A COMPARATIVE STUDY OF NATURAL CONTAMINATION WITH AFLATOXINS AND FUMONISINS IN SELECTED FOOD COMMODITIES FROM BOTSWANA AND ZIMBABWE

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DECLARATION

I declare that the study titled: “A comparative study of natural contamination with aflatoxins and fumonisins in selected food commodities from Botswana and Zimbabwe.” is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. It has been submitted and shall not be submitted in any form to any institution of higher learning for the award of any degree.

Signature of student Date

ACKNOWLEDGEMENTS

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DEDICATION

1. To my father, you never got the chance to say "…well done my son…", rest in peace.

2. In memory of Dr L S Teffo

ABSTRACT

Mycotoxins are toxic secondary metabolites produced by filamentous fungi. Aflatoxins and fumonisins are among the most toxic mycotoxins. They are a significant risk factor for a cocktail of chronic health conditions including cancer of the liver, oesophagus and kidney, teratogenicity, neural tube defects, interference with lipid metabolism, a weakened immune system and a negative impact on micronutrient absorption in both man and animals. This study compared natural contamination of peanuts, peanut butter and sorghum from Gaborone, Botswana and Bulawayo, Zimbabwe with aflatoxins and fumonisins. In total 34 peanut samples, 34 sorghum samples and 11 peanut butter samples were collected randomly from retail shops and informal markets in the two cities. Fungal contamination was determined using standard mycology methods. Aflatoxin and fumonisin contamination was determined using HPLC-FLD. *A. flavus/parasiticus* species were detected in 66% and 100% of randomly analysed peanut samples from Bulawayo and Gaborone respectively and 27% (3/11) of peanut butter samples from Bulawayo. 67% of randomly analysed sorghum samples from Bulawayo showed *A. flavus/parasiticus* and *Fusarium* species contamination while none of the randomly analysed sorghum samples from Gaborone showed any fungal contamination. Furthermore aflatoxins were not detected in any of the sorghum samples; however 61% (11/18) of the Bulawayo sorghum samples showed fumonisin contamination (Range: 8 – 187 ng/g). Three of the peanut samples from Bulawayo were contaminated with aflatoxins (range: 6.6 – 622 ng/g) and no aflatoxins were detected in Gaborone peanuts. All 11 peanut butter samples from Bulawayo were contaminated with aflatoxins (Mean: 73.5 ng/g, Range: 6.8-250 ng/g) and AFB1 was the most prevalent. These preliminary results indicate that peanut butter and peanuts from Bulawayo are contaminated with high levels of aflatoxins. Stricter policing of regulations should be implemented to ensure compliance by manufacturers and public health interventions implemented in vulnerable communities.

Key Phrases: mycotoxin, aflatoxins, fumonisins, *Aspergillus* species, *Fusarium* species, Zimbabwe, Botswana, peanuts, peanut butter, sorghum, PHRED, HPLC, AFPA, MEA+

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AFB:	Aflatoxin B
AFBO:	Aflatoxin B1-8,9-epoxide
AFG:	Aflatoxin G
AFM:	Aflatoxin M
AFPA:	*Aspergillus flavus* and *A.parasiticus* agar
CoA:	Co-enzyme A
CPA:	Cyclopiazonic Acid
CV:	Coefficient of variance
DON:	Deoxynivalenol
EU:	European Union
FA:	Fumonisin A
FB:	Fumonisin B
FC:	Fumonisin C
FP:	Fumonisin P
HBV:	Hepatitis B Virus
HBsAg:	Hepatitis B surface antigen
HCC:	Hepatocellular carcinoma
HCL:	Hydrochloric acid
HCV:	Hepatitis C virus
HGF:	Hepatocyte Growth Factor
HIV:	Human Immunodeficiency Virus
H_2O:	Water
HPLC:	High Performance Liquid Chromatography
IAC:	Immunoaffinity column
JECFA:	The Joint FAO/WHO Expert Committee on Food Additives

MEA^+:	Malt extract agar
MeOH:	Methanol
MRC:	Medical Reseach Council
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
NTD:	Neural tube defects
OTA:	Ochratoxin
OPA:	*o*-phthaldialdehyde
PDA:	Potato dextrose agar
PROMEC:	Programme on Mycotoxins and Experimental Carcinogenesis
SAX:	Strong Anion Exchanger
sIgA:	Secretory Immunoglobulin A
SPE:	Solid Phase Extraction
TLC:	Thin layer chromatography
TGF:	Transforming growth factor
UNISA:	University of South Africa
ZON:	Zearalenone

