts and beans) in both districts in the two year study. Generally, fungal analysis showed higher prevalence of *Fusarium spp*, in maize from the two areas. Fungal genera (*Fusarium* and *Aspergillus*) isolated in this study are among the most important mycotoxin producers associated with food and feed safety globally. These fungal species are known to produce a range of mycotoxins which were investigated in the subsequent chapter.

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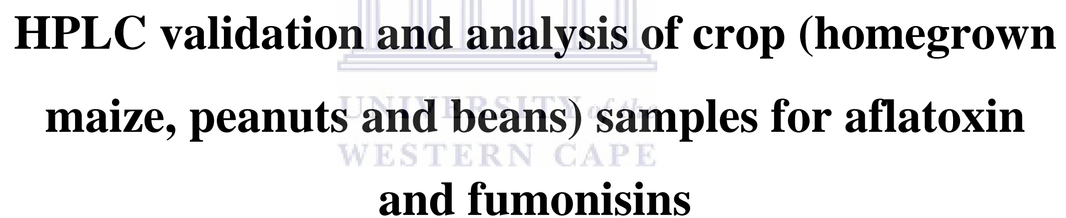
CHAPTER 5



HPLC validation and analysis of crop (homegrown maize, peanuts and beans) samples for aflatoxin and fumonisins

5.1 Introduction

Mycotoxins are found in various grain types and are unavoidable (EMANa; Schollenberger et al., 2006). Due to their toxic nature, current legislation has specified maximum limits particular for aflatoxins. The only way to limit these mycotoxins is to extensively monitor food and feed through research to reduce or destroy any occurrence of mycotoxigenic organisms that infect crops in the field and storage. Some African countries have extensive publications on the occurrence of fumonisins and aflatoxins (Ezekiel et al., 2012; Marasas et al., 2012; Shephard, 2008a; Wagacha and Muthomi, 2008).

Monitoring aspects usually include appropriate sampling, preparation and measurement and separation techniques with suitable specific detection ability (Shephard, 2008b). Therefore, highly sensitive and specific analytical techniques have been developed for quantitative and qualitative analysis of toxic fungal metabolites in food and feed (Shephard et al., 2011). There are various methods of detection presently used. The most common is high-performance liquid chromatography (HPLC) combined with fluorescence or UV detection, thin layer chromatography (TLC) and enzyme linked immunoassays (ELISA) (Gelderblom et al., 1988; Rice et al., 1995; Shephard and Sewram, 2004; Hussain, 2011).

One of the major objectives of this study was to investigate the extent of contamination of home-grown produce in the study areas with aflatoxins and fumonisins. This was achieved by using reversed-phase high-performance liquid chromatography with fluorescence detection (RP-HPLC-FLD). The two mycotoxins were analyzed separately because there is no HPLC method for their simultaneous analysis.

5.2 Materials and methods

5.2.1 *Chemicals and reagents*: All chemicals and reagents used in this study were of HPLC grade. Acetonitrile, methanol, glacial acetic acid, *o*-phosphoric acid (>

85%), 2 -mercaptoethanol (ME), toluene (methylbenzene), potassium dihydrogen phosphate, sodium chloride (NaCl), sodium dihydrogen phosphate, disodium tetraborate ($Na_2B_4O_7 .10H_2O$) and *o*-phthaldialdehdye (OPA) were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich. Water was distilled and deionized by a Milli-purification system (Millipore, Bedford, MA).

5.2.2 *Reference Standards*: Fumonisin (FB) B_1, B_2 and B_3 were obtained from PROMEC Unit (MRC, South Africa). Aflatoxin (AF) B_1, B_2, G_1, and G_2 were purchased from Sigma Aldrich (St. Louis, MO., USA).

5.3 Aflatoxin chemical analysis

Analysis of aflatoxins was done by post-column online photochemical derivatization with fluorescence detection for an enhancement of detector response (Joshua, 1995, Zang et al., 2005, Waltking & Wilson, 2006). Aflatoxins B_1 and G_1 cannot be detected (less fluorescent) under standard reverse phase HPLC conditions whereas aflatoxins G_2 and B_2 are readily fluorescent and can be detected at low levels (Muscarella et al., 2009). However, to enhance fluorescence of AFB_1, and AFG_1 for better sensitivity post-column derivatization and detection with fluorescent detector are required. Extracted samples were purified on AflaTest® immunoaffinity columns for better clean-up and to decrease matrix effects caused by complex matrices. The salting-out effect (addition of inorganic salts e.g. NaCl) was used in all samples in this study (maize, peanuts and beans) to improve extraction efficiency of aflatoxins. Salt added in test samples improves recovery.

5.3.1 Standard solutions

The preparation of aflatoxin standard solutions was carried out according to the methods described in AOAC 971.22 (AOAC, 2000). This is to determine the true analytical concentration of aflatoxin standard analogues. In brief each individual aflatoxin (received in a dry form) was diluted with toluene-acetonitrile (9:1, v/v) to obtain concentrations of between 8-10μg/mL as stock solutions.

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Concentrations of stock solutions were determined using a Uvikon 923 Double Beam UV/VIS spectrophotometer (Bio-Tek icontron, U.S). Aflatoxin concentrations were calculated at wavelengths of maximum absorption close to 350 nm using the equation (µg Aflatoxins/mL =A x MW x1000/Ɛ) and adjustments were made in order to obtain the appropriate working solutions. The absorbance (A) was measured and compared to the absorptivity (Ɛ) in calculation for the concentration of aflatoxin standard analogues. Then the mixtures were evaporated to dryness at 60°C with nitrogen gas in 4 mL amber vials. The residues were reconstituted in 2 mL methanol and stored at 4°C in darkness until the analysis.

5.3.2 Experimental apparatus

A Stuart® Orbital Shaker (Karlsruhe, Germany) and a model RC-3B refrigerated centrifuge (Sorvall, Bohemia, NY, USA) were used. Clean-up was done on a 12 port SPE manifold (Supelco, Bellefonte, PA, USA) utilizing AflaTest® immunoaffinity columns (IAC) purchased from Vicam (Watertown, MA, USA). An 1100 series HPLC Agilent system consisting of an auto-sampler, and a quaternary pump at 1.5 mL/min flow rate was used for chromatography. A Phenomenex Ultracarb 3µ ODS (20) (100 x 4.60 mm, 3 mm internal diameter Phenomenex, Torrance, CA) analytical column was used as HPLC column in connection to afluorescence detector (Darmstadt, Germany), set at 365 nm excitation and 440 nm emission wavelength. A UV lamp photochemical reactor for enhanced detection (PHRED^{TM}) - from AURA Industries, New York, USA was inserted between the HPLC column and the fluorescence detector. Agilent Chemstation - LC software was used for data collection and processing.

5.3.3 Sample extraction and cleanup using Solid Phase Extraction (SPE)

Samples were prepared and extracted according to the AOAC Method 991.31 (AOAC, 2000). A typical example was as follows: each 10g milled sample was mixed with 1g sodium chloride in 25 mL of the extraction solvent methanol: water (80: 20, v/v). The mixture was shaken using a Stuart® Orbital Shaker at 250 rpm for 10 minutes, then centrifuged at 4000 rpm at 5°C for 5 min. Extract was

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filtered through a Whatman No.4 filter paper, and 10 mL filtrate was diluted with 40 mL distilled water.

Ten (10mL) of the filtrate was loaded onto AflaTest® immunoaffinity columns (IAC) for cleanup at a flow rate of 1-2 drops/s. IAC used for aflatoxin cleanup, when compared to multifunctional cleanup columns (MFC) by a study done by Chen et al., 2005, provided increased extraction efficiencies, good recoveries, sensitivity and reproducibility and repeatability. The IAC columns were washed with 15 mL water followed by an elution of the analytes with 3 mL methanol into an amber vial. The elute was evaporated to dryness with a stream of nitrogen gas at <60°C, then re-dissolved with 200 µL of methanol and stored at 4°C prior to analysis. Photochemical reactor for enhanced detection - PHRED^{TM} is a post column derivatization procedure used to increase the sensitivity and selectivity of analyte response. It utilizes a reaction coil around a UV lamp (254 nm) located between the column compartment and the detector of the HPLC instrument to perform on-line post-column derivatization.

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5.3.4 Chromatographic analysis

Aflatoxin analysis was performed on HPLC- PHRED-FLD. Separation was achieved by a Phenomenex Ultracarb analytical column maintained at 35°C. Samples were isocratically eluted with mobile phase (0.01M KH_2PO_4: acetonitrile: methanol: acetic acid; 690: 150: 75: 20, v/v/v/v), at a flow rate of 1.5 mL/min. Fluorescence detection was set at excitation wavelength 360nm and emission wavelength 440nm. The standards (10µL) and extracts (20µL) were injected using an auto sampler into the HPLC system. The analytes were quantified by comparing peak areas of authentic standards with peak areas of investigated samples.

5.4 Method validation

5.4.1 In-house validation

An in-house evaluation of the aflatoxin method in maize, peanuts and beans was carried out according to procedures by Gnonlonfin et al., 2010 with slight modifications. These included the determination of recovery, repeatability (intra-day precision) and reproducibility (inter-day precision), accuracy, linearity, sensitivity and specificity. It essentially involved a 10 µL cocktail standard solution being injected in triplicate before the extracted samples are injected to perform any specific identification and monitoring of retention times. Commercial commodities purchased were used as blank samples with each sample being analysed and the results were incorporated in the calculation for recoveries.

Before extraction, dry maize, peanuts and bean samples were fortified at different concentrations (5; 10; 20 ppb) of each analogue standard as a result of diverse legal limits for mycotoxins in food and feed. Recoveries, intra-day precision and inter-day precision were evaluated and peak area versus concentration standard curves was plotted. HPLC system suitability was monitored using a freshly prepared working standard solution.

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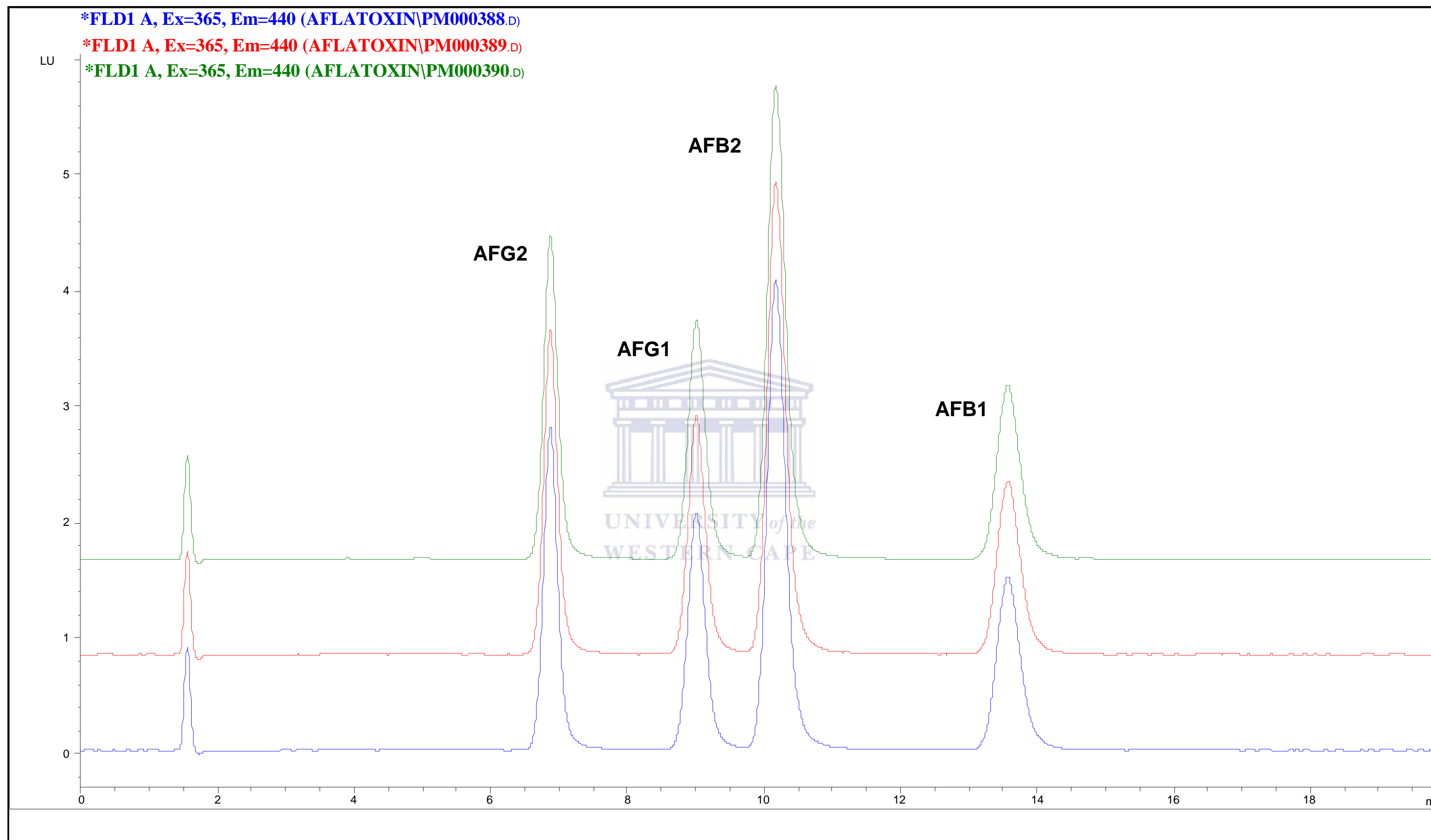

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Figure 5.1: Chromatograms of aflatoxin G_2, G_1, B_2, and B_1 working standard obtained for specificity.

5.4.2 Method performance

The resulting chromatographic elution order of aflatoxin G_2, G_1, B_2, B_1 was consistent and produced reproducible retention times of 7.0, 9.0, 10.3, 13.7 min (Figure 5.1), respectively.

Better, shorter retention times were achieved for the aflatoxin method with modification of column oven temperature to 35°C and flow rate to 1.5 mL/min, where it provided high throughput compared to the method previously reported by Gnonlonfin et al. (2010).

Specificity of the method presented in Figure 5.1 was obtained by injecting 10µl aliquots of AF working standard at concentrations of (102.00, 49.00, 261.12 and 61.39 ng/mL), AFB_1, AFB_2, AFG_1 and AFG_2, respectively in triplicate. Selectivity of the method was achieved on the basis of their comparative retention time of the matrix analyte to the retention time of reference standard. High degree of specificity and selectivity was achieved. There was a clear identification and quantification of all analytes, since no interfering peaks were demonstrated at the specific retention times of each chromatogram, indicating a high specificity of the analytical method.

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Figures 5.2 represents chromatograms of blank and individually spiked samples with aflatoxin B_1, B_2, G_1 and G_2 standard solutions. The chromatograms illustrated little background noise and no matrix interferences were observed during the analysis of blanks and standards. This shows the selectivity of the method for the analysis of the naturally contaminated samples.

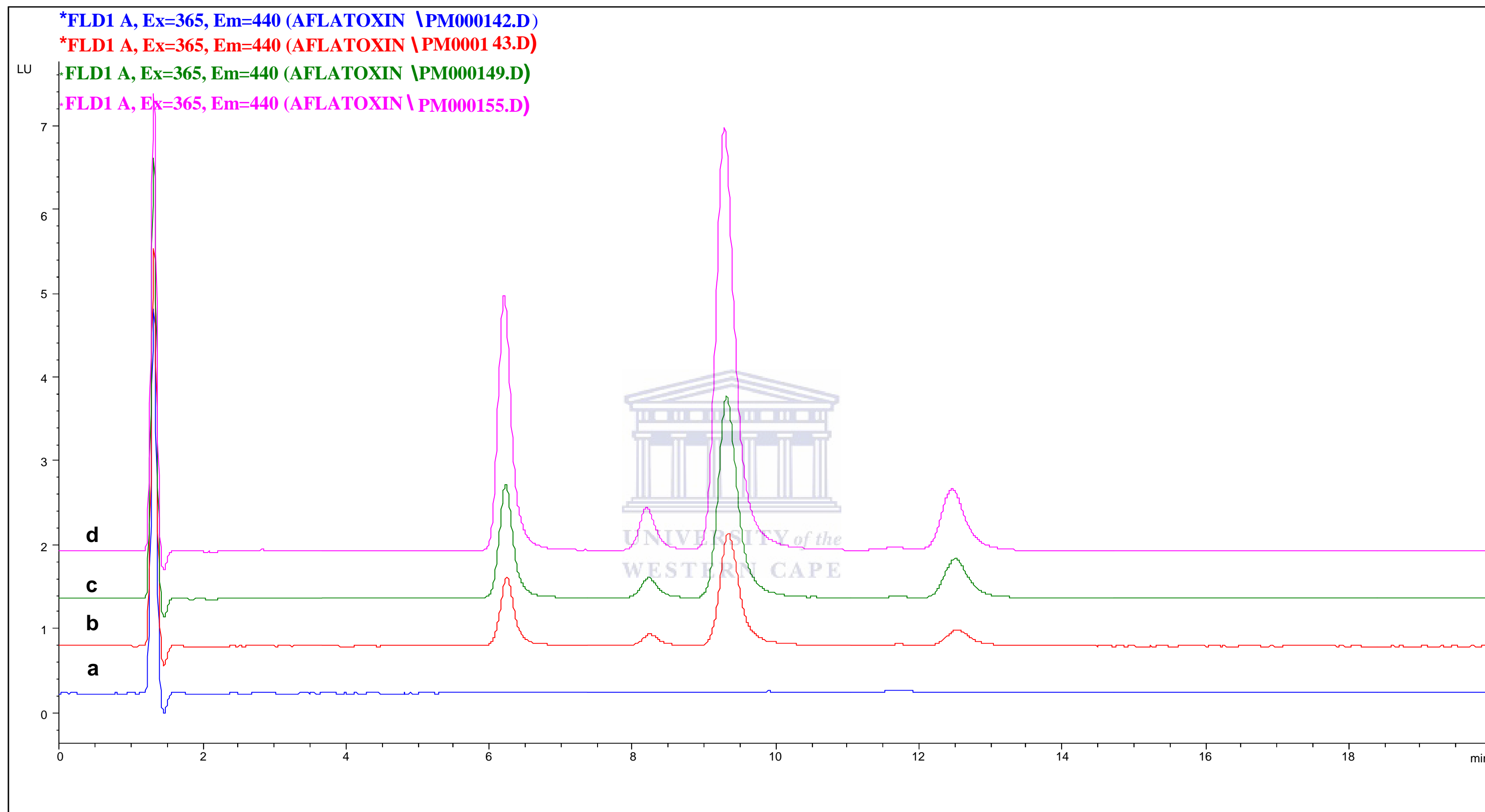

Figure 5.2: Chromatograms of maize samples spiked with aflatoxin working standard at different concentrations; (a) Blank; (b) 5 µg/kg; (c), 10 n/g; and (d) 20µg/kg. Similar results were obtained for the other two sample types.

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Good separation was obtained indicating good selectivity and specificity even at relatively low concentrations. Baseline resolution between all aflatoxin peaks was achieved. Short run time and low retention times were obtained with 1.5 mL/min flow rate and 0.01M KH_2PO_4: acetonitrile: methanol: AA (690: 150: 75: 20, v/v/v/v) mobile phase for AF analysis.

5.4.3 Method precision and accuracy

For the aflatoxin method, precision was evaluated for repeatability (intra-day) and reproducibility (inter-day) of the standard solution. Intra-day (n=3) was obtained by injecting three subsequent AF working standards within one day with variances of 0.01% to 1.5% RSD as illustrated in Table 5.1.

Table 5.1: Intra-day precision expressed as standard peak areas (µg/kg) of each aflatoxin analogue working standards (n=3).

	AFB_1	**AFB_2**	**AFG_1**	**AFG_2**
Standard 1	36	80	36	42
Standard 2	37	80	40	44
Standard 3	36	79	37	42
Mean	**36.6**	**79.6**	**37.6**	**42.8**
Stdev	**0.5**	**1.0**	**2.0**	**1.0**
%RSD	**1.5**	**0.0**	**0.1**	**0.0**

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Inter-day precision (n=12) was measured for four consecutive days from three injections with reported results using a mean relative standard deviation of the slope, which led to the values ranging from 1.7% to 2.8% RSD, Table 5.2. Both intra and inter-day results indicated good precision and repeatability.

Table 5.2: Inter-day precision expressed as standard peak areas (µg/kg) of each aflatoxin analogue standards (n=12, 3x working standard).

	AFB_1	**AFB_2**	**AFG_1**	**AFG_2**
	38	81	38	42
Day 1	37	81	38	42
	37	80	38	42
	36	80	38	42
Day 2	35	77	37	41
	35	77	37	41
	35	78	37	42
Day 3	36	80	36	42
	37	80	40	44
	36	79	37	42
Day 4	36	77	36	41
	37	80	38	43
Mean	**36.3**	**79.2**	**37.4**	**42.0**
SD	**1.0**	**1.5**	**1.1**	**0.7**
%RSD	**2.8**	**1.9**	**2.9**	**1.7**

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Accuracy (trueness) of the optimized method was determined by three replicate known standards at each of the three spiking levels (n = 12) of blank maize, peanuts and beans samples at 5, 10 and 20 µg/kg of each AFB_1, AFB_2, AFG_1 and AFG_2. From the experiments, acceptable accuracy of the method was achieved by calculating the reovery percentages, performed for each of the analytes (Table 5.3 to 5.5).

5.4.4 Linearity

To determine the linearity, calibration curves for each AF was constructed from the standards prepared in extracts of blank samples as shown in Figure 5.3. Linearity was performed in triplicate at different concentration levels where

calibration curves were determined by plotting the response factor of the peak area as a function of analyte concentration of 5, 10 and 20 µg/kg aflatoxin B_1, B_2, G_1 and G_2.

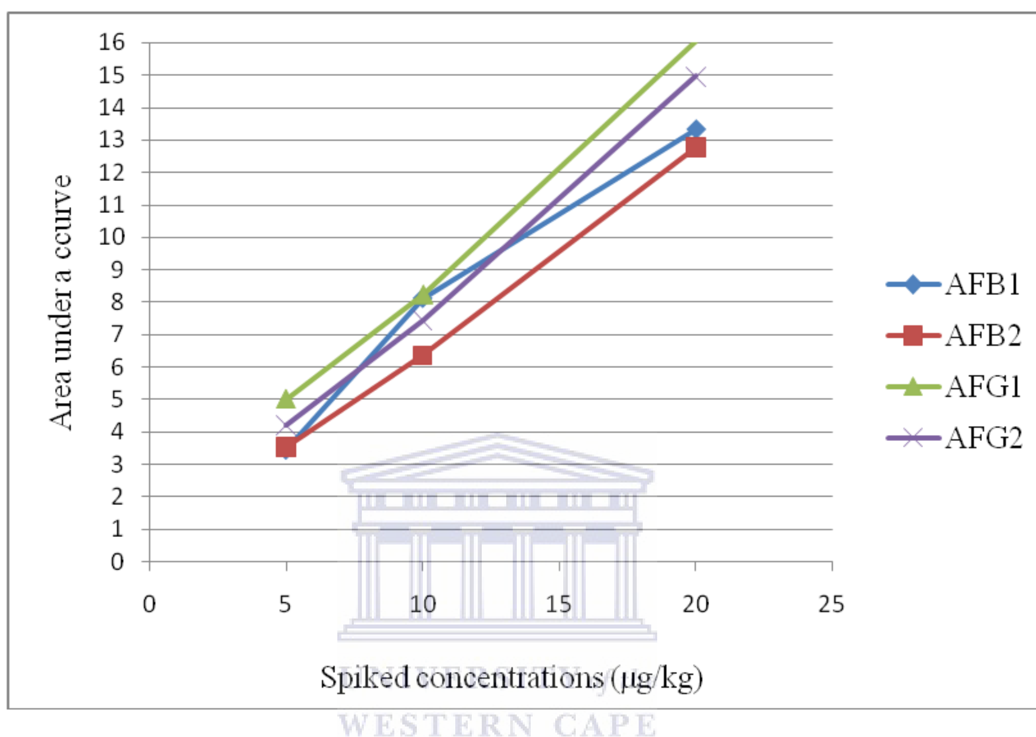

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Figure 5.3: Linear graph of samples spiked with aflatoxin standards: at 5, 10 and 20 µg/kg.

The correlation coefficients (R^2) for all the aflatoxin calibration curves ranged from 0.9749 to 0.9991, 0.984 to 0.9995 and 0.8314 to 0.9973 (Table 5.3 to 5.5) for maize, peanuts and beans, respectively. The method showed good linearity in beans after modification, by increasing the salt amount (Table 5.3). This was done to achieve a higher ionic strength and possibly improve extraction efficiency. This optimization decreased recoveries on the one hand but increased the correlation coefficient (R^2) (i.e. linearity) on the other hand, this was evident by the results in Table 5.3. It was clear that by increasing the salt (NaCl) content, better precision was achieved. These findings agreed with those of Gnonlonfin et.al, 2010 and show that salt quantity has an effect on both precision and recoveries. The limits

of detection (LOD) and quantitation (LOQ) were measured from signal to noise (s/n) ratio of the lowest aflatoxin standard concentration in spiked samples. The LOQ was determined with 2.0 μg/kg for aflatoxins.

5.4.5 Recoveries

Determination of the recoveries was achieved by spiking each dry milled commodity with individual analogues of aflatoxin standard at three concentration levels each as presented in Tables 5.3 - 5.5. The mean recoveries of aflatoxin from spiked maize samples were in the range of 65.9% to 87.6%; 9.2% RSD (Table 5.4) and peanuts (62.2 - 90.3%; 12.6% RSD; Table 5.5). In beans containing1g of salt the mean recoveries ranged from 24.8% to 62.0% with 4.6% RSD and in 5g salt added recovery ranged from 39.4% - 58.9% (3.2% RSD) as presented in Table 5.3. Increasing the NaCl quantity was done in order to improve the % recovery. The recoveries in beans were low even with 5 g of NaCl added. After investigation of chromatograms shown on Figure 5.2, aflatoxins were not detected in the blank samples.

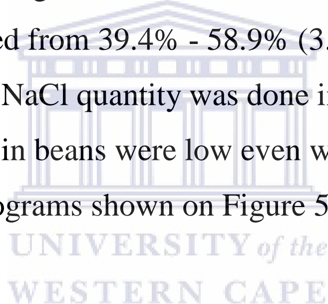
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Table 5.3: Recoveries and precision of beans spiked with aflatoxin standard at different levels with two levels of salt added, n=3.

	1g NaCl added					5g NaCl added			
AF Analogue	**Spiking level (µg/kg)**	**Mean recovered (µg/kg)**	**Recovered RSD (%)**	**Regression equation**	R^2	**Mean recovered (µg/kg)**	**Recovered RSD (%)**	**Regression equation**	R^2
	5	3.1	62.0 (4.2)			2.2	44.9 (5.6)		
AFB_1	10	6.8	67.9 (15.6)	y=0.528x+0.885	0.981	5.4	53.7 (0.8)	y = 0.756x-1.796	0.996
	20	11.2	56.2 (1.0)			13.5	67.3 (2.0)		
	5	3.1	61.6 (3.7)			2.6	52.3 (0.7)		
AFB_2	10	7.4	73.8 (10.0)	y=0.645x+0.285	0.987	5.7	57.2 (2.2)	y = 0.732x-1.259	0.997
	20	13	64.8 (1.5)			13.5	67.4 (0.8)		
	5	1.8	35.4 (2.9)			1.5	30.2 (11.5)		
AFG_1	10	2.1	21.2 (2.8)	y=0.097x+1.526	0.831	5.3	52.7 (5.6)	y = 1.024x-4.084	0.992
	20	3.6	18.0 (5.2)			16.5	82.3 (7.0)		
	5	3.1	62.0 (3.9)			1.4	28.6 (3.2)		
AFG_2	10	7.2	72.3 (1.8)	y=0.368x+1.848	0.929	2.9	28.9 (2.2)	y = 0.744x-3.193	0.958
	20	8.9	44.5 (7.8)			12.1	60.7 (2.2)		

Aflatoxins in blank beans samples were not detected.

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Table 5.4: Recoveries and precision of aflatoxin analogues in maize samples spiked with aflatoxin standard at different levels, n=3.

AF Analogue	Spiking level (µg/kg)	Mean recovered (µg/kg)	Recovery RSD (%)	Regression Equation	R^2
	5	3.4	68.9 (10.8)		
AFB_1	10	8.1	81.2 (22.1)	y=0.638x+0.843	0.975
	20	13.3	66.6 (11.3)		
	5	3.5	70.4 (5.7)		
AFB_2	10	6.4	63.6 (5.0)	y=0.621x+0.307	0.999
	20	12.8	63.9 (11.3)		
	5	5	100.2 (5.7)		
AFG_1	10	8.2	82.4 (5.1)	y=0.742x+1.105	0.998
	20	16	80.2 (11.5)		
	5	4.2	83.8 (8.1)		
AFG_2	10	7.4	74.4 (2.9)	y=0.723x+0.431	0.999
	20	15	74.8 (10.4)		

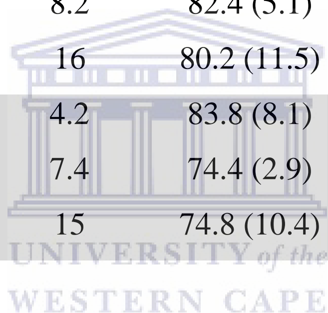
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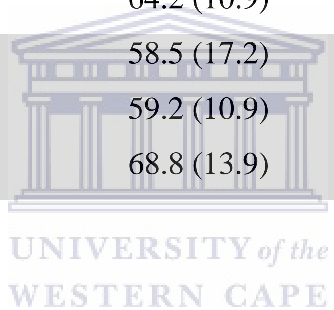
Table 5.5: Recoveries and precision of aflatoxin analogues in peanuts spiked with aflatoxin standard at different levels, n=3.

AF Analogue	Spiking level (µg/kg)	Recovery RSD (%)	Regression equation	R^2
	5	98.5 (10.9)		
AFB1	10	87.3 (6.7)	y = 0.811x+0.775	0.999
	20	85.2 (9.8)		
	5	80.5 (6.2)		
AFB2	10	61.7 (15.1)	y = 0.680x+0.120	0.984
	20	69.9 (24.1)		
	5	63.0 (10.2)		
AFG1	10	57.5 (15.5)	y = 0.654x-0.390	0.995
	20	64.2 (10.9)		
	5	58.5 (17.2)		
AFG2	10	59.2 (10.9)	y = 0.731x-0.995	0.996
	20	68.8 (13.9)		



5.5 Analysis of field samples

Eleven (24%) stored maize samples from VDM for both seasons were quantified with total aflatoxin from 1 - 52 µg/kg (Table 5.6). Five of thirty samples were found to be positive for aflatoxins whereas 4 out of the 5 positive samples were above both the maximum limit of 5 µg/kg for AFB_1 and 10 µg/kg for AFT, as set by the South African government (Rheeder et al., 2009). Only two out of six samples from the second season exceeded the limit of AFB_1 and AFT.

Table 5.6: Aflatoxin (µg/kg) contamination in maize samples collected from VDM in Limpopo over the two seasons (2011 and 2012) including maize from silos collected in November 2011.

Household no.s	AFB_1	AFB_2	AFG_1	AFG_2	Total AF	Household no.s	AFB_1	AFB_2	AFG_1	AFG_2	Total AF
	2011						2012				
1y	nd	nd	nd	nd	nd	**1(1)y**	nd	nd	nd	nd	nd
2y	nd	nd	nd	nd	nd	**1(2)y**	nd	nd	nd	nd	nd
3y	nd	nd	nd	nd	nd	**2y**	nd	nd	nd	nd	nd
4y(1)	19	2	57	9	87	**4y**	nd	nd	nd	nd	nd
4y(2)	nd	nd	nd	nd	nd	**5(1)y**	<loq	<loq	<loq	nd	<loq
5y	nd	nd	nd	nd	nd	**5(2)y**	<loq	nd	nd	nd	<loq
6y	nd	nd	nd	nd	nd	**5y**	<loq	nd	nd	nd	<loq
7y	nd	nd	nd	nd	nd	**6y**	4	<loq	13	<loq	19
8y(1)	nd	nd	nd	nd	nd	**7(1)y**	2	<loq	7	<loq	10
8y(2)	nd	nd	nd	nd	nd	**7(2)y**	<loq	<loq	1	<loq	<loq
9y	nd	nd	nd	nd	nd	**8y**	nd	nd	nd	nd	nd
10y	nd	nd	nd	nd	nd	**9y**	nd	nd	nd	nd	nd
11y	37	3	nd	nd	40	**10(1)y**	1	nd	<loq	nd	2
12(yw)	nd	nd	nd	nd	nd	**10(2)y**	<loq	<loq	nd	nd	<loq
13y	nd	nd	nd	nd	nd	**11y**	13	<loq	nd	nd	14
14y	nd	nd	nd	nd	nd	**12y**	nd	nd	nd	nd	nd
15y	nd	nd	nd	nd	nd	**13y**	nd	nd	nd	nd	nd
16y	28	2	<loq	<loq	30	**14y**	nd	nd	nd	nd	nd
17w(1)	nd	nd	nd	nd	nd	**15y**	<loq	<loq	3	<loq	3
17w(2)	nd	nd	nd	nd	nd	**16y**	nd	nd	nd	nd	nd
18y	4	<loq	nd	nd	4	**17y**	nd	nd	nd	nd	nd
19y	52	2	nd	nd	54	**18y**	<loq	<loq	nd	nd	<loq
09y(1)	nd	nd	nd	nd	nd	**19y**	17	<loq	22	<loq	40
09y(2)	nd	nd	nd	nd	nd						

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Household no.s	AFB$_1$	AFB$_2$	AFG$_1$	AFG$_2$	Total AF
			2011		
10y	nd	nd	nd	nd	nd
13y	nd	nd	nd	nd	nd
14y(1)	nd	nd	nd	nd	nd
14y(2)	nd	nd	nd	nd	nd
15y	3	<loq	nd	nd	3
20y	nd	nd	nd	nd	nd

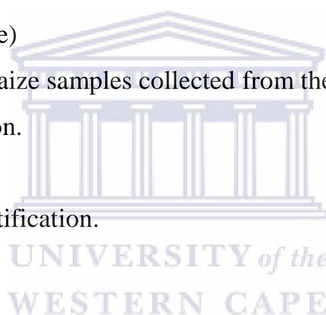
Y - Yellow maize

YW - mixed (yellow and white)

Highlighted area represents maize samples collected from the VDM during November 2011 after the July 2011 season collection.

Nd-Not detected

<loq - below the limit of quantification.



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One out of eight seed samples from VDM contained far less AFB$_1$ (3 µg/kg) and AFB$_2$ (0.4 µg/kg). Out of all maize from GSDM analysed for aflatoxin in the two seasons only six samples contained traces of aflatoxin below 0.6 µg/kg. Of the three maize foods sampled from the silo, one sample identified as 21R (0.6 µg/kg AFB$_1$) was contaminated with trace amounts of aflatoxin. Only 1/5 peanut samples collected from VDM was polluted with 3 µg/kg AFB$_1$ and 30 µg/kg AFG$_1$ in 2012. The peanut sample from GSDM did not contain aflatoxins.

5.6 Fumonisin chemical analysis

Fumonisins are analyzed by pre-injection derivatization. Once derivatized they have to be injected at room temperature within a 2 min period. The reason is that they have a short life time. The derivatized analogues become highly unstable

within a short time and rapidly breakdown into non fluorescent substances (Coppex, 2000).

5.6.1 Standard solutions

FB standards were obtained from the PROMEC Unit. Fumonisin stock standard solutions at concentrations of 256 µg/mL FB_1, 200 µg/mL FB_2 and 270 µg/mL FB_3 were diluted with 2.0 mL for FB_1, 2.5 mL for FB_2 and 2.0 mL for FB_3 of acetonitrile: water (1:1, v/v), to achieve concentrations of 55.04, 25.00 and 13.25 µg/mL working standard solutions for FB_1, FB_2 and FB_3, respectively. An *epi*-FB_3 that elutes prior to FB_3 on a HPLC chromatogram, occurs at levels of about 21 to 42%, (<20%) in FB_3 standard, but at lower levels than FB_3 (<20%) (Gelderblom et al., 2007).

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5.6.2 Experimental apparatus

A Uvikon 923 Double Beam UV/VIS Spectrophotometer (Bio-Tek icontron, U.S). Homogenizer was used, together with a Polytron PT 3100, Kinematica AG, Luzerne, Switzerland, an orbital shaker (Model STUART SSL1) from Karlsruhe, Germany, and a Model RC-3B refrigerated centrifuge from Sorvall, Bohemia, NY, USA. Extraction was carried out on an SPE manifold with 12 ports purchased from Supelco, Bellefonte, PA, USA. A Waters HPLC system was used: Model 515 isocratic pump, 1.0mL/min flow rate, fluorescence detector, 335nm excitation wavelength and 440nm emission wavelength. Agilent Chemstation - LC software was used for data collection system and processing. The chromatographic column used was a Phenomenex Luna 5µ C18 (2) (150 x 4.6 mm i.d.). The pH meter employed was a Beckman Model 70 or similar (calibrated).

5.6.3 Sample extraction and clean up using Solid Phase Extraction (SPE).

Representative, homogenized samples were analysed with slight modifications, by the method of Sydenham et al., 1996 which was used for sample extractions and

clean-up. A milled (20g) subsample in 100 mL of methanol-water (3:1, v/v) was homogenized at 5000 rpm for 3 min for maize, while peanut and bean samples were mixed (20g; 20 min) using an orbital shaker. The sample extracts were centrifuged at 4000 rpm for 10 min (20 min for beans and peanuts) at 4°C for extraction and the supernatant was filtered through MN 617 (185 mm) filter paper into a 250 mL Erlenmeyer flask.

An aliquot of the supernatant was adjusted to pH 5.8 - 6.0, depending on the ionic strength or the pH of the sample (Shephard, 1998, Sydenham et al., 1992) with 1M NaOH (or 1M HCl) to obtain better recoveries (Sydenham et al., 1992). Strong anion exchange cartridges (SAX, 500 mg packing Bond-Elut, Varian, Harbor City, CA, USA) were used for cleanup since this provides higher purification efficiencies (Sydenham et al., 1996). Cartridges on an SPE manifold were preconditioned with 5 mL MeOH followed by 5 mL MeOH: H_2O (3:1, v/v), eluted at <2.0 mL/min flow rate. An adjusted aliquot (10 mL) was purified on a SAX cartridge which offers greater purification over C18 columns (Stockenström et al., 1994; Visconti et al., 1996). Afterwards the column was washed with 5 mL MeOH: H_2O (3:1, v/v) then 3 mL MeOH (the SAX columns were always kept moist throughout), following an elution of analytes with 10 mL acetic acid: methanol (1:99, v/v) through gravity. Eluates were dried under nitrogen stream at <60°C in 4 mL glass vial and then stored at 4°C prior to analysis.

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5.6.4 Derivatization of samples and standards

Derivatization of fumonisins was achieved by a pre-column, *o*-phthaldialdehyde (OPA) derivatization reagent (prepared by dissolving 40 mg of OPA in 1 mL CH_3OH and 5 mL 0.1 M $Na_2B_2O_4$and diluted with 50 µl C_2H_6SO) that yields highly fluorescent compounds and forms easily isolated appropriate derivatives (Shephard et al., 1996; Shephard, 1998). FB standards were derivatized by using 20 µL of the fumonisin working standard containing concentrations of 55.04 µg/mL for FB_1, 25.00 µg/mL FB_2 and 13.5 µg/mL FB_3, respectively, mixing it with 200 µL OPA in a test tube, vortexing for about 30 seconds and directly injected into the HPLC system.

Dried samples were reconstituted with 200 µL of MeOH in a 4mL vial and vortexed for about 30 seconds. An aliquot of 50 µL was derivatised with 75 µL OPA and vortexed for about 30 seconds, then 20 µL of the mixture was directly injected into the HPLC system. Standards and samples were injected within an accurately determined time frame of two minutes after being mixed with the OPA. The syringe is cleaned with methanol several times after each injection.

5.6.5 Chromatography analysis

The reversed-phase HPLC separation was performed on a Phenomenex (Torrance, CA, USA) Luna C18 5µ particle size column (150×4.60 mm). The column was eluted isocratically at a flow rate of 1 mL/min with methanol: 0.1 M sodium di-hydrogen phosphate (78:22; v/v) mobile phase adjusted to pH 3.35 with ortho-phosphoric acid. The HPLC instrument was configured with an Agilent (Waldbronn, Germany) 1100 series fluorescence detector (FLD). Optimal FLD detection was set at excitation wavelength 335 nm and emission wavelength 440 nm. Derivatised fumonisin standard (10 µL) and sample (20 µL) were injected into the HPLC, within two minutes after adding the OPA. The quantification of each toxin was performed by comparing relevant standard calibration curve peak areas with sample peak areas.

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5.7 Method validation

Evaluation of the fumonisin method in maize, and peanuts was carried out with slight modifications according to Sydenham et al., 1996. Performance parameters for fumonisin recoveries, accuracy, linearity, repeatability (intra-day precision) and reproducibility (inter day precision), sensitivity and specificity were determined by spiking blank samples.

Commodities used as blanks (commercial) were analysed and the results were integrated in the recovery calculations. Blank samples before extraction (in dry form) were fortified with different concentration levels of 500; 1000; 5000 µg/kg for each FB analogue. The fortified blank samples were analysed and the results

were incorporated in the calculation for recoveries. Freshly prepared FB working standard solutions were prepared and injected in the HPLC.

5.7.1 Method performance

Elution order of the chromatograms when fumonisin working standards were injected in the HPLC were fumonisins B_1, epi-B_3, B_3 and then B_2. The stereoisomer *epi*-B_3 naturally occurs and separation from FB_3 at low levels when analysed using a RP-HPLC as reported by Gelderblom et al., 2007. The elution order and separation were effective, constant and reproducible from other matrix components. Retention times of FB_1, FB_2, *epi*-FB_3 and FB_3 were observed at 6.5, 14.7, 12.4 and 13.2 min (Figure 5.4), respectively, throughout the analysis.

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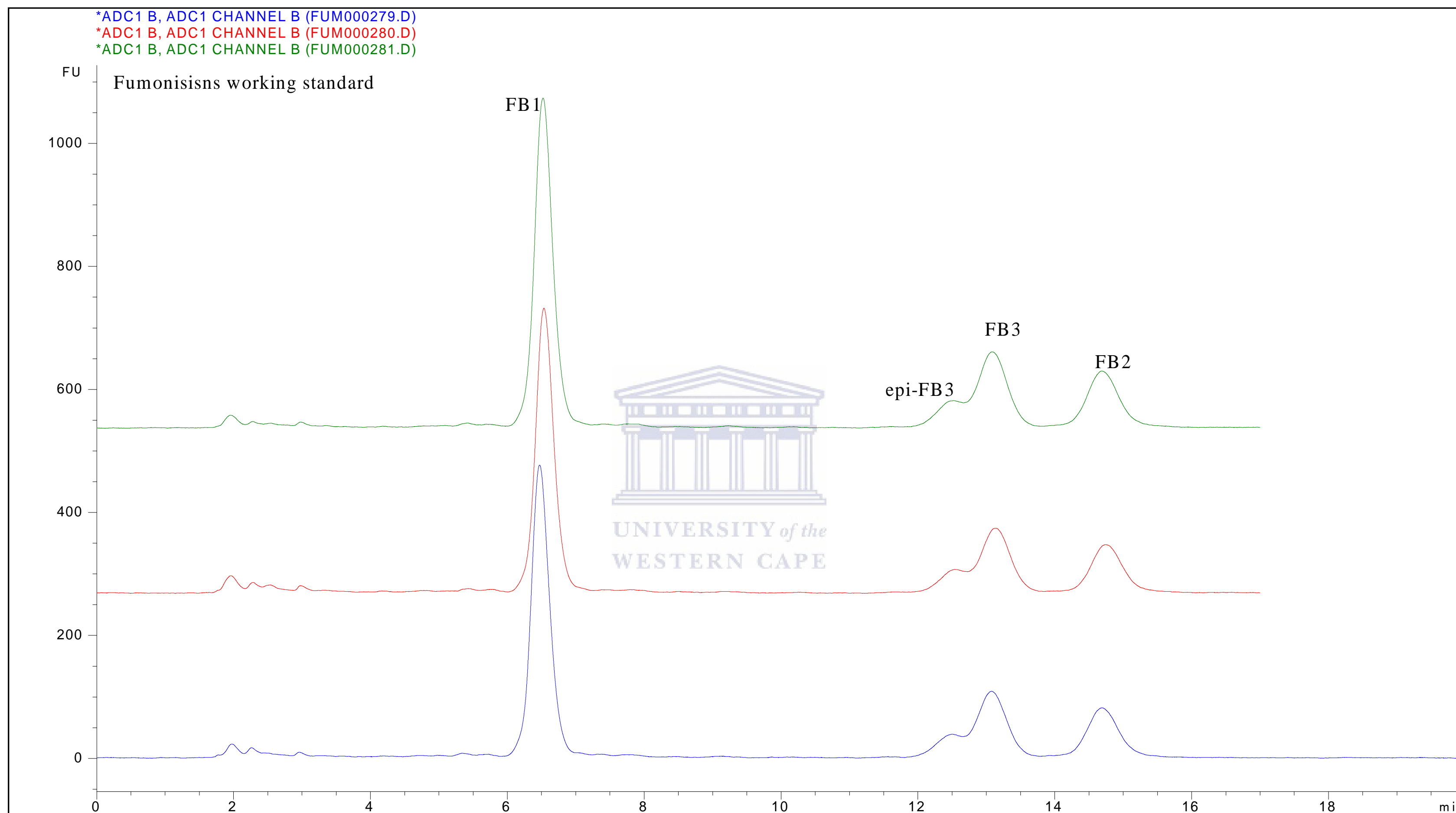

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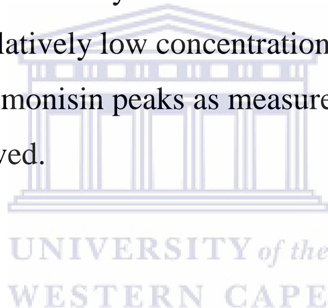
Figure 5.4: Three chromatograms of fumonisin B_1, *epi*-B_3, B_3 and B_2 working standard obtained for specificity

Method specificity was checked by injecting 20 µl of FB working standards (FB_1: 55.04, FB_2: 25.00 and 13.25 µg/mL for FB_3) in triplicate, respectively (Figure 5.4). This was achieved by comparing retention times of the matrix analyte and the reference standard. Sufficiently selective and specificity selectivity was obtained and there were no matrix interferences. Analytes were identified and quantified without any interference at the retention times of each chromatogram, resulting in good specificity of the analytical method.