CHAPTER 1: INTRODUCTION

1.1 Background

Mycotoxins are low molecular weight (MW ~ 700) natural products produced as toxic secondary metabolites by filamentous fungi under a wide range of climatic conditions (Richard, 2007; Bennet and Klich, 2003). The term mycotoxin is a combination of the Greek word for fungus *'mykes'* and the Latin word *'toxicum'* meaning poison (Turner et al., 2009). Mycotoxins are fungal products toxic to vertebrates and other animal groups in small concentrations, while fungal products toxic to bacteria are called antibiotics and those toxic to plants are called phytotoxins (Bennett and Klich, 2003; Bennett, 1987). The term phytotoxin can also refer to toxins made by the plants (Bennet and Klich, 2003). Other low molecular weight fungal metabolites that are only toxic in high doses are not considered mycotoxins. Mushrooms and other macroscopic fungi make mushroom toxins. Many of the mycotoxigenic fungi are also plant pathogens and a relationship between fungal infection and mycotoxin production has been demonstrated (D'Mello and Macdonald, 1997).

The role that mycotoxins play in the biology of the fungus is not clear, they are neither necessary for growth nor development of the fungus in normal situations but in certain situations they are useful for the producing organism (Fox and Hewlett, 2008; Hof, 2008). It is suspected that since mycotoxins weaken the host, fungi may use them as a strategy to better the environment for further fungal proliferation. Some mycotoxins like penicillin and patulin are antibiotic hence may help in blocking bacteria that may be competing for nutrients with the fungi in the same ecological niche (Fox and Howlett, 2008; Hof, 2008). The mycotoxin deoxynivalenol (DON) is also thought to enhance the virulence of the fungus *Fusarium Graminearum* (Richard, 2007). However most of the fungi producing the toxins do not rely on a host to complete their life cycles and some of the detrimental effects conferred by mycotoxins on the host, for example cancer, only occur after the fungus is dead which does not confer any benefit to the fungus that produced it (Fox and Howlett, 2008; Hof, 2008; Bennet and Klich, 2003).

Mycotoxins contaminate many types of food crops and food commodities throughout the food chain (Reddy et al., 2010; Turner et al., 2009). They occur under natural conditions in the soil, food, feeds and the environment (D'Mello and Macdonald, 1997). Once the crop becomes infected in the fields, fungal growth usually continues through harvest, storage and

food processing as long as temperature and moisture content are optimal for growth (Reddy et al., 2010). Mycotoxins can develop during production, harvesting or storage of grains, peanuts and other food crops (Krska et al., 2008; Bhat and Vasanthi, 2003). Insect infestation and tropical conditions which include high temperatures and humidity, drought stress, unseasonal rain during harvest, and flash floods lead to fungal proliferation and mycotoxin production (Bhat and Vasanthi, 2003; IARC, 2002; D'Mello and Macdonald, 1997). Poor harvest practices, improper storage conditions and poor methods of food handling during transport and marketing have also been shown to lead to fungal and mycotoxin occurrence. (Reddy et al., 2010; Shephard, 2008b).

Mycotoxins are some of the most potent mutagenic and carcinogenic substances known to man (Krska et al., 2008; Bhat and Vasanthi, 2003). They pose various chronic health risks. Tricothecenes have been implicated in gastrointestinal disturbances such as vomiting, diarrhoea and inflammation (EMAN, 2011; Bennett and Klich, 2003). Aflatoxins have been shown to be a risk factor for cancer of the liver and kidney, a weakened immune system, negative impact on micronutrient absorption, teratogenicity, mutagenicity and infectious disease modulation (Reddy et al., 2010; Katerere et al., 2008a; Williams et al., 2004; Wild and Hall, 2000; Hendricks, 1999; Hendrickse, 1997). Fumonisins have been shown to cause porcine pulmonary oedema and leukoencephalomalacia in horses as well as being a major risk factor for oesophageal cancer (Richard, 2007; Bennet and Klich, 2003). The symptoms of mycotoxicosis depend on a number of factors which include the type of mycotoxin, amount and duration of exposure, age, gender and health status of the exposed individual or animal, and many other poorly understood synergestic effects involving genetics and co-occurrence (Richard, 2007; Bennett and Klich, 2003; D'Mello and Macdonald, 1997; Bennett, 1987).