A blank sample was analysed and the obtained chromatogram was overlaid with that of individual fumonisin B_1, B_2 and B_3 spiked samples (Figures5.5). Both (analysis of blank and standards) chromatograms demonstrated little background noise with no matrix interferences at the retention times of the analyte. Satisfactory separation of the analytes was achieved indicating good selectivity and specificity even at relatively low concentrations. Appropriate baseline resolution between the fumonisin peaks as measured using their retention times was generally well achieved.



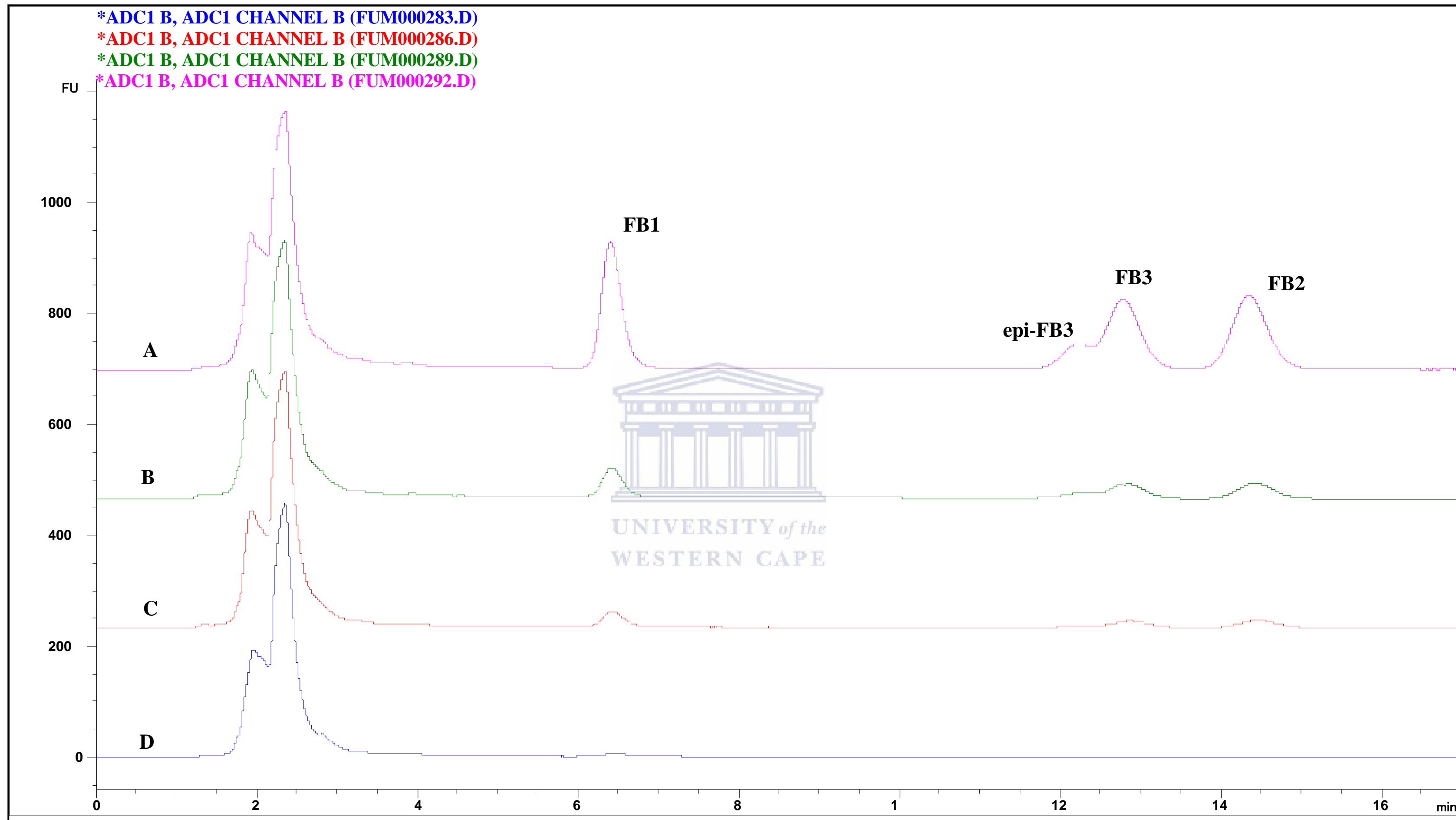


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Figure 5.5: Chromatograms of samples spiked with fumonisin working standard: (A) spiked at 5000 µg/kg; (B) spiked at 1000 µg/kg; (C) spiked at 500 µg/kg; and (D) blank sample.

5.7.2 Method precision and accuracy

The precision for fumonisin method was performed by measuring the intra-day and inter-day precision on working standards of 55.04 µg/mL, 25.00 µg/mL and 13.25 µg/mL for FB_1, FB_2 and FB_3, respectively. The intra-day data (n=3) was obtained by injecting three sequential FB working standards within one day and the inter-day was measured for five consecutive days. Results are stated in terms of relative standard deviation (RSD). Fumonisins intra- day and inter-day results ranged from 4.3 – 6.2% RSD, Table 5.7 and 5.0 – 7.6% RSD, Table 5.8.

Table 5.7: Intra-day precision: fumonisin working standards n=3, with standard peak areas.

	FB_1	FB_2	FB_3
Standard 1	9039	2560	4220
Standard 2	8839	2375	4118
Standard 3	9600	2690	4476
Mean	**9159**	**2542**	**4271**
Stdev	**395**	**158**	**184**
%RSD	**4.3**	**6.2**	**4.3**

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Table 5.8: Inter-day precision: fumonisin working standards (n= 12), expressed as standard peak areas.

	FB_1	**FB_2**	**FB_3**
	9039	2560	4220
Day 1	8839	2375	4118
	9600	2690	4476
	10207	3029	4853
Day 2	9796	2880	4645
	10471	3006	4894
	9935	2878	4666
Day 3	9802	2842	4639
	10005	3089	4804
	10454	3077	4908
Day 4	9703	2824	4591
	10019	2956	4740
Mean	**9822.5**	**2850.5**	**4629.5**
Stdev	**495.2**	**216.2**	**251.6**
%RSD	**5.0**	**7.6**	**5.4**

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The results obtained showed trueness of the method used by spiking blank maize, peanuts and beans samples at three levels (n = 9) with of 500, 1000 and 5000 µg/kg of each FB_1, FB_2 and FB_3 known standards. This was achieved by quantifying the percentage recoveries of each analyte which performed very well for each of the analytes.

5.7.3 Linearity

Determination of linearity was obtained as a result of the plot of fumonisins standards prepared in extracts of blank samples to construct a calibration curve as shown in Figure 5.6. This was performed and measured three times at different concentration levels where the calibration curves was determined by plotting the

response factor of the peak area versus the analyte concentration of 500, 1000 and 5000 µg/kg fumonisin B_1, B_2, and B_3.

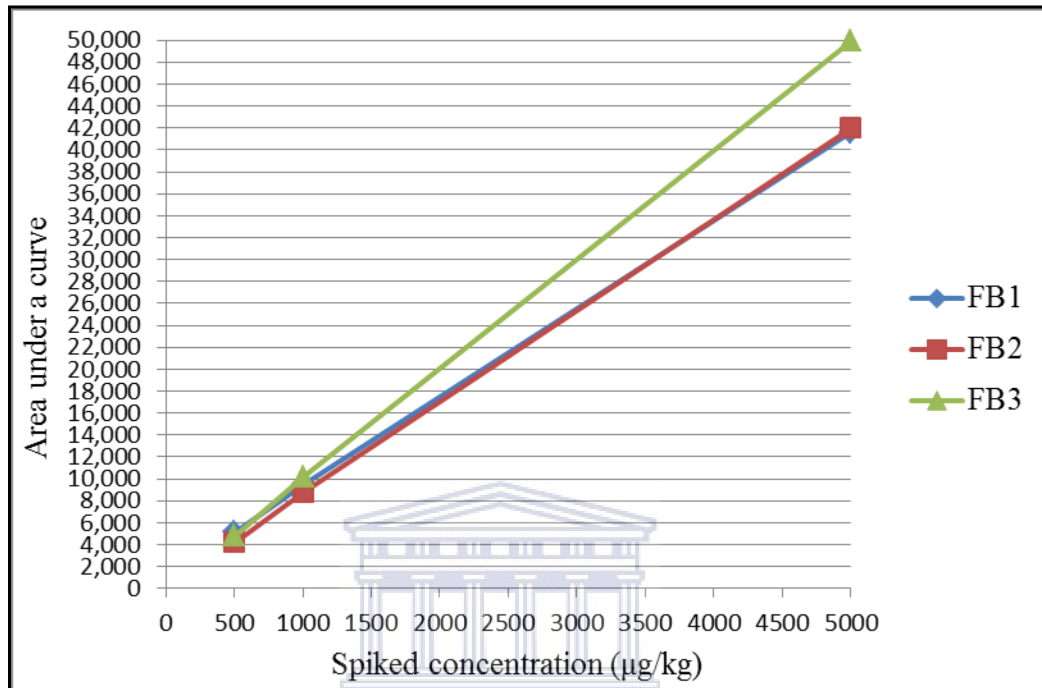

Figure 5.6: Linear graph for fumonisin spiked samples: at 500; 1000 and 5000 µg/kg.

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A regression coefficient (R^2) value for the fumonisins method with three different spiked concentration levels ranged from 0.9937 to 1.0 (Table 5.9), representing good linearity for all samples analysed. Individual linearity range for maize was 0.999; peanuts (1.0) and 0.9937 to 0.9987 for beans. A good quantification in fumonisin spiked samples was obtained. Detection limits (LOD) and quantitation limits (LOQ) were measured from signal to noise (s/n) ratio of the lowest fumonisin standard concentration in spiked samples. The LOQ was determined with 10 µg/kg for fumonisins.

Table 5.9: Recoveries and precision of each fumonisins analogues for maize and peanuts, spiked at different levels (n=3).

		Maize			**Peanuts**		
FB analogue	**Amount Spiked (µg/kg)**	**Recovery RSD (%)**	**Regression equation**	**R 2**	**Recovery RSD (%)**	**Regression equation**	**R^2**
	500	56.1 (2.5)			46.8 (0.5)		
FB_1	1000	58.5 (1.4)	y= 0.5643x+ 87.213	0.9999	44.8 (1.1)	y= 0.428x+22.673	1.0
	5000	56.6 (3.4)			43.2 (3.0)		
	500	84.4 (0.5)			67.1 (3.7)		
FB_2	1000	90.3 (2.3)	y= 0.8888x+20.875	0.9999	67.6 (5.5)	y= 0.656x+13.492	1.0
	5000	88.7 (1.8)			65.8 (5.3)		
	500	59.4 (2.7)			50.3 (4.9)		
FB_3	1000	63.8 (2.4)	y= 0.6319x-2.4889	0.9999	50.9 (3.7)	y= 0.485x+16.319	1.0
	5000	63.0 (2.7)			48.8 (3.2)		

*Maize blank (mean for FB_1 = 78.30, FB_2 = 25.82 & FB_3 = 4.81 µg/kg) were accounted for

*Peanuts blank samples were below the limit of quantification.

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5.7.4 Recoveries

Recoveries for fumonisins were obtained by spiking each dry milled commodity with individual toxins of each fumonisin standard solution at concentration levels of 500, 1000 and 5000 µg/kg as presented in Tables 5.9 and Table 5.10.

Average analyte recoveries produced ranged from 57. - 88% in maize and 45% to 67% in peanuts as shown on Table 5.9. FB_2 produced higher recoveries followed by FB_3 then FB_1 for both peanuts and maize commodities. In an AOAC-IUPAC Collaborative Study, average recoveries found in spiked blank maize ranged from 75% to 87%, with FB standard spiking solution of 100 to 8000 µg/kg) (Sydenham et al., 1996). For beans, recoveries were poor and ranged from 1.8 – 4.3% with equally poor precision (35 - 74%) (Table 5.10). Figure 5.5 show chromatograms of spiked samples with the blank samples and no apparent interferences.

Method development for analysis of fumonisin contamination of beans was discontinued due to time limitation. Beans are a poor matrix for fumonisins as natural contaminants, but can be infected with FB (Tseng et al., 1995a; Tseng et al., 1995b; Tseng & Tu, 1997). In a study by Scott et al. (1999), three types (white, adzuki and mung beans) of beans were not naturally FB contaminated but, the recovery of FB_1 and FB_2 from spiked (100ng/g) beans ranged from 60-98 depending on the extraction solvent. In a study by Siame et al. (1998) for the analysis of fumonisins, aflatoxins, and zearalenone in beans, peanuts, peanut butter used as food and for feeds in Botswana, no fumonisins were quantified in peanuts, and beans.

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Table 5.10: Recoveries and precision of each fumonisin analogue for beans spiked at different levels (n=3).

FB analogue	Amount Spiked (µg/kg)	Recovery RSD (%)	Regression equation	R 2
	500	3.6 (46)		
FB_1	1000	2.7 (35)	y= 0.029x+1.0663	0.9987
	5000	2.9 (41)		
	500	3.1 (45)		
FB_2	1000	1.8 (45)	y= 0.020x+1.757	0.9937
	5000	2.1 (43)		
	500	4.3 (61)		
FB_3	1000	2.4 (58)	y= 0.020x+8.133	0.9946
	5000	2.1 (74)		

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Commercial maize and peanut samples were used as blanks in the experiment and analysed for fumonisins. They were found to contain low levels of fumonisins and thus no true analytical blank was available for this study since it has been reported that fumonisins occur naturally and cannot be completely eliminated (Summerell and Leslie, 2011).

5.8 Analysis of field samples

Forty five stored maize samples collected in the first season (July 2011). Eight stored homegrown maize samples were also collected for the period of November 2011. Quantifiable levels of fumonisins in homegrown maize intended for human and animal consumption from VDM for both seasons were detected in 98% (44/45) of the samples collected (Table 5.11).

Table 5.11: Fumonisins concentrations (μg/kg) in maize samples collected from VDM in Limpopo over the two seasons (2011 and 2012) including maize from the silo collected in November 2011) using an HPLC.

Household no. s	**FB_1**	**FB_2**	**FB_3**	**Total FB**	**Household no.s**	**FB_1**	**FB_2**	**FB_3**	**Total FB**
	2011					**2012**			
1y	54	13	<loq	72	**1(1)y**	633	217	53	903
2y	42	14	<loq	55	**1(2)y**	22	<loq	<loq	31
3y	27	13	<loq	42	**2y**	18	<loq	<loq	25
4y(1)	530	126	32	688	**4y**	122	30	<loq	161
4y(2)	134	42	<loq	183	**5(1)y**	118	42	21	180
5y	275	79	19	373	**5(2)y**	84	25	<loq	116
6y	30	<loq	<loq	48	**5y**	144	40	<loq	184
7y	597	152	37	786	**6y**	66	27	<loq	98
8y(1)	6853	3104	734	10691	**7(1)y**	86	26	<loq	111
8y(2)	2025	1196	167	3388	**7(2)y**	25	<loq	<loq	33
9y	14	<loq	296	311	**8y**	1244	561	135	1940
10y	<loq	<loq	<loq	<loq	**9y**	116	43	<loq	167
11y	1857	498	226	2581	**10(1)y**	10	<loq	<loq	10
12(mix)	191	33	15	239	**10(2)y**	<loq	<loq	<loq	<loq
13y	<loq	<loq	<loq	<loq	**11y**	421	105	37	563
14y	<loq	<loq	<loq	<loq	**12y**	218	56	19	294
15y	78	29	8	115	**13y**	38	19	11	68
16y	520	178	80	778	**14y**	766	251	65	1081
17w(1)	159	85	<loq	249	**15y**	43	15	<loq	58
17w(2)	<loq	<loq	<loq	<loq	**16y**	<loq	<loq	<loq	<loq
18y	369	102	47	518	**17y**	73	30	<loq	102
19y	44	13	<loq	65	**18y**	156	46	10	213

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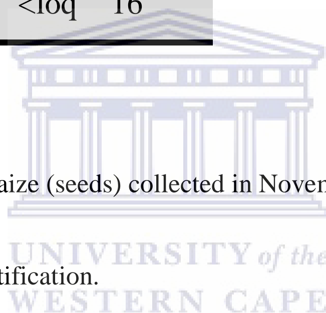
Household no. s	FB_1	FB_2	FB_3	Total FB
		2011		
9y(1)	11	<loq	<loq	11
19y	436	103	41	579
9y(2)	96	19	<loq	115
10y	13	<loq	<loq	13
13y	15	<loq	<loq	15
14y(1)	<loq	<loq	<loq	<loq
14y(2)	94	22	<loq	119
15y	<loq	<loq	<loq	<loq
20y	16	<loq	<loq	16

W - white maize

Y- yellow maize

Highlighted area represents maize (seeds) collected in November 2011after the July 2011 collection.

<loq - below the limit of quantification.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Twenty nine (29) out of thirty (30) samples contained FB_1 (ranging from 0 - 6853 µg/kg) during season one and 23/23 with the range of 9 - 1244 µg/kg in season two. During 2011 and 2012 July season's maize from VDM was contaminated with FB_1 at a range of 0 to 6853 µg/kg (total fumonisin (FUMT) ranged from 2 – 10691 µg/kg) and 0-1244 µg/kg (9 -1940 µg/kg FUMT) respectively (Table 5.11) with no significant difference ($p > 0.05$) between the seasons in FB_2 and FB_3 as well. Contamination level in maize collected in November ranged from 0-96 µg/kg.

In 70% (42/60) of maize samples from GSDM households over both seasons (2011 and 2012), fumonisins were contaminated in a range of 0 to 5724 µg/kg (Table 5.12). Three samples from the silo were also sampled in November 2011

and the average ranged from 30 to 6692 μg/kg FB_1. Fumonisins in all prepared maize samples were present in 16/31 (ranging from 0 - 3498 μg/kg); and 28/31 with the range of 0 - 5724 μg/kg.

Table 5.12: Fumonisins (μg/kg) detected in home-grown maize collected from GSDM in Mpumalanga over the two years (2011 and 2012), including maize collected in November 2011.

Household no.s	**FB_1**	**FB_2**	**FB_3**	**Total**	**Household no.s**	**FB_1**	**FB_2**	**FB_3**	**Total**
		2011					**2012**		
1w	12	<loq	<loq	18	**1(1)w**	<loq	<loq	<loq	<loq
2w	3499	349	<loq	3848	**1(2)w**	62	25	<loq	88
3w	<loq	<loq	nd	<loq	**3w**	<loq	<loq	nd	<loq
4w	<loq	<loq	nd	<loq	**4w**	<loq	<loq	nd	<loq
5w	212	148	22	583	**5w**	13	<loq	nd	13
6w	108	67	15	190	**7w**	<loq	<loq	nd	<loq
7w	<loq	<loq	nd	<loq	**8w**	14	<loq	nd	14
8w	<loq	<loq	<loq	<loq	**9w**	5724	4976	1161	11861
10w	2050	878	61	2989	**10w**	32	14	<loq	46
13w	19	<loq	<loq	19	**12w**	91	24	16	130
14w	414	165	18	597	**13w**	10	<loq	nd	10
15w	<loq	<loq	nd	<loq	**14w**	<loq	<loq	nd	<loq
16w	<loq	<loq	nd	<loq	**15w**	12	<loq	nd	12
17w	<loq	<loq	nd	<loq	**16(1)w**	12	<loq	nd	12
18w	177	17	<loq	198	**16(2)w**	<loq	<loq	nd	<loq
19w	<loq	<loq	nd	<loq	**17(1)w**	<loq	<loq	nd	<loq
20w	129	48	21	199	**17(2)w**	<loq	<loq	nd	<loq
3y	49	14	<loq	62	**18w**	12	<loq	nd	12
4y	178	69	<loq	246	**19(1)w**	63	22	8	93

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Household no.s	FB_1	FB_2	FB_3	Total	Household no.s	FB_1	FB_2	FB_3	Total
		2011					**2012**		
5y	<loq	<loq	nd	<loq	**19(2)w**	25	9	<loq	35
6y	880	463	102	1445	**20w**	14	<loq	nd	14
8y	<loq	<loq	nd	<loq	**21w**	23	<loq	nd	23
9y	<loq	<loq	nd	<loq	**2y**	11	<loq	nd	11
11y	<loq	<loq	nd	<loq	**3y**	17	<loq	nd	17
13y	<loq	<loq	nd	<loq	**5y**	6	<loq	nd	6
12y	15	<loq	<loq	15	4y	<loq	<loq	nd	<loq
13y	<loq	<loq	nd	<loq	5y	6	<loq	nd	6
16y	<loq	<loq	nd	<loq	**8y**	<loq	<loq	nd	<loq
17y	<loq	<loq	nd	<loq	**9y**	<loq	<loq	nd	<loq
19y	<loq	<loq	<loq	<loq	**11y**	<loq	<loq	<loq	<loq
21-M	30	<loq	<loq	30	**13y**	11	<loq	<loq	11
21-K	230	73	<loq	311	**17y**	<loq	<loq	nd	<loq
21-R	6692	2946	1147	10785					

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W - white maize

Y- yellow maize

Highlighted area represents maize samples collected from the GSDM Community Silo during November 2011 after the July 2011 season collection.

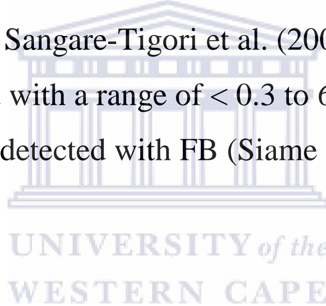
Nd-Not detected

<loq – below

In the year 2012, maize sample number 9 was the only sample highly contaminated with FB (5724 µg/kg B_1, 4976 µg/kg B_2 and 1161 µg/kg B_3). The rest of the detected samples were contaminated at low levels (< 100 µg/kg FB_1) and both FB_2 and FB_3 were ≤ 25 µg/kg.

In the analysis of peanut samples, from five peanut samples collected from VDM two were found to contain fumonisins at very low levels (39 µg/kg FB_1 and 17 µg/kg FB_2; the other with only 9 µg/kg FB_1). The two peanut samples contaminated were sampled (2/3) during the second season at different places from the same VDM household. However in GSDM only one household had enough peanuts to be sampled in 2011 with concentration of 21 µg/kg FUMT and 7 µg/kg FB_1 detected.

Peanuts are generally not contaminated with fumonisins but fumonisins can be associated with stored peanuts as a result of cross contamination. There were 45% *Fusarium spp.* isolated fungal species compared to 24% *Aspergillus* in peanuts from Nairobi (Kenya) markets (Figure 5.7.) but these species identified were *F. oxysporum*, *F. equiseti* and *F. torulosum* which are not FB producers (Gachomo et al., 2004). In a survey by Sangare-Tigori et al. (2006), 7/10 peanut samples from Cote D'ivoire were found with a range of < 0.3 to 6 mg/kg FB_1 where as peanuts from Botswana were not detected with FB (Siame et al., 1998).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5.9 Statistical data analyses

Since the maize data analyses performed for all 7 parameters (FB_1, FB_2, FB_3, AFB_1, AFB_2, AFG_1, and AFG_2) had skewed distributions and no transformations were possible, non-parametric analyses techniques were used. The non-parametric Wilcoxon Rank Sum Test was used to analyse the data obtained on fumonisin and aflatoxin content in stored maize samples quantified by an independent instrument (HPLC) in 2011and 2012 seasons. Statistical significance was calculated to compare the results at the 5 % significance. Differences in each parameter are statistically significant only if the levels are $p < 0.05$. A statistical analysis was performed at the Medical Research Council (MRC) by the Biostatistics Unit.

5.10 General results and discussion

Maize from one household in VDM was quantified with maximum FB_1 at 6853 µg/kg (2011); Figure 5.8 and 1244 µg/kg (2012). This sample represents a typical VDM sample with the highest FB in all tested samples. The same household number 8 from VDM was observed with samples which were highly contaminated with fumonisin during both seasons (6853 µg/kg, 2011 and 1244 µg/kg, 2012) due to poor storage. On average, maize during the first season was quantified with 469 µg/kg fumonisins B_1 (741 µg/kg FUM) while season two was detected with 211 µg/kg FB_1 (302 µg/kg FUM).

Maize products (1000 µg/kg) intended for human consumption, 4000 µg/kg for unprocessed maize and 200 µg/kg for maize-based foods and baby foods are various fumonisin limits set by the European Union (EU) as maximum tolerated limit (MTL) (EC, 2007, Kimanya et al, 2012). There are no maximum limits set for fumonisins in South Africa. Four samples were detected with fumonisin levels above the maximum limit of 1000 µg/kg set by the EU, with three samples from the first season and only one in the second season. For fumonisin exposure, the US Food and Drug Administration (FDA) has recommended levels to be put in place to decrease FB in food and feed and to also reduce human exposure to FB (FDA, 2001a; FDA, 2001b). Depending on maize use, regulations by the FDA are at maximum levels of 2 - 4 mg/kg (total of B_1, B_2 and B_3) for human consumption (FDA, 2001). According to the FDA regulations 95% of the contaminated maize samples in season one (2011) and 100% during 2012 exceed the maximum limit of 2 mg/kg.

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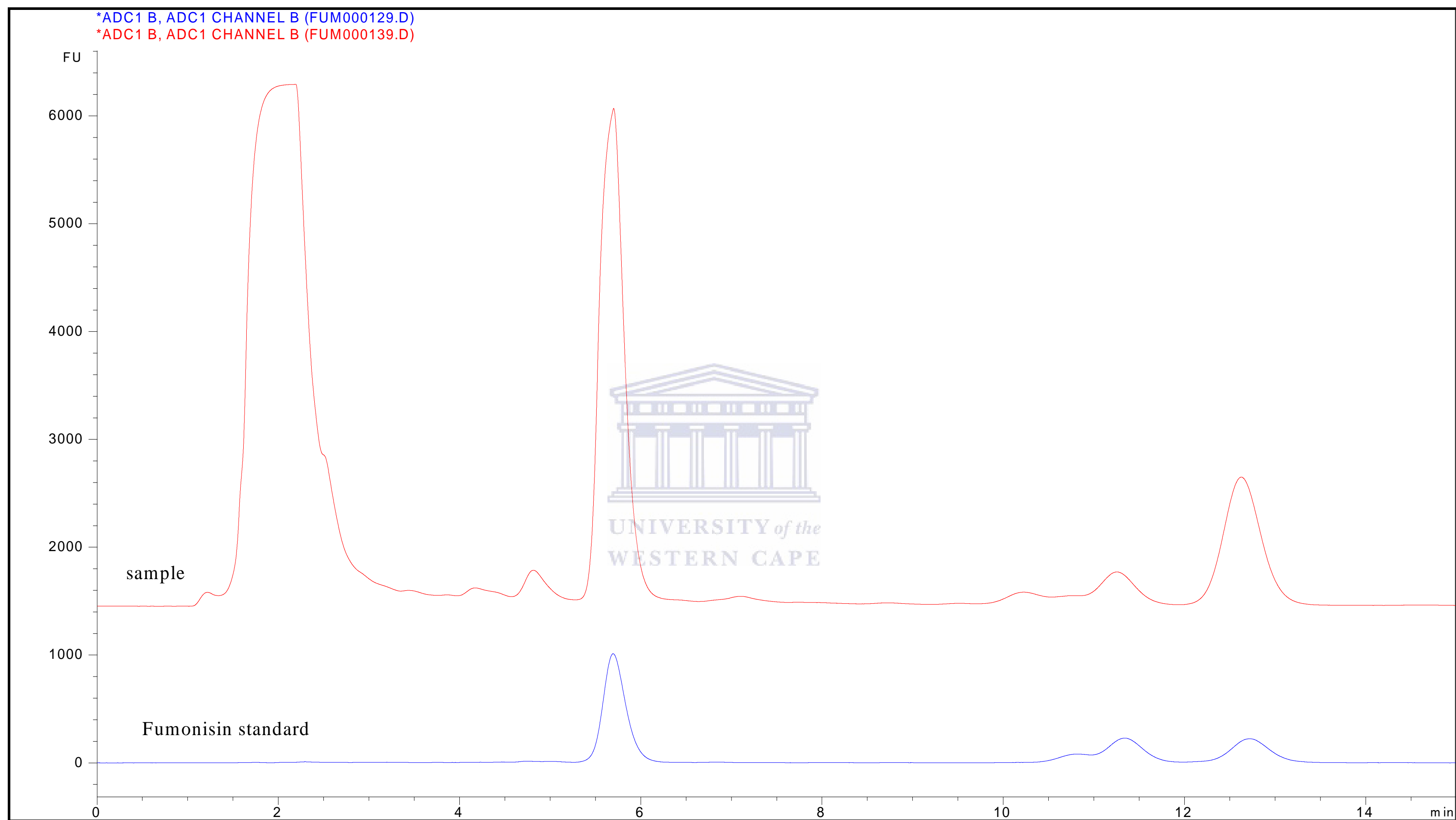

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Figure 5.7: Chromatogram of the home-grown maize from VDM observed with the highest FB (6853µg/kg) in the first season compared to the FB standard.

Aflatoxin contaminated samples (24%) from VDM for both seasons were quantified with aflatoxin from 1 - 52 µg/kg. Six out of the eleven contaminated samples exceeded the maximum regulated limit of AFB_1 (5 µg/kg) and AFT (10 µg/kg) limit set by South Africa with three 20 µg/kg set as a maximum by the FDA (Abbas et al., 2012). Four of five samples contaminated with AF from season one exceeded both AFB_1 and total aflatoxin ranging from 4 µg/kg to 52 µg/kg. A maximum level (ML) of AFB_1 (2 µg/kg) and 4 µg/kg total aflatoxin intended for direct human consumption has been set by the European Union (EU) for nuts and cereals (EC, 2006) while in baby food products it set at very low levels of 0.10 µg/kg AFB_1 (EC, 2004).

Two (13 µg/kg and 17 µg/kg) of six samples from the second season exceeded the limit of AFB_1 and total AF. Maximum AFB_1 detected was 10-times higher (52 µg/kg) than the SA maximum permitted level of 5 µg/kg FB_1. Most (4/6) of the highly contaminated samples were encountered in the first season with an average concentration of 28µg/kg AFB_1 and 43µg/kg AFT, while in 2012 AFB_1 was less frequently detected at 6 µg/kg (15 µg/kg total AF). In a study done from Malawian household by Matumba et al. (2009) it was found that in 45.3% AFB_1 detected in stored maize only 12.3% (106 samples) exceeded the 5 µg/kg AFB_1 MTL set by FAO, 2004.

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All highlighted eight maize samples on Table 5.11 stored as seeds by the VDM households in November 2011 showed FB_1 levels ranging from 3 µg/kg to 96 µg/kg FB_1 far below the EU regulated limits. In comparing the seeds and maize sample collected in July, fumonisin contamination varied but did not differ. The reason might be the storage of seed, they were stored in enclosed plastic bottles when they were sampled. In most African countries, smallholder farmers greatly depend on previous harvest to keep as seed, farmer-to-farmer sales and informal market and in Southern Africa they access 10% of seeds from formal markets (Smale et al., 2009; Baiphethi & Jacobs, 2009). The previous harvest seeds have been known in many countries to be suitable if stored appropriately but, the

productivity of agricultural crops will be greatly increased by the use of good quality seeds (Baiphethi & Jacobs, 2009).

Highest FB_1 level from GSDM was 3499 µg/kg (3848 µg/kg FUMT) in 2011 (Figure 5.9) and in 2012 (5724 µg/kg; 11861 µg/kg FUMT). These were different households which show that some household may either have improved their storage or some worsened in FB contamination. One was detected at low levels for the second sample and the other the FB contamination has increased. Average levels of FB_1 occurrence in maize were (267 µg/kg, 2011) and 0 - 215 µg/kg (year 2012) without any significant difference ($p > 0.05$) between seasons but marginally significant ($0.05 < p < 0.10$) for FB_2 and FB_3.

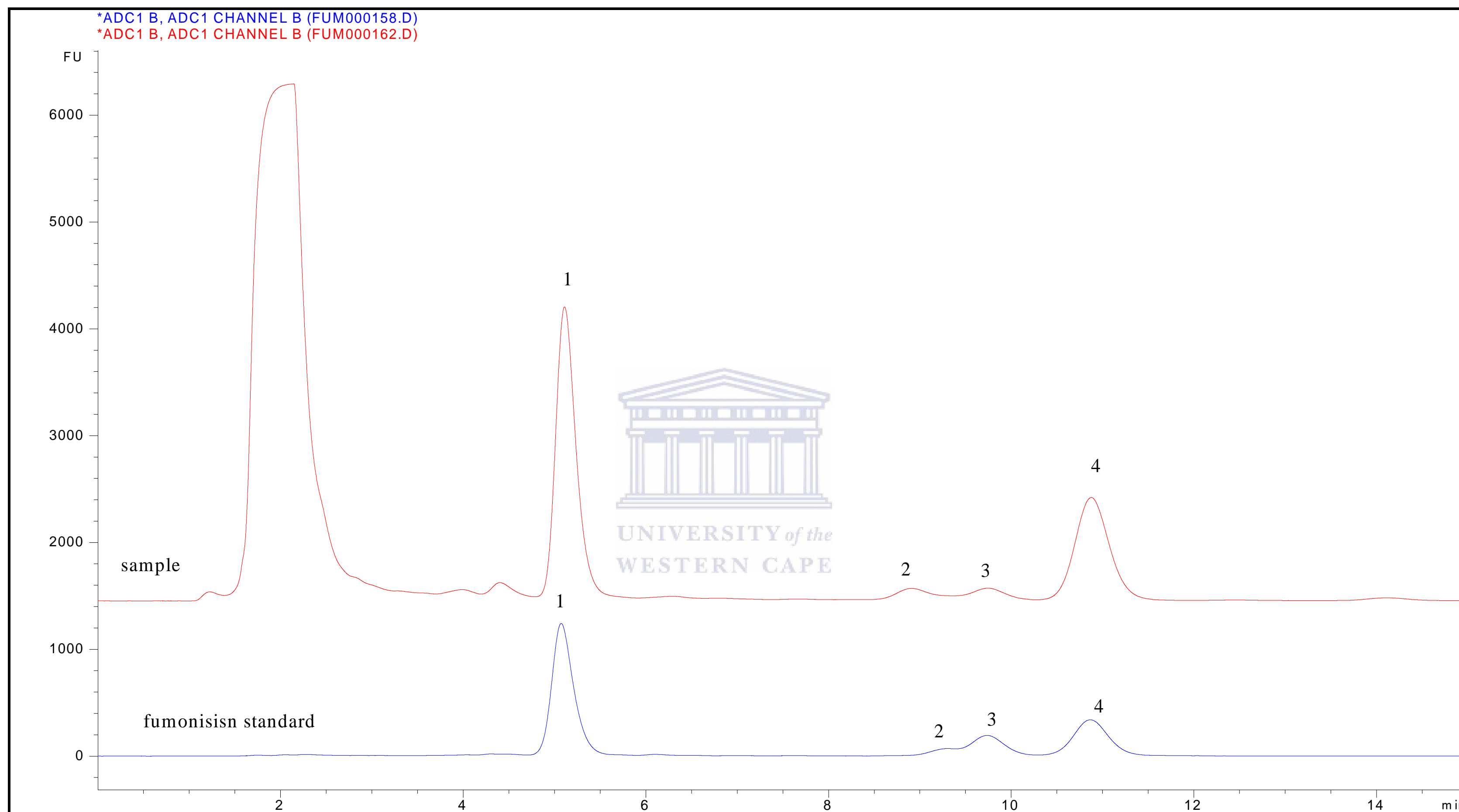

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Figure 5.8: Highest fumonisins:(1)B_1, (2)*epi*-B_3, (3) B_3 and (4) B_2 detected from contaminated home-grown stored maize from GSDM (3499 µg/kg) in the first season compared to the FB standard.

Two maize samples in the first season and one sample during 2012 exceeded the EU MTL of 1000 µg/kg, but 55% and 90% of samples exceeded the US FDA limit of 2 mg/kg, respectively. The 2012 sample exceeded the EU MTL by more than 5-times while the other two samples were exceeded two and three times.

All three silo samples were recovered with fumonisins ranging from 30 - 6692 µg/kg (30-10785 µg/kg total FB range) (Table 5.12). Two of three samples from GSDM collected in November showed low FB contamination. However, sample 21R (6692µg/kg FB_1 and 10785µg/kg, FUMT) was collected from the silo in GSDM which contained mouldy maize. This was as a result of the silo not being ventilated appropriately. The reason was that communities could not afford to keep it ventilated continuously.
Contamination of the peanuts with FB from both households maybe due to cross-contamination where the households kept the peanuts in the same sack previously used to store maize or in the same storage area. Maize in the two households was found with considerable levels of FB concentrations. One peanut sample from five collected from VDM was detected below the South African limit of 5µg/kg AFB_1 and a peanut sample from GSDM was not detected with aflatoxin.

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All peanut samples detected with aflatoxins were predominantly found with AFB_1. The prevalence of AFB_1 in peanuts has also been reported by Kamika (2012) in peanuts sold at markets in Kinshasa, Democratic Republic of Congo, and Pretoria, South Africa. In another study by Alam et al. (2010), it was reported that in food and feed products, AFB_1 generally predominates. Recently in other countries, studies have shown AFB_1 exceeding the 20 µg/kg U.S. maximum limit in over 86% of 29 peanut cake samples examined from Nigeria (Ezekiel et al., 2012). In Benin, fifteen peanut samples were detected with a total aflatoxins concentration content of 10 µg/kg to 346 µg/kg (Ediage et al., 2011). None of the beans collected over 2011 and 2012 during both seasons from both districts were quantifiable with fumonisins and aflatoxins.

5.10.1 Variety of maize collected from Mpumalanga (GSDM)

Two maize types were sampled in this study, a) white (planted for human consumption) and b) yellow (planted as animal feed) (Table 5.13). White (n=17) and yellow (n=12) maize samples were collected in the first season and in the second season, white (n=22) and yellow (n=9) maize samples were collected. Quantification of FB in the two varieties of maize varied. During season one, 9/17 white and 5/12 yellow maize samples were observed with FB contamination in comparison to 19/22 white and 8/9 yellow maize samples of season two.

Mean levels of FB_1 detected in white maize edible for humans were found in a concentration of 389 µg/kg (maximum 3498 µg/kg), year 2011 and 308 µg/kg (maximum 5724 µg/kg), year 2012 as shown in Table 5.13. Animal feed (n= 12 yellow maize) was found with a mean concentration of 93 µg/kg FB_1; with a maximum of 880 µg/kg in 2011 and 8 µg/kg FB_1 (n= 9; 17 µg/kg) in 2012.

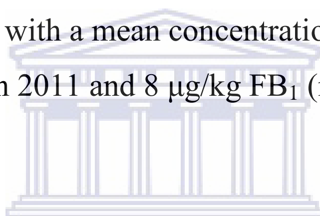

Table 5.13: HPLC results on average distribution of fumonisins (µg/kg) levels in white and yellow home-grown maize produce intended for human (wm) and animal (ym) consumption collected of both seasons from Mpumalanga (GSDM).

Maize varieties	**year**	**Fumonisin** (µg/kg)			**Total**
		FB_1	**FB_2**	**FB_3**	
Yellow maize	2011	93	45	9	147
	2012	<loq	<loq	nd	<loq
White maize	2011	389	78	29	508
	2012	308	230	54	564

Nd- not detected

<loq - below the limit of quantification

All highly FB contaminated maize (exceeding the FB MTL established by the European Commission by more than 2-times) samples collected during the two

seasons were observed in white maize. All contaminated animal feed samples were (range 1-880 µg/kg) below the 1000 µg/kg EU regulation limit.

Average detection levels of all analogues in all maize were observed with variations between the two seasons as shown in Table 5.14. In this regard, FB contamination from GSDM ranged from 20 to 267 µg/kg in the year 2011 and 38 to 215 µg/kg in 2012. All maize samples were recovered at a mean range of 77 - 628 µg/kg (2011) and 18 – 211 µg/kg (2012) below the EU MTL of 1000 µg/kg as shown on Table 5.14 but a few individual samples (n=4) exceed the MTL. AF contaminated samples (23% of the samples) in 2011 had higher levels of contamination than (26% of samples) in 2012. Mean concentrations of FB_1 and AFB_1 contained in stored home-grown maize from Limpopo in 2011 showed a reduced concentration in the 2012 season.

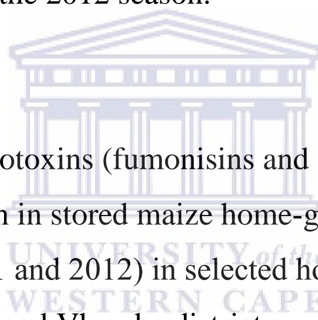
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Table 5.14: Average mycotoxins (fumonisins and aflatoxin) (µg/kg) contamination in stored maize home-grown maize over two seasons (2011 and 2012) in selected household's villages of Gert Sibande and Vhembe districts.

	GSDM			**VDM**		
	2011		**2012**	**2011**		**2012**
	µg/kg	**p-value^{a}**	**µg/kg**	**µg/kg**	**p-value^{a}**	**µg/kg**
FB_1	267^{a}	0.4592	215^{a}	628^{a}	0.8478	211^{a}
FB_2	65^{b}	0.0860	164^{b}	258^{a}	0.9278	72^{a}
FB_3	20^{b}	0.0704	38^{b}	77^{a}	0.1454	18^{a}
AFB_1	<loq	*	<loq	28.1^{a}	0.3784	6.2^{a}
AFB_2	<loq	*	<loq	2.0^{a}	0.8259	<loq
AFG_1	<loq	*	<loq	11.4^{a}	0.0070	7.7^{a}
AFG_2	<loq	*	<loq	1.8^{a}	0.1046	0.3^{a}

[a]p - value – Statically comparing the two years in each province

*Samples from GSDM were no statically analysed as most of the samples were not contaminated

with aflatoxin.

*No statistically significant differences recovered between two years (p>0.05).

*Marginal statistically significant differences recovered between two years (0.05< p>0.10).