As well as their role in disease etiology, mycotoxins also pose food safety risks, especially in developing countries where there is a lack of food security and quality is subsumed by food insufficiency (Katerere et al., 2008a; Shephard, 2003). In many developing countries, the combination of poor storage practices/infrastructure, climatic conditions and compromised food safety oversight mechanisms result in unacceptable/hazardous levels of mycotoxins entering the food chain (WHO, 2006). It is also known that mycotoxin exposure is a function of both contamination levels and food consumption thus consuming highly contaminated foods in moderate amounts or moderately contaminated foods in high amounts will lead to

high exposures (Shephard, 2008b). Most rural subsistence farming communities are faced with double adversity in that the staple foods they consume in high amounts are also contaminated at significant levels (Wild and Gong, 2010; Shephard, 2008b).

There are also financial and economic losses associated with mycotoxins (Bennett, 1987). Contaminated food is usually sold at very low prices and sometimes may not be traded on the formal market and/or has to be destroyed (Reddy et al., 2010; Bhat and Vasanthi, 2003). Other losses include reduced livestock production when animals are fed contaminated feed. The mycotoxin effects on animals include reduced productivity of milk, eggs, meat, weight gain, an inability to resist diseases as well as high mortality (Richard, 2007; Bhat and Vasanthi, 2003; Bennett, 1987). Both the quality and quantity of animal products are reduced resulting in huge financial losses for farmers (Bhat and Vasanthi, 2003). The contaminated animal products may also be passed on to the human diet (Krska et al., 2008; Richard, 2007), resulting in protein-energy malnutrition, especially in young children (Katerere et al., 2008a; Gong et al., 2002).

Additionally the cost of exporting mycotoxin safe food especially to the United States and the European Union is increased by investments to reduce mycotoxin contamination (Williams et al., 2004). The stringent maximum mycotoxin tolerable levels set by the industrialised countries, especially the European Union disadvantages many developing countries that rely on food exports for economic growth. Developing countries also export the least contaminated food leaving the contaminated food to be consumed at home; this may result in an increase in health care costs and the burden of disease including infectious diseases and cancers (Williams et al., 2005; 2004).

Mycotoxin exposures are generally lower in the developed world compared to the developing world (Wild and Gong, 2010; Reddy et al., 2010; Shephard, 2008a; Wu, 2004; Williams et al., 2004; Wild and Hall, 2000). This might be due to the fact that in the developed world diets are varied, the majority of the population obtain their food from formalized supply chains for example retail markets. Commercial food suppliers are rigorously monitored for quality and the legislated maximum tolerable levels for mycotoxins are widely enforced (Reddy et al., 2010; Shephard, 2008a; Wu, 2004). In contrast, the diets in the developing countries are less varied and the majority of the population rely on food from subsistence farming which is informally traded and impossible to monitor (Shephard, 2008a; Katerere et al., 2008a; Williams et al., 2004). To compound matters, food produced on a subsistence

basis is usually consumed irrespective of quality due to food scarcity problems as a result of poverty (Wild and Gong, 2010; Shephard, 2008a; Williams et al., 2004). Sometimes the alternative staple diets are also heavily contaminated with mycotoxins (Shephard, 2008b).

Even when food is sourced from commercial markets, quality of products is rarely monitored, and there is less emphasis on legislating maximum tolerable levels for mycotoxins (Liu and Wu, 2010; Shephard, 2008a). Even those countries that have legislation in place usually struggle to enforce these due to capacity constraints (Shephard, 2008a; Wu, 2004), especially lack of technological infrastructure (Williams et al., 2004). Also the analytical methods used to monitor mycotoxins are time consuming; require highly skilled personnel and expensive equipment, which use expensive reagents with short shelf lives (Turner et al., 2009; Abbas et al., 2004).

The growth of organic farming has led to debate on the possibility of high mycotoxin levels in organic food grown in the developed world (Benbrook, 2005). Organic farmers rely on organic matter which encourage fungal proliferation and do not use synthetic pesticides. This has led to some researchers postulating that organic farming produce might contain higher mycotoxin levels than conventional farming produce (Benbrook, 2005). However several comparative studies have detected mycotoxins up to 1.5 times more frequently in conventional farming systems than in organic farming and in most studies mycotoxin levels in conventional food exceeded those in organic food by a factor of 2.2 (Benbrook, 2005).