*<loq - below the limit of quantification

Mycotoxin concentrations in maize between the two growing seasons were measured for fumonisins and found to have no significant variation in the levels of FB_1, FB_2 and FB_3 in maize samples from Limpopo. The *p*-values measured for fumonisin content in VDM between the two years were 0.8478 FB_1, 0.9278 FB_2 and FB_3 (0.1454). Quantification of fumonisins in maize from GSDM between the growing seasons was not significantly different ($p > 0.05$) for FB_1 ($p = 0.4592$). However, the contamination levels were marginally significantly different ($0.05 < p < 0.10$) between the two years for FB_2 ($p = 0.0860$) and $p = 0.0704$ FB_3.

In the two seasons, few maize samples (n = 4, VDM and n = 3 GSDM) were quantified with FB at extremely high levels. These samples far exceeded the maximum limit of 1000 µg/kg fumonisins set by the EU MTL. The maximum permitted level of FB in food has not yet been set in South Africa. Even though only a few maize samples from VDM were contaminated with aflatoxin, most of those observed far exceeded the maximum regulated limits set by SA and the EU. One of the households had extremely high concentration levels of 52 µg/kg AFB_1. The matrix, maize was detected more with AF than peanuts. In a recent study of mycotoxins in food and feed from Burkina Faso Mozambique, aflatoxin was mostly found in maize than peanuts (Warth et al., 2012). In Tanzania and the republic of Congo, maize was the main source of aflatoxin contamination compared to cassava and was found to be a chronic problem in those areas (Manjula et al., 2009).

Maize samples from Limpopo were prevalently recovered with FB and AF compared to samples from Mpumalanga. Fumonisins and aflatoxins recovered from maize samples in Limpopo was statistically significantly different ($p < 0.05$) at values of < 0.0001 when compared to contamination levels of Mpumalanga for

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each year and for both years combined. Insect damage in stored maize causes fungi infection and may increase mycotoxin contamination (Meikle et al., 2002). Aflatoxin and fumonisin are the two mycotoxins which are mostly associated with insect injury (Dowd, 2003). Maize from VDM was visually infested with *Sitophilus zeamais* and it was stored directly from field into the polystyrene bags with no further drying. This fact, in all likelihood, caused the higher levels of aflatoxin and fumonisin contamination. GSDM maize was further dried in aerated wooden cribs after harvest which is why it is less contaminated with mycotoxins although the storage facilities were exposed to extreme conditions such as the sun and rodents.

Fumonisin results in this study correspond with the recent outcome of the study done by Ncube et al. (2011) in comparing FB levels in maize from Eastern Cape, KwaZulu-Natal, Limpopo and Mpumalanga provinces of South Africa. In that study Zululand (range 1.3 - 10.9 µg/kg) and Limpopo (range 0.5 - 3.3 µg/kg) were shown to have higher levels of fumonisins compared to Mpumalanga (maximum 0.7 µg/kg) and the Eastern Cape (maximium 2.6 µg/kg) in the growing seasons 2006 and 2007.

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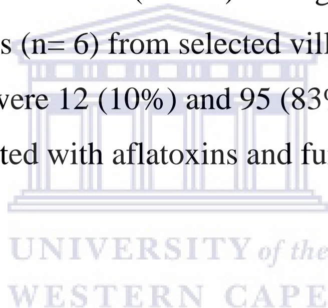
Levels of contamination might also be as a result of lack of knowledge on health effects caused by these mycotoxins, crop rotation, appropriate drying and storage methods to be used, and the importance of early harvesting (Negedu et al., 2011). Climatic conditions (temperature and relative humidity) also affect the growth of fungi and mycotoxin production. Temperatures of 20 - 25°C have been reported by Mogensen et al. (2009) to be the highest for production of fumonisins. High production of aflatoxin occurs at 33°C and even up to high temperatures of 37°C (EMANb). During the harvest seasons of 2011 and 2012, temperatures in the field were observed to range from 18°C to 30°C and 17 - 30°C respectively for the two seasons with maximum rainfall of 434 mm and 202 mm, and relative humidity of 33% to 93% and 35% to 87% in Limpopo also for the same two seasons.

In Mpumalanga temperatures of between 15 - 33°C and 14 - 32°C were measured while relative humidity ranged from 31 - 91% and 29 - 88% with 221 mm and 206 mm maximum rainfall being measured over both seasons 2011 and 2012. This shows that both provinces have climatic conditions which are susceptible for fumonisin and aflatoxin production in the field even with the limited rainfall which was noted to have decreased in the 2012 season. However poor storage methods could be the reason for elevated contamination levels in the 2012 season.

Fumonisin contaminated samples were substantially greater in maize compared to peanuts from both areas and in both 2011 and 2012. Greatly contaminated and very high FB_1 (6853 µg/kg; 2011) levels in maize were frequently found in VDM. The suitability of the methods were assessed for natural contamination of fumonisins and aflatoxins in maize (n=116) homegrown as food and/or feed, beans (n= 15) and peanuts (n= 6) from selected villages in Vhembe and Gert Sibande districts. There were 12 (10%) and 95 (83%) maize and peanut (17% and 50%) harvests contaminated with aflatoxins and fumonisins, respectively.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5.11 Conclusion

The efficiency of the HPLC method has been demonstrated by the excellent chromatographic separation of the analogues and linearity with repeatable injections for stored maize and peanut samples. Even though recoveries for FB_1 were found to be lower and FB_2 found to be higher in all spiked samples, they were still within the acceptable range. These results thus established that the methods were successfully validated.

In this present research study, the quantitative results obtained indicated that Vhembe subsistence farmers experience higher aflatoxin and fumonisin contamination in their stored home-grown crops compared to Gert Sibande. No beans or peanut samples from Mpumalanga were found to be contaminated with both toxins above the regulation limit. The Vhembe district had only a few peanut

samples that were contaminated with AF and FB. The FB levels of maize contamination between sample types (white and yellow), demonstrated that white maize was more contaminated in comparison to yellow maize. Significant different levels of infection with fumonisins and aflatoxins in stored home-grown maize reported between Limpopo and Mpumalanga in this study in terms of the amount recovered is related to their different storage facilities and practices. This also shows the relevance of this study and future regular monitoring studies on the natural occurrence of mycotoxins in staple foods stored in different traditional storage forms of rural areas in South Africa.

None of the ‘seed’ (maize collected in November 2011) obtained showed quantifiable aflatoxin levels while fumonisins were quantified in all samples, but were below the limit regulated by the EC and FDA. These samples were kept as seeds for the next season and had no apparent visual mould contamination. However, some samples contained low levels of fumonisins (lower than the lowest regulated limit by the European Commission (EC) of 200 μg/kg for maize-based baby foods) and a high percentage of *A. flavus* contamination. This indicates that mycotoxin production does not only occur on visually mouldy crops.

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Inhabitants of both districts might be at risk with fumonisin contamination as they mostly consume and depend on home-grown maize. Fumonisins and aflatoxin were mostly found in maize as compared to other commodities. Therefore, comprehensive data on the severity and quantity of mycotoxin contaminated in home-grown commodities using highly sensitive and selective methods could provide clear evidence on the level of contamination.

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CHAPTER 6

Multi-toxin quantification of homegrown agricultural produce in selected rural areas of Mpumalanga and Limpopo

6.1 Introduction

Inhalation or ingestion of mycotoxins may cause health problems (Pitt 1996). Food or feed matrices can be contaminated by several different mycotoxins. Traditionally, matrices were analysed for one compound at a time from an analytical point of view (Stroka et al., 2000; Shephard et al., 1990; Entwisle et al., 2000; Cahill et al., 1999). Recently however, there have been multifunctional sample clean-up columns developed to determine more than one mycotoxin simultaneously (Göbel and Lusky, 2004; Chan et al., 2004).

Liquid chromatography-triple quad mass spectrometry (LC-MS/MS methods have become widely used analytical techniques (Sulyok, et al., 2006; Spanjer, et al., 2008) for the analysis of mycotoxins. It has been used for detection and quantification of food and feed contaminants such as mycotoxins in a broad spectrum (Shephard, 1998; Spanjer et al., 2008; Streit et al., 2013). Recently the LC-MS/MS methods have been developed and validated to detect and quantify simultaneous multiple mycotoxins, pharmaceuticals and pesticides of different matrices in one extract (Sulyok, et al 2006; Mol et al. 2008; Wille et al 2010; Romero-González et al., 2011; Sulyok et al., 2010). LC-MS/MS allows for sensitive and specific multi-mycotoxin analysis without time consuming sample preparation i.e. clean-up extraction, pH modification and pre-concentration of analytes because of the diverse chemistry involved (Shephard, 2008; Richard et al., 1993). In this study LC-MS/MS with matrix-matched calibration standards (for maize analysis) and standard addition methods (for beans and peanut analysis) (Mol et al. 2008; van Asselt et al., 2012) were used to validate the HPLC methods.

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6.2 Materials and methods

Samples were subsampled and analyzed using a validated multi-toxin analysis method for feed (Van Asselt et al., 2012) to quantify for mycotoxins in selected commodities.

6.2.1 ***Chemicals and reagents*****:** All chemicals and reagents used in this study were of LC analytical grade and deionized water was purified with a Millipore Milli-Q Plant system. Methanol, acetonitrile and LC/MS-grade water were obtained from Biosolve (Valkenswaard, the Netherlands). Acetic acid was obtained from Merck (Darmstadt, Germany). Formic acid (CH_2O_2; 99-100%), ammonium formate (NH_4CO_2H) and internal standard (IS) ^{13}C-Caffeine (10µg/ml; 99%, $C_8H_{10}N_4O_2$) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

6.2.2 ***Apparatus:*** Samples were milled to 1mm particle diameter using a Retsch® mill (Haan, Germany). Samples were weighed in 50ml polypropylene, Greiner centrifuge tubes from Sigma-Aldrich (Zwijndrecht, The Netherlands). A horizontal shaker device purchased from Edmund Bühler SM30 control, Hechingen, Germany was used for the shaking stages. An MSE Falcon 6/300 laboratory centrifuge purchased from Lower Sydenham, London, UK was employed for all centrifuge work. Autosampler vials with a built-in syringeless filter device (Mini-UniPrep, 0.45 μm, Whatman, Forham Park, NJ) were used to remove any possible solid materials from the final extract. The vials were closed using a six position compression pressing equipment (Whatman,'s-Hertogenbosch, The Netherlands). A Restek Ultra Aqueous C18, 3 µm, 100 x 2.1 mm i.d. LC column obtained from Restek Corporation, Bellefonte, USA was used in the chromatographic stages of purification. A Shimadzu LC Prominence system (Shimadzu, 's-Hertogenbosch, The Netherlands), connected to a triple quadrupole mass spectrometer AB SCIEX QTRAP® 5500 obtained from Applied Biosystems, The Netherlands was used for the MS/MS measurements.

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6.2.3 ***Reference Standards*****:** Mixed mycotoxin standard solutions containing 37 individual mycotoxins each were purchased from the following commercial suppliers: Biopure, (Romerlabs, Austria); Sigma-Aldrich (Zwijndrecht, The Netherlands) and from MRC, PROMEC unit, Cape Town, South Africa. Multi-component standards were prepared in acetonitrile from authentic reference standards. From this stock solution, different working solutions were prepared for calibrations and these multi-analyte standard solutions were termed (1v) with 10

mycotoxin components and (2v) consisting of 27 mycotoxin components. These multi-analyte working standards were subsequently used in the analysis of maize and legumes. The reference analytes were of utmost purity, with greater than 95% total purity. All mycotoxin standard solutions and stocks were stored in amber vials at +4 °C in the dark with a shelf life of six months (Appendix 5.1). Before use, the standards were brought to room temperature.

6.3 Multi-mycotoxin methods

All stored samples were homogenized and milled to a less than 1 mm particle diameter. They were prepared and analyzed with no chemical derivatisation in a wide range of analytes with different polarities. Samples were analyzed using two different methods according to the sample type being analyzed.

6.3.1 ***Matrix matched standard*** (maize samples)**:** Matrix-matched standards were used to compensate for matrix effects in the analysis. Linear calibration curves were prepared by spiking a blank matrix extract with increments of known analyte concentrations. During the LC-MS/MS analysis, MMS4 sstandard was used as a bracketed calibration solution.

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6.3.2 ***Standard addition method*** (legumes samples)**:** This procedure is used to determine the quantitation of an analyte in a complex matrix. This involved a test (unspiked) sample divided in 3 or more even aliquots where one was analysed as a blank sample. Others were spiked with increasing known concentrations of analytes. This was done to construct a calibration curve. Fumonisins were spiked in dry samples before extraction and the supernatant was spiked with aflatoxin after extraction due to differences in extraction efficiency of both mycotoxin groups.

Matrix effects like ionization suppression differ from matrix to matrix, and are well known to commonly occur in LC-MS/MS. They can be caused by

compounds of different origins and may have a negative effect on quantitative method performance characteristics.

6.4 LC-MS/MS Instrument

Analyses were performed using an LC-MS/MS with a TurboIonSpray electrospray ionization (ESI) ion source for determination of analytes. Separation was performed on a Restek, Ultra Aqueous column with a flow rate of 0.4 mL/min and maintained at a column temperature of 35 °C. The instrument was comprised of an online degasser, pump, auto-sampler spectrometer (maintained at 11 ˚C) and column oven. Samples were injected at 5µL resulting in 15.0 min run time. This method analysis was set-up to simultaneously separate and quantify 27 mycotoxins in a single injection. The analytical instrument was connected to the ANALYST® software. Vials were positioned in a sequence of 8 sample vials and bracketed by the fourth matrix matched calibrant (MMS4) (section 6.5).

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Electrospray Ionization (ESI) was performed for all mycotoxins using selective and sensitive multiple reaction monitoring (MRM) transitions, both in positive and negative polarities. For each sample, two separate chromatographic runs were observed by scanning two fragmentation reactions per analyte. The following parameters were used: source temperature 400 ˚C; curtain gas 10 psi; collision gas (argon) medium; ion spray voltage (IS) of -5000 V and +5000 V (depending on the ionization mode); ion source gas1 (sheath gas) 35 psi and ion source gas 2 (drying gas) 40 psi. Analysis in an MRM mode per analyte was performed both as qualitative and as quantitative determination.

6.5 Maize sample analysis

6.5.1 Matrix-Matched Standard (MMS) Calibration preparation

An MMS calibration curve was prepared from a previously analysed blank matrix sample (2.5g ± 0.02g) and was extracted and made up as in procedure 6.5.2. Six

aliquots (250 µl) from the control blank matrix extract were used to prepare six MMS reference solutions (table 6.1). The extracts were spiked at different concentrations with multi-analyte standard solutions (125 µl (1v): 500 µl (2v): 1375 µl H_20; v/v/v), and diluted with dilution solvent (625 µl 84% ACN + 1375 µl H_2O; v/v) as described in appendix 5.2 to obtain different MMS concentration levels as illustrated in Table 6.1. Dilution and extraction solvent contain acetonitrile (ACN) instead of methanol, for the reason that ACN can reduce the co-extraction of sample matrix components than MeOH (Kokkonen, 2011).

Table 6.1: MMS series of fumonisins and aflatoxins concentrations (ng/ml) final dilution in the vials.

MMS range	FB_1	FB_2	FB_3	AFB_1	AFB_2	AFG_1	AFG_2
MMS0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MMS1	1.25	1.25	1.25	0.625	0.625	0.625	0.625
MMS2	2.50	2.50	2.50	0.125	0.125	0.125	0.125
MMS3	6.25	6.25	6.25	0.3125	0.3125	0.3125	0.3125
MMS4	12.5	12.5	12.5	0.625	0.625	0.625	0.625
MMS5	25.0	25.0	25.0	1.250	1.250	1.250	1.250
MMS6	62.5	62.5	62.5	3.125	3.125	3.125	3.125

Fumonisin B_1, FB_2 and FB_3
Aflatoxin B_1, B_2, G_1 and G_2

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In the analysis sequence, MMS4 (0.625 ng/mL) was repeatedly used as a quality control standard (analysed after every 6-8 injections) (Mol. et al, 2008). The MMS range was analysed for linearity response versus concentration.

Spiking of test samples was done to determine the recoveries of the specific analytes. This was achieved by weighing approximately three blank samples each of 2.5g ± 0.02g spiked with appropriate amounts at low concentrations levels and

1.0g ± 0.02g spiked at high levels with aflatoxin and fumonisin into separate 50ml plastic Greiner tubes as shown on Table 6.2.

Table 6.2: Quality Control sample preparation

	BL-L	L_{-1}	L_{-2}	BL-H	H_{-1}	H_{-2}
Mass (g)	2.5	2.5	2.5	1	1	1
Aflatoxin B_1, B_2, G_1, G_2 (µg/kg)	N/A	1	1	N/A	5	5
fumonisin B_1, B_2, B_3 (µg/kg)	N/A	20	20	N/A	100	100
Extraction solvent	10	10	10	4	4	4
Spike solution - (Iv) µl	N/A	25	25	N/A	50	50
Spike solution - (2v) µl	N/A	100	100	N/A	200	200

BL - Blank samples

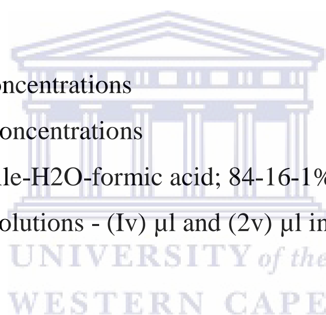
$L_{-1\&2}$ - two low levels spike concentrations

$H_{-1\&2}$ - two high levels spike concentrations

Extraction solvent - (acetonitrile-H2O-formic acid; 84-16-1%; v/v/v)

Multi-analyte standard spike solutions - (Iv) µl and (2v) µl in appendix 5.1.

N/A - Not Applicable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The samples were spiked and diluted in different volumes as listed in Table 6.2, and subsequently prepared as outlined in section 6.5.2 for the shaking stage of the samples on the horizontal shaker onwards.

6.5.2 Extraction and sample preparation

Approximately 2.5 g ± 0.02 g of each maize sample was weighed in duplicate into 50ml plastic Greiner tubes.

Internal Standard (25 µl) followed by 10 ml of extraction solvent (acetonitrile: H_2O: formic acid; 84:16:1%; v/v/v) was added and the resulting mixture was mixed by hand end-over-end. Samples were subsequently thoroughly mixed on a horizontal shaker at 200 cycles / min for 2 hrs, and then centrifuged (3100 rpm; 10

min). A 250 µl aliquot extract from each sample was transferred into auto-sampler vials and diluted with 250 µl dilution solvent (26.25% (v/v) of acetonitrile in H_2O) then capped and vortexed for approximately 3 seconds. After appropriate mixing, samples were stored at 4 °C for 30 minutes. Samples were then filtered by using a six position compression pressing equipment. Subsequently samples were stored at 4 °C until the next day. Before injection samples were brought to room temperature. Finally, 5 µ extract was injected into the LC-MS/MS.

6.5.3 LC-MS/MS separation

Chromatographic separation was performed on a Restek Ultra Aqueous C18, 3 µm (100 x 2.1 mm i.d.) at 35 °C with 5 µL injection volume. Mycotoxin separation was achieved using a gradient elution composed of Mobile phase A and Mobile phase B at 0.4 ml/min flow rate.

***Mobile phase* A**: 100% H_2O (1 L) with 1 mM ammonium formate and 10 ml of 1% formic acid.

***Mobile phase* B**: H_2O: methanol, (5:95; v/v) containing 1 mM Ammonium formate and 10 ml of 1% formic acid.

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Gradient composition started at 100% A from 0 – 1 min and in 2 – 3 min elution was equal at 50% A and 50% B. The proportion of B was increased linearly to 100% within 10 min. Finally, it was switched back to 100% A over 10.5 – 15 min followed by an equilibration of 2 min before the next injection. Equilibration was carried out by running a blank solution, mycotoxin standard termed 'standard C' and sequentially bracketing with MMS4 after every 6-8 injection. Retention times and specific analyte sensitivity were examined.

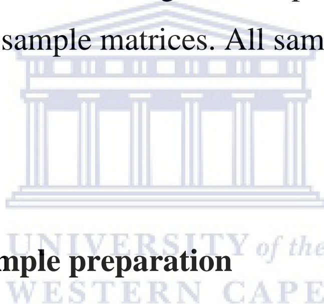
6.6 Peanuts and beans sample analysis

6.6.1 Standard Addition Method preparation:

In using this method for bean and peanut samples, a previously known blank

(beans and peanuts) sample was obtained and weighed into six sub samples. It was fortified at different concentration levels of known mycotoxin analytes before and after extraction due to different extraction efficiencies. The concentrations were quantified with a non-spiked sample.

Recoveries for each mycotoxin were determined by spiking at different levels of standard analyte. To 2.5 g of blank matrix weighed into six separate plastic Greiner tubes as follows; (i) two samples were spiked with 125 µl multi-analyte standard (1v) (containing 100 µg/kg total FB) before extraction then 500 µl multi-analyte standard (2v) (containing 5 µg/kg AFT) after extraction. (ii) Two other samples were spiked with 250 µl standard (1v) (100 µg/kg total FB) before extraction and 10 µl standard (2v) and 190 µl H_2O after extraction with no internal standard added. The remaining two samples were treated as blanks and were analysed before the sample matrices. All samples were extracted as in section 6.6.2.



6.6.2 Extraction and Sample preparation

Homogenised matrices were weighed in duplicate at 2.5 g ± 0.05 g into 50ml Greiner tubes, and an extraction solvent (10 ml, acetonitrile-H_2O-formic acid; 84-16-1%; v/v/v) was added. One of the duplicate samples was fortified with 125µl of multi-analyte standard (1v) (containing 100 µg/kg FUM) before adding an extraction solvent. Then in all samples, 25µl of Internal Standard (10 µg/ml) was added. The mixtures were first manually thoroughly mixed end-over-end, followed by 200 cycles / minute on a horizontal shaker for 2 hours, and centrifugation at 3000 rpm for 10 minutes. The extracted supernatant (200 µl) from the fortified samples were transferred into auto-sampler vials and spiked with 10 µl of multi-analyte standard (2v) (5µg/kg total AF: H_2O, 10 µl:190 µl, v/v) and an additional 190µl H_2O was added.

Unspiked aliquots (200 µl) were diluted with 200 µl dilution solvent (1 ml 84% ACN in 20 ml H_2O, v/v) into Whatman auto-sampler vials. Final volumes (400 µl)

of both spiked and unspiked samples were capped and thoroughly mixed for approximately 3 seconds and refrigerated at 4-8 °C for 30 min. Samples were thereafter filtered by using the six position compression pressing equipment for analysis. Samples were then stored at 4 °C until the next day. Before analysis, they were brought to ambient temperature and subsequently, 5 µl of the extract was injected into the LC-MS/MS system.

6.6.3 LC-MS/MS separation

Chromatographic separation was carried out at 35 °C and a flow rate of 0.4 ml/min using a RESTEK Ultra Aqueous C18, 3 µm (100 x 2.1 mm i.d.). Two elution solvents were used, Mobile phase A contained 1 mM ammonium formate and 1% (v/v) formic acid in water and Mobile phase B contained1 mM Ammonium formate and 1% (v/v) formic acid in H_2O: methanol, (5:95; v/v).

Mobile phase composition and the gradient were carried out as in section 6.5.3 with the injection volume of 5 µL. For column equilibration, a solvent and a standard C solution were analyzed before and after the quality control samples. Sequentially, standard C was repetitively analysed after every 6-8 injections of sample matrices.

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6.7 Data processing

All the LC-MS/MS data acquired were processed using the ANALYST®–MultiQuant software (AB SCIEX) and quantified using Microsoft™ Excel to assess mycotoxin occurrence in the samples analyzed. Quantitation of analytes was performed against matrix matched standards. Matrix interferences were greatly reduced due to the MRM mode transitions. All mycotoxins were identified according to retention time and the product ion ratio between two transitions; the quantification and the confirmatory (qualifier).

6.8 Statistical processing of data

Quantification data obtained from the maize analysis was subjected to the statistical analyses where non-parametric analyses techniques were used. Natural mycotoxin (fumonisin and aflatoxin) concentrations found in home-grown maize from Vhembe and Gert Sibande districts over two years (2011 and 2012) were compared in order to evaluate the difference in contamination levels in each FB and AF parameter. All individual content levels were compared between the districts and the years (2011 and 2012) using the non-parametric Wilcoxon Rank Sum Test with statistical significance at 5%. Individual values between the two years and two districts using the LC-MS/MS were significantly differend if $p<0.05$.

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6.9 Quantification of fumonisins and aflatoxin

The limit of quantitation (LOQ) and the limit of detection (LOD) were determined from a signal to noise (s/n) ratio of the lowest matrix-matched standard concentration in spiked samples and differed between aflatoxin and fumonisins. The LOQ was determined at 10:1 signal-to-noise (*s/n*) ratio, with 10 µg/kg for fumonisins and 1.0 µg/kg for aflatoxins.

Recoveries were calculated by comparison of the response obtained for each mycotoxin detected with that of known spiked mycotoxin levels, expressed as a percentage. Method performance characteristics in appendix 5.3 lists recoveries and regression coefficients observed at the range of 90 to 104%; R^2 = 0.9992 to 0.9999 for aflatoxins and 85 to 95%; R^2 = 0.9993 to 0.9997 for fumonisins. Both MMS calibration curves of fumonisins and aflatoxins proved to be linear across both calibration ranges(appendix 5.4).

6.10 Analysis of stored commodities in selected rural areas of Vhembe District.

Fumonisins levels in maize collected from selected Vhembe district areas in July (n=45) and November (n=8) were detected in 91% (48/53; 0 - 13203 µg/kg total fumonisins) of the samples over the two years combined (Table 6.3). Contamination rate was 20/22 in season one (2011) and 100% (23/23,) in the second season (2012).

Table 6.3: Fumonisin (µg/kg) concentrations in maize samples collected from Vhembe district municipality in Limpopo over the two seasons using LC-MS/MS detection.

Household no. s	**FB_1**	**FB_2**	**FB_3**	**Total FB**	**Household no.s**	**FB_1**	**FB_2**	**FB_3**	**Total FB**
	2011					**2012**			
1y	152	33	38	223	**1y(1)**	949	335	59	1342
2y	98	17	10	126	**1y(2)**	51	18	<loq	78
3y	22	<loq	<loq	22	**2y**	37	12	<loq	53
4(1)	964	181	107	1251	**4y**	133	42	19	193
4(2)	140	21	<loq	161	**5y(1)**	10	<loq	<loq	15
5y	352	98	56	506	**5y(2)**	147	31	31	208
6y	78	12	34	123	**5y(3)**	161	71	20	252
7y	773	237	119	1129	**6y**	82	38	13	132
8y(1)	8514	3654	1035	13203	**7y(1)**	127	36	<loq	172
8y(2)	1575	743	158	2476	**7y(2)**	33	12	<loq	48
9y	75	37	14	126	**8y**	1584	671	168	2422
10y	<loq	<loq	<loq	<loq	**9y**	148	45	24	216
11y	1502	539	238	2279	**10y(1)**	10	<loq	<loq	15
12y	220	49	44	313	**10y(2)**	14	<loq	<loq	20

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Household no. s	FB$_1$	FB$_2$	FB$_3$	Total FB	Household no.s	FB$_1$	FB$_2$	FB$_3$	Total FB
	2011					2012			
13y	15	<loq	<loq	15	11y	522	138	56	715
14y	14	<loq	<loq	14	**12y**	400	100	57	556
15y	38	<loq	<loq	38	**13y**	103	34	11	147
16y	851	231	115	1197	**14y**	1241	361	100	1701
17w	69	30	<loq	99	**15y**	77	23	10	110
17w	<loq	<loq	<loq	<loq	**16y**	15	<loq	<loq	23
18y	1193	270	193	1655	**17y**	108	39	14	161
19y	174	87	67	328	**18y**	195	67	25	286
9(1)y	51	14	<loq	65	**19y**	640	173	110	923
9(2)y	351	101	27	480					
10y	<loq	<loq	<loq	<loq					
13y	<loq	<loq	<loq	<loq					
14(1)y	12	<loq	<loq	12					
14(2)y	52	11	<loq	62					
15y	<loq	<loq	<loq	<loq					
20y	49	10	<loq	60					

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Y - Yellow maize

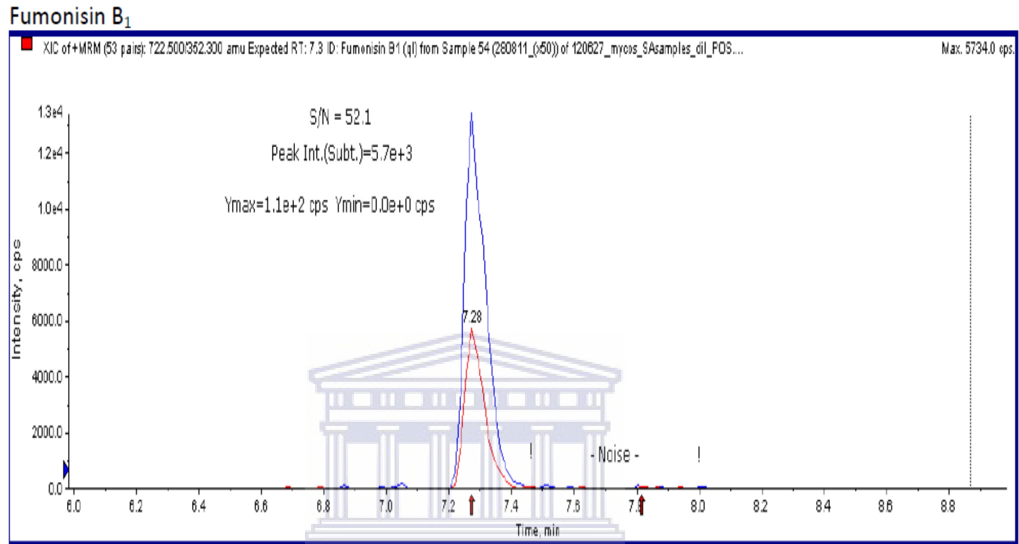
W - White maize

*Highlighted area - samples collected in November 2011 from households with enough samples to be collected after the July 2011 collection.

<loq - below the limit of quantification.

Levels of contamination ranged from 12 µg/kg to 8514 µg/kg the first year and 10 - 1584 µg/kg in the second year. There were no significant differences ($p > 0.05$) between the two seasons for all fumonisin B1 ($p = 0.6439$), B$_2$ ($p = 0.8300$)

and p = 0.8122 for B_3 content. Maize samples from the same household (no. 8) contained high amounts of FB_1 and FB_2 for both seasons. It was found to be 8514 µg/kg as illustrated with Figure 6.1, over year 2011 and 1584 µg/kg for 2012 season.



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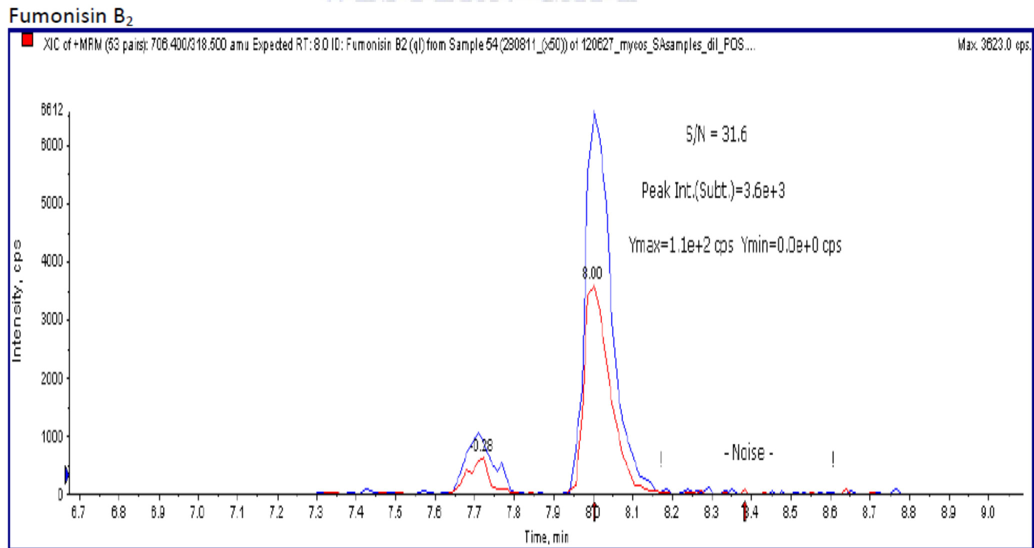

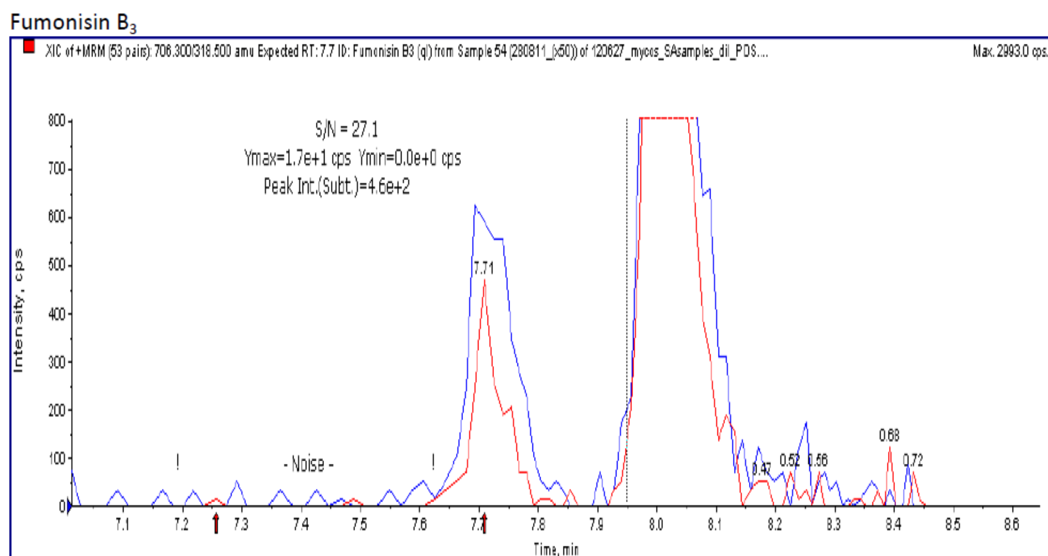

Figure 6.1: Extracted fumonisin ion chromatograms obtained from LC-MS/MS analysis of highly quantified yellow maize sample no. 8 found during season one (2011) from VDM. FB_1: 8514 µg/kg, 3654µg/kg FB_2 and 1035 µg/kg FB_3.

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In South Africa currently, there are no legal regulation guidelines or maximum limits set in place for fumonisin contamination in food or feed. Therefore, an EU guideline of 1000 µg/kg (sum of FB_1 and FB_2, EC/1881/2006) for maize products intended for human consumption was used as a maximum regulatory limit in this study (EC, 2007). FDA has regulations of 2000 - 4000 µg/kg maximum levels allowed in maize (van Egmond et al., 2007). Baby (infants and young children) foods (maize-based foods) are regulated at lower fumonisin limits of 200 µg/kg (EC, 2006). In subsequent years, four and two maize samples exceeded the EU guideline of 1000 µg/kg (sum of FB_1 and FB_2). The most contaminated samples exceeding the accepted levels ranged from 1193 - 8514 µg/kg which were observed as FB_1 in the 2011 and 2012 seasons. Of the maize 'seeds' (n = 8) collected in November 2011, 63% were quantified with FB levels ranging from 12 to 351 µg/kg. The highest level detected was 351µg/kg FB_1, which is still below the respective European maximum limit of 1000 µg/kg in maize.

Twenty nine % of maize samples collected from Limpopo were contaminated with aflatoxin (maximum 133 µg/kg AFB_1) (Appendix 5.5). Aflatoxin B1 contamination ranged from 1-133 µg/kg (n = 6/22) and 1 – 73 µg/kg (n = 7/23) with no significant difference ($p > 0.05$) between 2011 and 2012, respectively. Aflatoxin analogue B_1, with the p-value of 0.3784, B_2 ($p = 0.8259$) and G_2 ($p = 0.1046$) were measured with no significant difference ($p > 0.05$) whereas G1 had a significant difference of $p = 0.0070$ between the two seasons.

Aflatoxin content in maize is regulated at 5 µg/kg AFB1 and 10 µg/kg total AF under the South African national regulations (Act No. 54 of 1972, as amended by Government Notice No. R. 1145 of 8 October 2004) (Rheeder et al., 2009).
The EU regulates maize contaminated with aflatoxins at a maximum limit of 5 µg/kg AFB_1 and 10 µg/kg total AF (EC, 2006). The majority of positive samples (8/12) had high levels of AFB_1 and total AF. All eight AFB_1 positive samples contained levels above the RSA maximum limit of 5 µg/kg with six of the eight exceeding the total AF limit of 10 µg/kg in food. Samples from two households (no.11 and no.19) were highly contaminated with aflatoxin over the two seasons. Sample no.11 was the highest AF contaminated sample for the two consecutive seasons with AFB_1 levels more than 14-times above the maximum levels stipulated by S.A. and the EU.

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Aflatoxin B_1 was detected in ≤30% of samples collected from both seasons. AFB_2, AFG_1 and AFG_2 concentrations for positive maize samples (n=5; n=8 and n=3) ranged from 2.0 to 15µg/kg; 1.0 to 93µg/kg and 2.0 to 12 µg/kg respectively. In all the samples quantified for both seasons, AFB_1 was the dominant analogue found while AFG_1 was found in higher concentration levels compared to AFB_2 and AFG_2. This is in line with the studies that published AFB_1 in food and feed as the prevalent toxin (Weidenborner, 2001; Alam et al., 2010; Kamika, 2012) as well as AFG_1 found in higher concentrations than AFB_2 and AFG_2 (Weidenborner, 2001). Aflatoxins in all maize samples collected inNovember (seeds material') was detected below the LOQ.

None of the beans (over the two seasons of the study) and peanuts (first season) had quantifiable levels of either aflatoxins or fumonisins. However, two of three peanut samples collected during the second season were found to contain levels of 41 µg/kg aflatoxin G_1 and 257 µg/kg fumonisin B_1. Aflatoxins in peanuts are regulated under the South African national regulations (Act No. 54 of 1972) at 5 µg/kg aflatoxin B1 and 10 µg/kg total aflatoxins content (Rheeder et al., 2009). For the domestic market the European Commission has established legal limits of 5 µg/kg AFB1; 10 µg/kg total AF and 15 µg/kg total AF content for aflatoxin in peanuts both in foodstuff for human consumption and peanuts intended for further processing, respectively (EC, 2009). Limit of 2 µg/kg for AFB_1 and 4 µg/kg for total AF in peanuts has been set for direct human consumption EC, 2006. The Codex Alimentarius Commission has recommended a maximum level (ML) of 15 µg/kg total aflatoxin to peanuts intended for further processing as well as prevention and reduction practices base on good agricultural practices (GAP) and good storage practices (GSP) (Codex, 2010).

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6.11 Analysis of stored commodities in selected rural areas of Gert Sibande District.

For both seasons, fumonisins were found in 97% (60/62; range 0 - 28272 µg/kg total fumonisins) of maize samples from GSDM rural households (Table 6.4). This includes two of three samples collected in November 2011, where one was not analysed as it had badly decomposed. Level of FB_1 contamination for season one (2011) ranged from 1 - 2732 µg/kg and for the second season (year 2012; 3 - 18924 µg/kg) with fumonisin analogues significantly different ($p < 0.05$) between the two growing seasons. Between the two years, the significant difference (p-value) was found to be $p = 0.0066$ for FB_1, $p = 0.0038$ for FB_2 () and $p = 0.0222$ for FB_3.

Table 6.4: Contamination of fumonisins (µg/kg) in homegrown maize collected from Mpumalanga over the two seasons using an LCMSMS.

Household no. s	**FB_1**	**FB_2**	**FB_3**	**Total FB**	**Household no.s**	**FB_1**	**FB_2**	**FB_3**	**Total FB**
		2011					**2012**		
1w	<loq	<loq	<loq	11	**1(1)w**	<loq	<loq	<loq	<loq
2w	2732	1866	285	4883	**1(2)w**	74	25	12	110
3w	<loq	<loq	<loq	<loq	**3w**	<loq	<loq	<loq	<loq
4w	<loq	<loq	<loq	<loq	**4w**	23	<loq	<loq	34
5w	140	54	14	209	**5w**	16	<loq	<loq	23
6w	129	81	15	225	**7w**	<loq	<loq	<loq	14
7w	<loq	<loq	<loq	<loq	**8w**	14	<loq	<loq	21
10w	1370	392	374	2136	**9w**	18924	7191	2158	28272
13w	<loq	<loq	<loq	12	**10w**	24	<loq	<loq	37
14w	525	178	38	741	**12w**	81	32	12	125
15w	<loq	<loq	<loq	<loq	**13w**	<loq	<loq	<loq	10
16w	<loq	<loq	<loq	<loq	**14w**	<loq	<loq	<loq	11
17w	<loq	<loq	<loq	<loq	**15w**	<loq	<loq	<loq	<loq
18w	<loq	<loq	<loq	<loq	**16(1)w**	<loq	<loq	<loq	<loq
19w	<loq	<loq	<loq	<loq	**16(2)w**	12	<loq	<loq	18
20w	279	125	79	483	**17(1)w**	<loq	<loq	<loq	<loq
8w	<loq	<loq	<loq	<loq	**17(2)w**	18	<loq	<loq	26
3y	<loq	<loq	<loq	10	**18w**	27	11	<loq	41
4y	419	114	17	550	**19(1)w**	47	18	<loq	69
5y	<loq	<loq	<loq	<loq	**19(2)w**	50	26	<loq	82
6y	188	106	14	308	**20w**	21	<loq	<loq	30
8y	<loq	<loq	<loq	<loq	**21w**	11	<loq	<loq	17
9y	<loq	<loq	<loq	<loq	**2y**	<loq	<loq	<loq	<loq
11y	<loq	<loq	<loq	10	**3y**	<loq	<loq	<loq	<loq

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Household no. s	FB_1	FB_2	FB_3	Total FB	Household no.s	FB_1	FB_2	FB_3	Total FB
		2011					**2012**		
12y	12	<loq	<loq	18	4y	22	<loq	<loq	31
13y	<loq	<loq	<loq	<loq	5y	191	63	14	268
16y	<loq	<loq	<loq	<loq	**8y**	12	<loq	<loq	17
17y	<loq	<loq	<loq	<loq	**9y**	<loq	<loq	<loq	<loq
19y	<loq	<loq	<loq	<loq	**11y**	<loq	<loq	<loq	<loq
21-M	26	<loq	<loq	36	**13y**	<loq	<loq	<loq	11
21-K	175	49	10	235	**17y**	15	7	<loq	22

Y - Yellow maize

W - White maize

*Sample 21-M and 21-K represents maize collected in November 2011 from the community silo situated in Dondonald.

<loq - below the limit of quantification.

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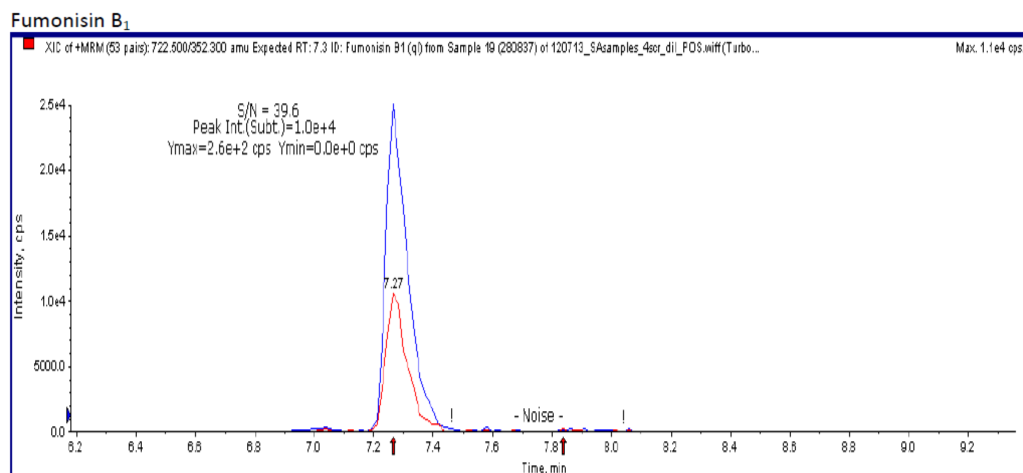
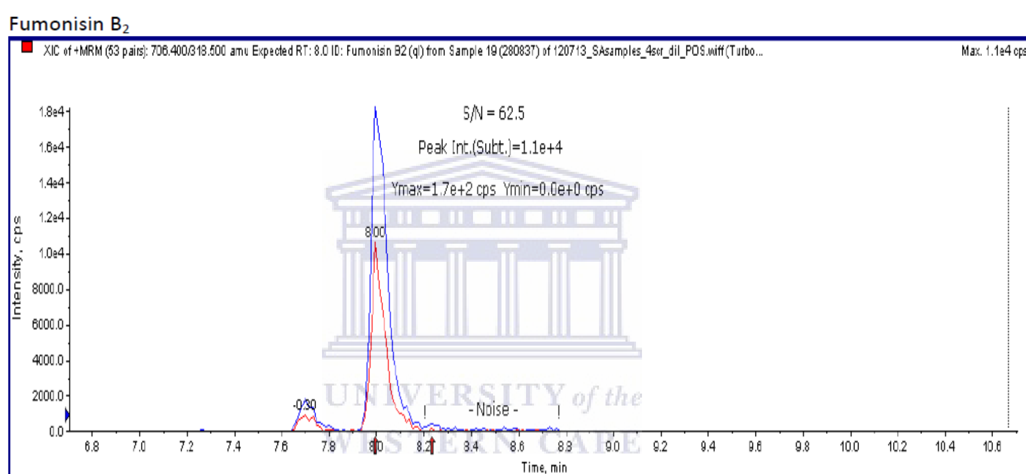
Eleven out of twenty nine FB1 positively tested maize samples found in 2011 contained levels above the limit of quantification. However, in the second season, 58% (18/31) of the samples were observed with FB1 were above the LOQ. Only fifteen and five samples were not detected with FB_3 in 2011 and 2012 growing seasons. In the year 2011, one of the samples (household no. 2) was observed with alarmingly maximum 2732 µg/kg FB_1 level (Figure 6.2), but the maize sample from the same household in the second season was detected at very low level (6 µg/kg). Two of three samples collected from the silo were consecutively observed at 26 and 175 µg/kg FB_1.





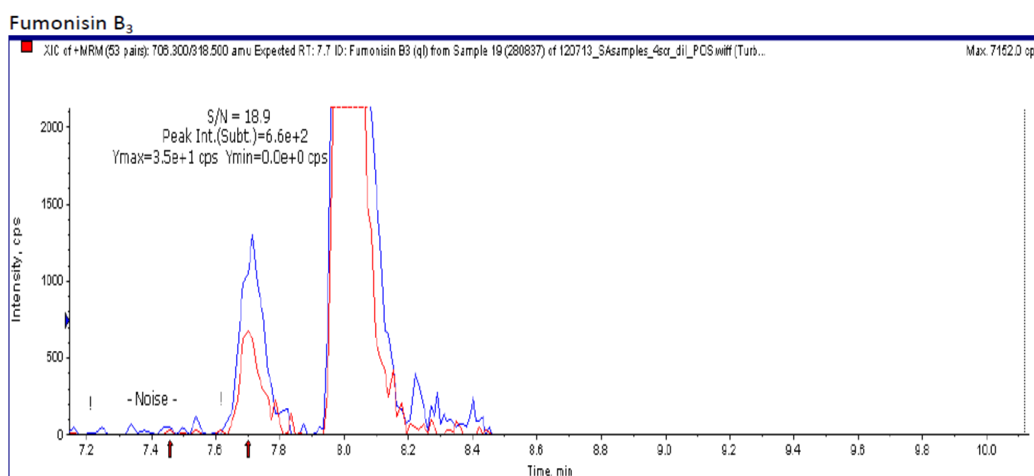


Figure 6.2: Extracted fumonisin ion chromatograms obtained from LC-MS/MS analysis of highly contaminated white maize sample no. 2 from GSDM in 2011. F B_1= 2732 µg/kg; 1866 µg/kg B_2, and 258 µg/kg B_3.

Maize from household number 9 in the following year was the only sample extremely contaminated with fumonisin (18924 µg/kg). Other individually analysed samples were generally low (less than 200 µg/kg; range 3.0 – 191 µg/kg) and well below the EC guidance value. Only 3 maize samples from Gert Sibande district exceeded the EU limit of 1000 µg/kg (sum of FB_1 and FB_2). Two contamination levels were exceedingly high viz., 2732 µg/kg and 1370 µg/kg in year 2011 while in 2012 an alarmingly high level of 18924 µg/kg was detected.

Of the samples collected from GSDM in both seasons, only one sample of yellow maize was contaminated with 1.0 µg/kg AFB_1. This was sample number five observed in the second season. All beans (n=1) and peanuts (n=10) samples analysed did not contain aflatoxins or fumonisins levels above the limit of quantification (LOQ) and other mycotoxins analysed were also below the LOQ.

6.11.1 Maize varieties found in GSDM

Yellow maize (YM, n = 21) and white maize (WM, n = 39) in both seasons from Mpumalanga (GSDM) were collected and measured for AF and FB. Only one yellow maize sample was observed with 1.0 µg/kg AFB_1, below the legal limit of RSA. Table 6.5 represents the mean fumonisin B_1, B_2 and B_3 levels of each maize type for each season's collection.

Table 6.5: Average distribution of fumonisins levels in white and yellow home-grown maize samples intended for human (WM) and animal (YM) consumption collected of both seasons from Mpumalanga (GSDM).

		Fumonisins (µg/kg)			
GSDM	**Years**	**FB_1**	**FB_2**	**FB_3**	**Total**
Yellow maize (ym)	2011	52	18	2.6	73
	2012	30	10	2.4	42
White maize (wm)	2011	305	159	47	510
	2012	881	335	117	1317

On average, fumonisin concentration in yellow maize (range 2.6 – 52 µg/kg) during the year 2011 was higher whereas in 2012 white maize (range 117 – 881 µg/kg) was the highly contaminated maize type (Table 6.5).
Both sample types in year 2012 were 100% contaminated with FB. In season one 88% (15/17) and 100% (12/12) were observed to be contaminated with FB but, only 35% and 25% of white and yellow maize samples, respectively were above the LOQ.

For the two growing seasons, yellow maize with FB was found in lower concentration levels as compared to white. This is in line with a report by Shephard et al. (1996) who found lower mean fumonisin levels in South African yellow maize compared to white maize over four years (1989 – 1992), but the reversed situation occurred in the last season (1993). Over the two year period the highest contaminated samples were observed in white maize.

Fumonisin levels in yellow maize demonstrated a relative decrease whereas in white there was an increase in 2012. In white maize (510 – 1316 µg/kg) FB contamination more than doubled as illustrated on Table 6.5. Two out of three (2732 and 1370 µg/kg) FB prevalent samples were white maize. One extremely contaminated yellow maize sample (18924 µg/kg FB_1) was found to be more than 18-times the EU limit, while other samples contained less than 74 µg/kg during 2012.

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6.12. General results and discussion

6.12.1 Comparisons of mean levels of mycotoxin observed in maize from different districts.

The mean levels of FB1 were found higher in 2012 with634 µg/kg than 193 µg/kg found in 2011 from GSDM while in VDM the opposite occurred, i.e. 578 µg/kg (2011) and 295 µg/kg (2012) (Table 6.6). Diseased maize from Vhembe district in the first season had higher average AFB1 concentration of 48 µg/kg compared to

that of the second season which was 20 µg/kg. Table 6.6 shows the mean levels of AF and FUM from all positive maize samples stored by Gert Sibande and Vhembe districts over a two year season.

Table 6.6: Average total fumonisins and aflatoxin content in stored home-grown maize intended for human and animal consumption over two seasons in selected household’s villages of Gert Sibande and Vhembe districts.

	GSDM		**VDM**	
Analogues	**2011**	**2012**	**2011**	**2012**
		Fumonisins (µg/kg)		
FB_1	193	634	578	295
FB_2	96	241	213	98
FB_3	27	86	75	32
		Aflatoxins (µg/kg)		
AFB_1	<loq	<loq	13	6.0
AFB_2	<loq	nd	1.3	<loq
AFG_1	<loq	nd	1.6	5.7
AFG_2	<loq	nd	<loq	<loq

*Nd-not detected – samples were not contaminated.
*<loq - below the limit of quantification

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Overall, samples from Limpopo (VDM) were found to be the more highly contaminated region with both AF and FB compared to Mpumalanga (GSDM). Considering that six samples from VDM compared to three from GSDM contaminated with FB_1 exceeded the maximum tolerable limit by a factor of more than one. Fumonisin B1 and B2 contamination levels for the rest of the samples from VDM (70%; range 12 - 964 µg/kg and 91%; 10 - 949 µg/kg) were much higher than that of GSDM (29%; range 12 - 525 µg/kg and 55%; 12 - 191 µg/kg) in year 2011 and 2012 respectively. VDM samples ranged from 1010 to 1216 8 µg/kg for (FB1 and FB2) indicating high exposure levels of fumonisins.

Frequent detection of fumonisins in Limpopo compared to Mpumalanga was also reported by Ncube et al., 2011. Although there were few samples from VDM contaminated with aflatoxin, the levels were extremely high exceeding both S.A. and EU maximum limit of 5 µg/kg AFB_1 and 10 µg/kg total aflatoxin. This may be due to inappropriate sanitation and storage practices used particularly sample no. 11 which was contaminated with fumonisins and aflatoxin on both seasons although lower in the second season but still excessively highly contaminated. The consumption of maize, containing high levels of aflatoxins may cause acute toxicity and could result in death. There have been reports of aflatoxicosis cases and aflatoxicosis outbreaks caused by high levels of aflatoxin found in maize consumed by humans (Krishnamachari et al., 1975; Ngindu et al., 1982; Chao et al., 1991; CDC, 2004).

Another contributing factor may be environmental conditions in the field before harvest. Climate in the Vhembe and Gert Sibande sampling areas during the growing season ranged from 18 - 30°C and 15 - 33°C temperatures with 33 - 93% and 31 - 92% relative humidity and rainfall of 19 - 434 and 19 - 221 mm in the first season, respectively. Over the second season, Vhembe temperature ranged from 17 - 30°C, relative humidity (35 - 87%) and 202 mm max. rainfall while GSDM climate was measured at 14 - 32°C with 29 - 89% and 206 mm max. rain. These parameters are known to encourage mycotoxin production.

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6.13. Occurrence of other mycotoxins found in the stored commodities

Of the thirty seven mycotoxins measured, 20 other mycotoxins were detected from the samples in the study include the deoxynivalenol (DON), sum of 3 &15-acetyl-DON, ochratoxin A, HT-2 toxin, T-2 Toxin, α-zearalenol, ß-zearalenol, zearalenon, agroclavine, alternariol, alternariol-methylether, beauvericin, diacetoxyscirpenol (DAS), moniliformin, mycophenolic acid, 3-nitropropionic acid, roquefortine C, sterigmatocystin, nivalenol and DON-3-Glucoside. Four toxins; ochratoxin A, HT2 toxin, T-2 toxin and agroclavine were detected at low levels (less than the lowest LOQ 1.0 µg/kg) in the second season, but in the first

season were respectively frequently found at levels of 433; 146; 60 and 0.288 µg/kg, respectively. These toxins were quantified only in samples from GSDM except for agroclavine which was found in samples from both districts. Ochratoxin A forms part of the most important mycotoxins and contamination in grain has been report as directly influenced by storage conditions after harvest (Petzinger and Weidenbach, 2002).

Other recovered mycotoxins such as beauvericin were frequently encountered at 62% and 79% and 3-nitropropionic acid 19%; and 29% of the samples with maximum levels of zearalenone at 546 µg/kg and 3082 µg/kg and DON (430 µg/kg and 105 µg/kg) found in 2011/2012, respectively. Frequent co-occurrence of mycotoxins was observed in most home grown agricultural commodities from the surveyed areas. The co-occurrence of wide variety of mycotoxins in different commodities increases the probability of interactions (additive or synergistic effects), which may increase the risk to human health (Alborch et al., 2012). Maize in these regions is consumed as staple food and the health risks associated with high consumption of the FB in maize and maize-based food and feed depends on the extent to which they are consumed (Shephard et al., 1996).

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6.14. Conclusions

Sampled staple foods from rural households have been shown to be contaminated by extremely high levels aflatoxin and fumonisins, specifically AFB_1 and FB_1. These two toxins are regretfully known to cause the most devastating effects on human and animal health. Maize was the most contaminated commodity with both aflatoxin and fumonisins. The VDM based samples were greatly contaminated by both mycotoxins compared to Mpumalanga household samples. Aflatoxin positive samples originate from the Limpopo province only. Statistically, there was no significant difference in the fumonisins and aflatoxin contamination levels found in VDM maize samples between the two seasons.

High levels of fumonisins found in maize samples from Limpopo and Mpumalanga rural areas suggest that these areas may be at risk of negative effects of fumonisins through maize consumption. Given the fact that maize is the staple grain for both areas, the risk of fumonisins exposure is unavoidable with 91% (VDM) and 68% (GSDM) of samples contaminated. Home-grown maize crops also showed high prevalence of beauvericin, zearalenone, DON, (sum of 3&15acetyl-DON, moniliformin, 3-nitropropionic acid and ochratoxin A. Contamination during both seasons may have been as a result of uncontrolled environmental factors, late harvest and poor storage management.

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CHAPTER 7

General Discussion, Recommendations and Conclusions

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7.1 General Discussion

Subsistence farmers in South Africa contribute significantly to food production, food security and employment but are faced with high crop production losses because of the effects of improper storage practices (WWF, 2011) which result in mycotoxin and mycological contamination. Maize grown by these farmers is prone to contamination because of the agro-ecological zone and the post-harvest storage conditions. Agriculture consists mainly of production of staple foods such as maize for household consumption and only a few households are able to sell small quantities of their products to street vendors as a means of subventing their income. We set out to assess productivity, agricultural storage practices, fungal and mycotoxin (fumonisins and aflatoxin) occurrence of home-grown stored grains and comparing two areas with different agroecological climates over two growing seasons (2011 and 2012).

For two consecutive harvesting seasons 2011 and 2012, maize, peanut and bean samples were collected in two districts of Gert Sibande in Mpumalanga and Vhembe in Limpopo provinces of South Africa. The two districts have a high concentration of subsistence and small scale farmers which lie in the sub-tropical climatic zone. Twenty households from each district were randomly recruited and the number of samples collected depended on the availability of crops cultivated. Limpopo samples consist of maize (n=45), peanuts (n=5) and beans (n=5) and an additional homegrown maize (n=8) stored as seeds, collected in November 2011 over the two growing seasons. Samples collected from Mpumalanga were maize (n=60), peanut (n=1) and beans (n=10) over the two year period (2011 and 2012) and three maize samples stored at the community silo sampled in November 2011.

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Production output of subsistence farmers in rural South Africa is poorly understood since no production data is available. Households in these areas depend mainly on maize as their primary source of food and income, and to some extent, upon groundnuts and beans as their secondary source of food.

For both districts, arable land was limited (less than 4 hectares per farmer) with significantly different maize productivity between Vhembe (average = 0.6 tonnes) and Gert Sibande districts (average = 2.4 tonnes) and self-sufficiency (25 to 50%) of three months per year. Two maize varieties in GSDM were cultivated, yellow (as feed) and white (for humans) while VDM only produced yellow maize for both food and feed. Both districts indicated that damaged grains are sorted and given to livestock and poultry. Smallholder farmers (per household) in Limpopo on average were mostly women of the age 10 years and older with a statically significantly lower mean ± 6.5 of residents compared to Mpumalanga where mostly men with larger (mean ± 9.2) residents were observed.

All surveyed households practised monoculture and leave maize to dry out for duration of up to three months before storage. This very practice may result in high mycotoxin levels in maize produce. Maize storage structures differed between the two districts with difference capabilities to safeguard grains from fungal and insect infection. All the storage forms used were prone to either grain spoilage caused by insect infestation or fungal infestation. In addition other factors such as environmental conditions and storage practice impact on all aspects of grain production (Shephard et al., 1996; Marasas et al., 2001a; Fandohan, 2004).

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In Mpumalanga, the majority of farmers used traditional open wooden cribs for drying before sending the produce to the community silo for storage of which 25% comprised of steel tanks for storage. The majority of Limpopo farmers used polypropylene sacks kept inside the houses on a cement floor stacked up against the wall which will allow moisture damage to the grain and 22% used sealed store houses. Weevils' *(Sitophilus zeamais)* infestation on harvested crops in the polypropylene bags was observed. Stalk-borers *(Prostephanus truncates)*, and weevils in Vhembe areas and rodents in GSDM were the most common pests attacking stored maize. Kankolongo et al (2009) has reported weevils and stalk-borers as the most damaging pests in maize stored for human consumption in Zambia. All samples were investigated for the incidence of fungal species and

quantification of fumonisin and aflatoxin using both HPLC with fluorescence detection and LC-MSMS instruments.

Fusarium and *Aspergillus* fungal genera were the prevalent genera found in homegrown maize kernels sampled from the rural areas. Maize is known to be commonly infected by these two genera (Dutton and Kinsey, 1996; Odhav and Naicker, 2002). VDM maize kernels were observed to be infested with a high incidence of *F. verticillioides* (93%) throughout the two seasons while GSDM kernels were predominately infested with *F. subglutinans* (89%) (Table 7.1). Samples having an infestation with *F. verticillioides* did not necessarily also result in having high frequencies of *A. flavus*. Studies have reported that maize kernels contaminated with *F. verticillioides* were less likely to be infested with *A.flavus* and have been shown to be negatively associated with other fungal species (Wicklow, 1988; Marasas et al. 1979; Rheeder et al., 1990a).

Maize kernels from VDM were isolated with high occurrences of *F. verticillioides* which corroborates with previous reports on South African maize (Gelderblom et al., 1988; Rheeder et al., 1990b, 1993; Marasas, 2001b and recently Ncube et al., 2011). The high occurance of *F. subglutinans* in Mpumalanga maize kernels compared to kernels in Limpopo is in agreement with Ncube et al., 2011. Maize samples from VDM kept as seeds for the next season were collected in November 2011, and these samples containe both *F. verticillioides* (100%) and *A. flavus* (35% maximum). Incidence in these samples kept as seed for cultivation shows that the apparently visibly clean maize can contain toxin-producing fungi, which has been reported by Thomas and Buddenhagen, 1980; and CAST, 2003.

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Table 7.1: Overview of the study data for the percentage isolated fungal infection in stored samples (maize, peanuts and beans) collected from selected VDM (Limpopo) and GSDM (Mpumalanga) rural areas over two seasons.

Seasons	Locality	Sample type	No. of samples[a]	*F. vert*[b]	Mean(max) % freq.[c, d]	*A. flavus* (*MEA*)[b]	Mean(max) % freq.[c, d]	*A. flavus* (*AFPA*)[b]	Mean(max) % freq.[c, d]
2011	**VDM**	M	18	16	15 (56)	8	5(18)	13	7(32)
		P	2	0	0	0	0	1	(3)
		B	3	0	0	0	0	1	(2)
		Nov.	8	8	17	1	9(9)	8	8(35)
	GSDM	M	31	11	8 (52)	0	0	2	9(10)
		Nov.	2	1	3	1	8	1	10
2012	**VDM**	M	15	14	9 (23)	10	8 (16)	10	19(69)
		P	3	0	0	0	0	1	3
		B	2	0	0	0	0	1	2
	GSDM	M	30	9	10 (75)	4	9 (27)	8	6(16)

[a] Total number of analysed samples; [b] Total number of infected samples; [c] Mean of positive samples (% frequency); [d] Maximum infection level (% frequency).

Nov. - maize samples collected in November 2011.

F. vert. - F. verticillioides

AFPA= *Aspergillus flavus* / *parasiticus* agar and MEA.- malt extracts agar.

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0 - not isolated

7/7 - ground and coarse maize samples as well as peanuts and beans were dilution plated onto AFPA and found with10 to 700cfu/g range.

Peanuts and beans from GSDM - there was no incidence of *Aspergillus flavus.*

Of the *Aspergillus* species identified, *A. flavus* occurred in 55% of maize samples from Limpopo but occurred at a lower frequency viz., 13% in maize from Mpumalanga. The relatively higher occurrence of *Fusarium spp*. in comparison with *Aspergillus* species in maize has been reported by (Dutton and Kinsey, 1996). No incidence of *A. flavus* occurred in peanuts and beans throughout the regions studied. High incidences of *F. verticillioides* in samples from VDM is of grave concern considering that it produces secondary metabolites (fumonisins) which inevitably are the cause of animal and human diseases (Shephard et al., 1996; Rheeder et al., 2002).

In maize samples, fumonisins were observed in 91% and 68% of the samples from Limpopo and Mpumalanga, respectively (Table 7.2). However aflatoxin contamination (30%) was observed in VDM maize samples only and alarmingly, at unacceptably high concentrations which exceeded the South African and European regulatory limit by a factor of 2. Positive maize samples (<30%) in the VDM areas exceeded the 5 µg/kg AFB_1 and 10 µg/kg total AF limit stipulated by the South African national regulations and (FB_1 and FB_2) from both areas exceeded 1000 µg/kg set by the European Commission (Rheeder et al., 2009; EC, 2006). Three samples from GDSM exceeded the fumonisins limit, two in 2011 and only one in 2012. Detected FB_1 levels were significantly different (p = 0.0116) whereas FB_2 (p = 0.2414) and FB_3 (p = 0.2414) were not significantly different between the two seasons for both methods employed for the analyses. However, VDM (n=6) maize samples exceeded the limit, four in 2011 and two in 2012 and showed no significant difference (p >0.05; range 0.2811-0.9871) in the fumonisins contamination levels between the two seasons for both methods used.

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Table 7.2: Summary data of fumonisin and aflatoxin concentration from positive grain samples (maize, peanuts and beans) taken at selected areas of Limpopo and Mpumalanga over two years (2011and 2012).

Season	Locality	Sample type	No. of samples	FB_1[a]	FB_1 (total FB) µg/kg Mean[b]	Maximum[c]	AFB_1[a]	AFB_1 (total AFB) µg/kg Mean[b]	Maximum[c]
2011	**VDM**	M	22	20	841(1264)	8,514(13203)	6	48(60)	133(149)
		P	2	2	<loq	<loq	2	<loq	<loq
		B	3	3	<loq	<loq	3	<loq	<loq
		Nov.	8	5	117(155)	351(480)	1	<loq	<loq
	GSDM	M	29	9	644(1061)	2732(4883)	3	<loq	<loq
		B	7	7	<loq	<loq	7	<loq	<loq
		Nov.	2w	2	101(136)	175(235)	1	<loq	<loq
2012	**VDM**	M	23	23	295(425)	1584(2422)	13	20(41)	73(78)
		P	3	1	257	257	1	<loq	<loq
		B	2	2	<loq	<loq	2	<loq	<loq
	GSDM	M	31	18	1088(1624)	18924(28272)	1	1.0	1.0
		P	2	2	<loq	<loq	2	<loq	<loq
		B	3	3	<loq	<loq	3	<loq	<loq

*Sample types; M - maize; P - peanuts; B – beans.

[a]A number of positive samples.

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[b] Mean of positive samples (µg/kg).

[c] Maximum infection level (µg/kg).

<loq - below the limit of quantification

Peanut samples from GSDM were not detected.

All beans and peanuts in the first season were not detected

The aflatoxin tolerated limit was exceeded in seven samples, with four from 2011 and three in 2012 with only a significant difference of $p = 0.0070$ in AFG_1 contamination between the two years for both methods used in the determinations. There was no significant difference ($p > 0.05$; range 0.3135 - 0.9340) in the analysis of aflatoxin contamination between HPLC and LC-MS/MS over the year 2011 and 2012

The two maize varieties found in GSDM differed in mean fumonisin levels viz, white maize was more contaminated compared to yellow maize in the two sampled seasons. This is in line with the findings of Shephard et al. (1996) where the detected levels of FB in yellow maize were lower compared to white maize for four consecutive years but in the next year the opposite occurred. None of the beans and peanuts from Mpumalanga were infected with *A. flavus* or with quantitative aflatoxins, but from Limpopo 2/5 peanuts were detected with only one peanut sample contaminated with aflatoxin G_1 (41 µg/kg) and the other fumonisin B_1 (251 µg/kg). VDM (Limpopo) and GSDM (Mpumalanga) areas are not negatively exposed to mycotoxins through consumption of the legumes as there were no high levels of toxic contamination.

This outcome presents evidence that the homegrown samples (maize) from the households are contaminated with mycotoxins which coincides with the high incidence of fungal species found. Specifically the few samples from VDM found with aflatoxins at dangerously high levels. Mycotoxin contamination differences between regions maybe expected due to climatic and environmental differences. Rainfall variations in Limpopo (434 mm in 2011 and 202 mm max. in 2012) may have had an influence in the production of fumonisins in maize in the field during

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the growing seasons compared to Mpumalanga (221 mm max. in 2011 and in 2012; 206 mm max.). In addition, temperatures and relative humidity conditions in Limpopo and Mpumalanga during the growing seasons may allow for fungal development and mycotoxin production and may have further been encouraged by poor climatic conditions during storage. In these subsistence farms, regulations to limit mycotoxin presence to ensure food and feed safety are ineffective. The presence of fumonisins was significantly higher in maize as staple food compared to other grains, which was in agreement with the report by Summerell and Leslie (2011).

Overall Limpopo farmers appear to experience greater mycotoxin contamination in maize produce and also lower harvests which may be attributed in part to the inadequate poor storage facilities available, weevil infestation, the climate in the region and lack of grain varieties. Both areas experience higher levels of total fumonisins. Only some VDM maize samples contained aflatoxin levels. Co-occurrence of aflatoxins and fumonisins in maize as staple food may potentially induce liver cancer (Gelderblom et al., 2002).

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Further work needs to be done to understand the role of cultural and storage practices on production and mycotoxin occurrence and what interventions can be put in place to improve the situation. This accentuates the need for constant monitoring of these toxins in food products especially maize which is the main staple food for most African populations especially those within the general rural communities. These results have also revealed the co-occurrence of mycotoxins in food commodities from the two districts.

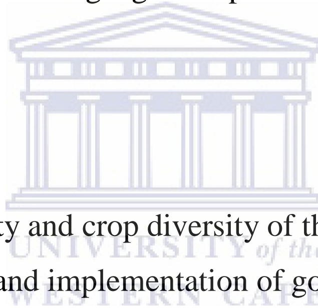
7.2 Recommendations

- Frequent investigation, documenting the type and extent of contamination is an important step in developing strategies which are relevant and culturally acceptable for the reduction of contaminants.

- Subsistence farmers need to be made aware of the very serious health risks caused by the presence of mycotoxins in their home-grown grain as well as being knowledgeable about food safety problems.

- A study needs to be done on the health and wellbeing of the animals which are fed the mouldy and often contaminated crops by the subsistence farmers as well as exposure studies on people consuming products from these animals.

- There needs to be an introduction of other crops to balance the current maize diet. Planting of different food crops has advantages such as counteracting environmental hazards and maintaining the fertility of the land and thereby ensuring a greater productivity to ensure food security.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7.3 Conclusion

In conclusion, productivity and crop diversity of these farmers need to improve through proper planning and implementation of good policies, training and farmer support programmes. In terms of crop productivity, GSDM households have a significantly ($p = 0.0184$) higher yield compared to VDM which most likely may be attributed to the farmers starter pack (seed, fertilizer, plough and planting of 1 hectare per household) support received from the government. Comparison of the natural occurrence of mycotoxins (fumonisin and aflatoxin) in stored home-grown grain in between the two provinces was statistically significantly different (p values < 0.0001) over both seasons. Although GSDM subsistence farmers do not experience much fumonisin and aflatoxin contamination, their storage practices need to improve far more significantly due to the climatic conditions which are to a large extent unpredictable. Crop diversity for farmers needs to be encouraged and improved through the design and implementation of good farming practices and sound policies.

Few samples in this study, i.e. sample 2 & 9 from GSDM and 8 & 11 from VDM illustrated in Chapters 4, 5 & 6 are not suitable for human consumption. This was due to the large amounts of mycotoxin contamination agreeing with the high prevalence of fungal infection results (*Fusarium* species). The presence of mycotoxin-producing fungi is however, known not to always favour mycotoxin contamination. Mycology results show high occurrence of *Aspergillus* and *F. verticilliodes* genera in VDM maize samples whereas GSDM maize samples contained lower amounts. These mycotoxin results thus illustrate high amounts of *Aspergillus* and *F. verticilliodes* in VDM maize samples whereas the GSDM experiences lower amounts. The significantly higher occurrence of mycotoxins (fumonisin and aflatoxin) in VDM may be attributed to poor storage practices which include factors such as high weevil infestation, stalk-borers (signs of spoilage were obvious on visual inspection), environmental and seasonal factors such as humidity and moisture. Mycological and chemical analysis results of all evaluations corresponded well.

High levels of mycotoxins in the homegrown maize samples in these two rural areas over two seasons are of concern since maize is the staple diet of the inhabitants and is consumed almost every day as well as the prolonged health effects in humans and animals caused by mycotoxins exposure. Efforts to reduce mycotoxin contamination of agricultural products must include the following: preventing preharvest contamination and exposure to mycotoxins; minimizing postharvest contamination or growth of harvested products and the highest degree of degradation and destruction of all these contaminants during food processing. All these interventions need to be implemented immediately and even drastically. Even though South Africa is generally regarded as a food secure country, it still faces challenges such as to ensure both food safety and quality which may to a large extent be associated to mycotoxin contamination. Although the extent of the study was somewhat limited and with only a limited number of samples taken, a too high percentage of them were contaminated with aflatoxins and the results suggest that there is a need for a serious intervention both at policy and marginal field level in order to develop methods to reduce mycotoxin

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contamination. Results of this study may help to inform the sampled households on the numerous negative and quite dangerous effects (mycotoxins) to humans and livestock caused by consuming contaminated crops. Effective education of the subsistence farmers about the correct and essential hygienic storage methods of their harvested crops will also go a long way in reducing contamination. Further research in the determination of the extent of the mycotoxin dietary exposure to contaminated crops should be done on a larger scale to include as many subsistence farming sectors as possible in order to derive a statistically more valid model.

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APPENDICES

APPENDIX 1

Appendix 1.1

ETHICS COMMITTEE

12 May 2011

Dr D Katerere
PROMEC Unit
MRC Cape Town

Dear Dr Katerere

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Protocol ID: EC11-002
Protocol title: Preliminary investigation of aflatoxins and fumonisins contamination and dietary exposure in rural South Africa
Meeting date: 28 February 2011

Thank you for your response to the Committee, dated 29 March 2011. The response was found to be acceptable. I am pleased to inform you that ethics approval is now granted for the study.

Wishing you well with your research.

Yours sincerely

PROF. D DU TOIT
CHAIRPERSON: MRC ETHICS COMMITTEE

MRC Ethics Committee: Prof D du Toit (chairperson), Prof A Dhai, Dr N Khaole, Dr NE Khomo, Prof D Labadarios, Ms L Mpahlwa, Prof H Oosthuizen, Dr L Schoeman, Prof AA van Niekerk

APPENDIX 2

Appendix 2.1:

A semi-structured questionnaire translated into the vernacular (Tshivenda and siSwati).

Questionnaire: **Mycotoxin (aflatoxin and fumonisins) study**

<u>Household Details:</u>

Village name…………………………………………………………….....

Household code………………………………………………………….

How many people live in the household?..

Adults……………………….Children under 12 years…………………....

<u>Storage History:</u>

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1. Do you cultivate maize with other crops?

   If yes list the crops

   When do you store harvested maize?, Directly after harvest or Pre-storage

2. Why do you pre-store?

3. Where do you pre-store?

4. How long is the maize crop left in the field to dry

5. On average how much maize do you get / harvest

6. On average how long does a season's harvest last

less than a month, 3 months, 6 months, to the next harvest (1 year)?

7. In what state is the maize stored

   Cob, loose grain, milled, other

   Where there is a storehouse in use (describe the type of storehouse and take a photograph)

8. For how many seasons have you used the storehouse?

9. When do you clean the storehouse

10. Do you sort damaged and mouldy maize before storage?

11. If yes, what do you do with damaged / mouldy maize

12. Do you remove old grains from storage before putting new harvest in?

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13. What other crops/items are kept in the storehouse

14. Do you encounter problems in storing the plants? What problems (if any) do you experience during storage?

15. When do you observe this problem:

   At the beginning of storage? After, a few months? At the end of storage?

   How do you solve the problem?..

   What pesticides do you apply to the maize to ensure that they do not rot, if any?..

16. Where do you get your maize seed from?

   Previous season, shop or government

Consumption:

1. What are the foods you eat on a regular basis?
2.
3. How often do you consume maize?

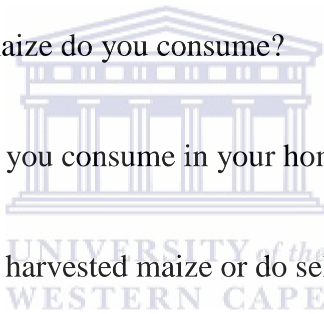
    Daily, Weekly, Monthly

4. How much do you consume?

5. Is the maize you consume home-grown or not?

6. How do you prepare your maize?

7. What proportion of the maize do you consume?



8. Do you plough the maize you consume in your home yard or not?

9. Do you consume all your harvested maize or do sell some?

10. Where do you sell your maize?

APPENDIX 3

Appendix 3.1: Subsistence farmers grain duration and consumption of the harvest.

	Mpumalanga		Limpopo	
	2011	**2012**	**2011**	**2012**
<6months	5	0	10	9
6-12 months	0	7	0	3
>12 months	15	10	9	5
consume all	9	16	11	16
sell some	9	1	8	3
exchange ±5bags	n/a	n/a	1	n/a

APPENDIX 4

Appendix 4.1: Isolated maize (Ground and milled), peanuts and beans samples expressed in colony forming units / g (cfu/g).

Districts	Household no.s	Commodity	*A. flavus* (cfu/g)
		Year 2011	
VDM	11	maize	20
GSDM	21	maize	600
		Year 2012	
VDM	01	maize	10
VDM	05	maize	100
VDM	07	maize	10
VDM	10	maize	700
VDM	17	maize	400
VDM	18	maize	200
VDM	19	maize	50
VDM	14	peanuts	3
VDM	16	beans	2

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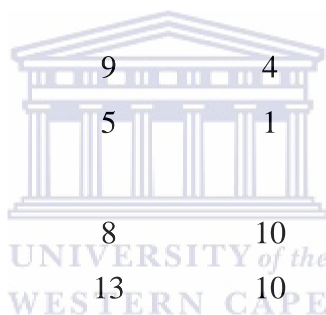
Appendix 4.2: Total fungal isolation with frequency on occurrence of mycotoxigenic fungi isolated from maize kernels sampled from selected villages in two districts VDM and GSDM for 2011 and 2012 seasons.

Fungal species isolated	Number of isolates					
	VDM			GSDM		
	Genus	2011	2012	Genus	2011	2012
Fusarium	43			135		
F. verticillioides		16	14		11	9
F. subglutinans		4	2		31	24
F. graminearum		0	0		23	14
Other *Fusarium*		5	2		17	6
Diplodia	19			34		
D. maydis		9	4		17	13
D. macrospora		5	1		3	1
Aspergillus	41			14		
A. flavus (MEA)		8	10		0	4
A. flavus (AFPA)		13	10		2	8



APPENDIX 5

Appendix 5.1: Standard (1v) and (2) LC-MS/MS spiking multi-component mixture (ng/ml).

(1v) components	Spiking level (ng/ml)	(2v) components	Spiking level (ng/ml)
Citrinin	187.5	15-acetyl-deoxynivalenol	500
Fumonisin B1	125	Aflatoxin B1	6.25
Fumonisin B2	125	Aflatoxin B2	6.25
Fumonisin B3	125	Aflatoxin G1	6.25
Moniliformin (-)	156.25	Aflatoxin G2	6.25
Beauvericin	25	Agroclavine	6.25
Enniatin A	125	Alternariol	125
Enniatin A1	125	Alternariol methylether	31.25
Enniatin B	125	citreoviridin	500
Enniatin B1	125	Deoxynivalenol	1250
		Diacetoxyscirpenol	125
		Fumagillin	500
		HT-2	125
		Mycophenolic acid	500
		Neosolaniol	125
		Nitropropionic acid	125
		Ochratoxin A	62.5
		Penicillic acid	625
		Roquefortine C	25
		Sterigmatocystin	25
		T-2	125
		Verruculogen	2500
		ZON (-)	62.5
		α-Zearalenole	312.5
		ß-Zearalenole	312.5
		Nivalenol	625
		deoxynivalenol-3-glucoside	250

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Appendix 5.2: Preparation of the Matrix-Matched Standard (MMS) range.

Solutions (µl)	MMS_0	MMS_1	MMS_2	MMS_3	MMS_4	MMS_5	MMS_6
Spike std	0	5	10	25	50	100	250
Dilution solv.	250	245	240	225	200	150	0
Extractant	250	250	250	250	250	250	250

*MMS - matrix matched standard

*Spike standard - Multi-analyte (1v) - (125µ1 (1v), 500µl (2v) + 1375µl H_20)

*Dilution solvent - (625µl 84% ACN + 1375 µl H_2O)

*Extractant – volume extracted

Appendix 5.3: Linearity and recovery results of the MMS calibration curve.

	Retention time (min)	Recoveries (%)		Regression equation	R^2
		MMS-low	MMS-high		
AFB1	5.0	90	96	y= 323702x-7218	0.9992
AFB2	4.8	104	93	y= 306525x-2922	0.9999
AFG1	4.4	92	95	y= 343570x-5519	0.9996
AFG2	4.3	75	96	y= 295982x-3345	0.9993
FB1	5.4	95	85	y= 22522x-2594	0.9993
FB2	6.2	91	87	y= 32567x-537	0.9997
FB3	5.9	90	90	y= 20266x-5996	0.9993

*MMS –low - Matrix-Matched Standard spiked at low concentration.

*MMS –high - Matrix-Matched Standard spiked at higher concentration.

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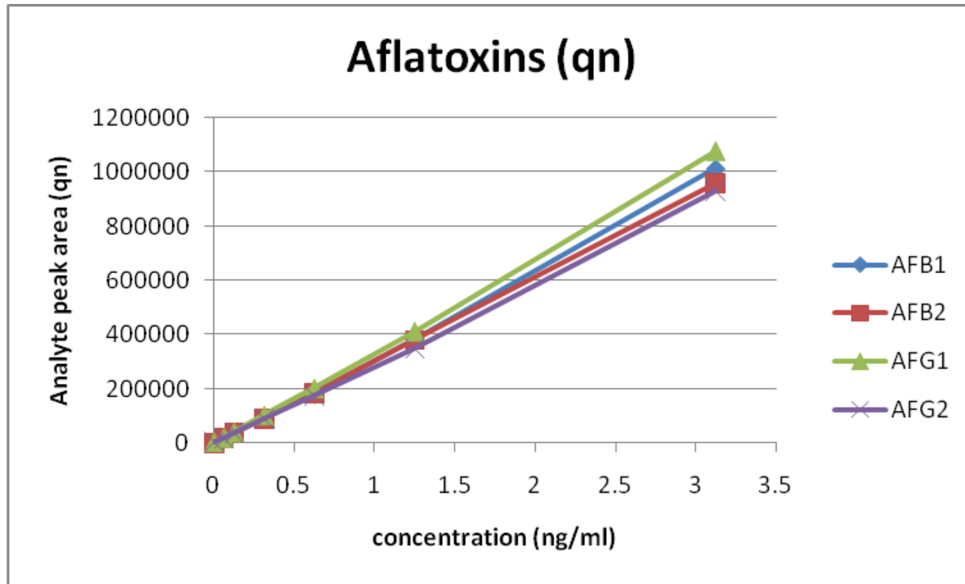
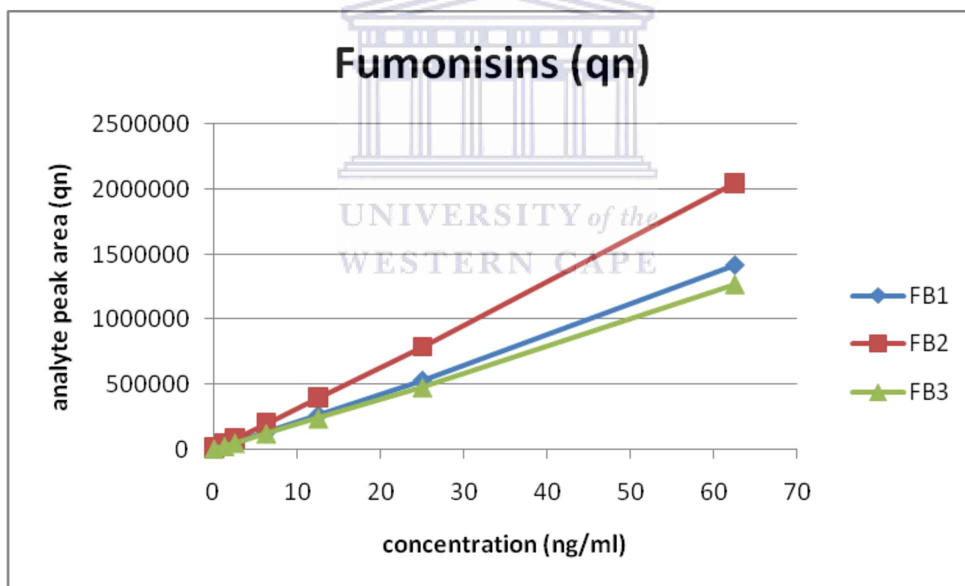
Appendix 5.4: MMS calibration curve was analysed by using linear regression.





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Appendix 5.5: The occurrence of aflatoxins (µg kg^{-1}) concentration in maize samples collected from Limpopo over the two seasons using an LC-MS/MS.

Household no.s	AFB_1	AFB_2	AFG_1	AFG_2	Total AF	Household no.s	AFB_1	AFB_2	AFG_1	AFG_2	Total AF
	2011						2012				
1y	nd	nd	nd	nd	nd	**1y(1)**	nd	nd	nd	nd	nd
2y	nd	nd	nd	nd	nd	**1y(2)**	nd	nd	nd	nd	nd
3y	nd	nd	nd	nd	nd	**2y**	nd	nd	nd	nd	nd
4(1)	14	2	33	4	54	**4y**	nd	nd	nd	nd	nd
4(2)	nd	nd	nd	nd	nd	**5y(1)**	nd	nd	nd	nd	nd
5y	nd	nd	nd	nd	nd	**5y(2)**	1	nd	7	2	10
6y	nd	nd	nd	nd	nd	**5y(3)**	1	nd	1	nd	2
7y	nd	nd	nd	nd	nd	**6y**	39	nd	93	12	143
8y(1)	nd	nd	nd	nd	nd	**7y(1)**	nd	nd	nd	nd	nd
8y(2)	nd	nd	nd	nd	nd	**7y(2)**	1	nd	1	nd	2
9y	nd	nd	nd	nd	nd	**8y**	nd	nd	nd	nd	nd
10y	nd	nd	nd	nd	nd	**9y**	nd	nd	nd	nd	nd
11y	133	15	1	<loq	149	**10y(1)**	nd	nd	nd	nd	nd
12y	nd	nd	nd	nd	nd	**10y(2)**	nd	nd	1	<loq	1
13y	<loq	nd	nd	nd	<loq	**11y**	73	5	<loq	<loq	78
14y	nd	nd	nd	nd	nd	**12y**	nd	nd	nd	nd	nd
15y	1	<loq	nd	nd	2	**13y**	nd	nd	nd	nd	nd
16y	70	6	nd	nd	76	**14y**	nd	nd	nd	nd	nd
17w	<loq	nd	nd	nd	<loq	**15y**	nd	nd	nd	nd	nd
17w	<loq	nd	nd	nd	<loq	**16y**	nd	nd	nd	nd	nd
18y	6	1	<loq	<loq	7	**17y**	nd	nd	nd	nd	nd
19y	67	4	3	<loq	73	**18y**	2	<loq	<loq	<loq	2
9(1)y	<loq	<loq	nd	nd	<loq	**19y**	23	<loq	29	<loq	51

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Household no.s	AFB_1	AFB_2	AFG_1	AFG_2	Total AF
		2011			
9(2)y	nd	nd	nd	nd	nd
10y	<loq	<loq	<loq	nd	1
13y	nd	nd	nd	nd	nd
14(1)y	<loq	nd	nd	nd	<loq
14(2)y	nd	nd	nd	nd	nd
15y	nd	nd	nd	nd	nd
20y	<loq	nd	nd	nd	<loq

W - White maize

Y- Yellow maize

Highlighted area represents yellow maize

Sample 21-M and 21-K represents maize collected in November 2011.

Nd - not detected

<loq - below the limit of quantification