1.2 Problem Statement

Mycotoxins are a global problem. They are some of the most potent mutagenic and carcinogenic substances known (Bhat and Vasanthi, 2003). They pose a cocktail of chronic health risks including cancer of the liver and kidney, a weakened immune system, negative impact on micronutrient absorption, teratogenicity, mutagenicity and infectious disease modulation (Reddy et al., 2010; Katerere et al., 2008; Williams et al., 2005; 2004; Hendrickse, 1997). Their presence in food meant for human consumption poses food safety risks as well as financial and economic losses associated with poor quality food produce (Shephard, 2003). It is estimated that more than 5 billion people in the developing world are at risk of chronic exposure to mycotoxins through contaminated food (Sun et al., 2011; Williams et al., 2004; Shephard, 2003). Other than infectious agents, mycotoxins are ranked as the most important chronic food safety risk factor, and of greater risk than synthetic food

contaminants, plant toxins, food additives or pesticide residues (Reddy et al., 2010; Kuiper-Goodman, 1998).

It is virtually impossible to eliminate mycotoxins from the food chain because of the unpredictable, heterogeneous nature of mycotoxin contamination, hence the only practical solution is minimising the amount that ends up on the table and this can be achieved through proper regulation and intervention strategies (Reddy et al., 2010; Shephard, 2008b; Williams et al., 2004; Bennett and Klich, 2003; Peraica and Domijan, 2001; D'Mello and Macdonald, 1997;). However for Governments to regulate the levels of aflatoxins and fumonisins there is a need for constant surveillance to generate accurate data (Reddy et al., 2010; Shephard, 2008b). Such a surveillance study was last carried out in the late 1990's in Zimbabwe (Shephard, 2003; Gamanya and Sibanda, 2001; Henry et al., 1998; Nyathi et al., 1987; Wild et al., 1987) and in Botswana there are only three surveillance studies with the last one being in 2005 (Nkwe et al., 2005; Mpande et al., 2004; Siame et al., 1998).

1.3 Motivation of the study

From the information available it is clear that surveillance reports on Botswana and Zimbabwe are very few and outdated. This study endeavoured to generate current data on the extent of aflatoxin and fumonisin contamination in selected food commodities sourced from Gaborone, Botswana and Bulawayo, Zimbabwe. The study outcomes will inform the relevant authorities and policy makers how the two cities are faring in terms of food control in general and aflatoxin and fumonisin control in particular. This information would be instrumental in justifying broader country wide and sub-regional surveillance studies, relevant policy changes and possible public health interventions.

1.4 Aims and Objectives

The main aim of this study was to compare natural contamination of peanuts, peanut butter, and sorghum from Bulawayo, (Zimbabwe) and Gaborone, (Botswana) with aflatoxins and fumonisins. The specific objective of this study was to assess and compare natural occurrence of aflatoxins and fumonisins in selected food commodities in Botswana and Zimbabwe. This involved collection of peanut, peanut butter and sorghum samples, analysis and quantification of test samples for aflatoxins and fumonisins as well as analysis of fungal contamination in randomly selected test samples.

In sub-Saharan Africa peanuts are an important oilseed for local consumption and for export (Omer et al., 2001). Peanut butter is one of the cheap sources of protein especially among the poor socioeconomic groups. Peanut butter is a popular ingredient in children's food including sandwiches, porridge and vegetables. In Zimbabwe and Botswana peanuts are mainly grown by small scale farmers for household consumption and processing. Sorghum grows in very harsh environments where other crops do not usually grow easily. It is a major cereal and food source for Sub-Saharan Africa and India.

1.5 Limitations of the study

The high cost of sample collection and analysis as well as time constraints meant that only a limited number of samples were processed in this study. Thus the study is preliminary in nature. But it is significant in that the results can be used to build a case for more frequent surveillance studies with a broader reach in terms of sample size and geographical area.

1.6 Chapter Layout

Chapter 2 contains the review and critical analyses of relevant literature concerning aflatoxins and fumonisins. The incidence, economic, social and health effects and the mechanism of toxicity of aflatoxins and fumonisins are also covered. The chapter concludes by looking at the current legislation to limit aflatoxins and fumonisins in food and feed.

Chapter 3 outlines the methodology used to carry out the study, from sample collection, sampling methods, analytical method validation, sample analysis (including sample preparation, mycotoxin extraction, clean-up and analysis and mycological analysis). Background information on the study area is also covered.

Chapter 4 presents and discusses the results of mycological analysis of samples, method validation and chemical analysis of samples. The results are summarized in form of tables and graphs.

Chapter 5 gives a general discussion of the results and how the current study contributes to the body of knowledge. Conclusions and recommendations of the research are also made in this chapter.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Shephard (2008a) notes that human mycotoxicoses have probably existed since the development of settled agricultural communities reliant on grain stores. Mycotoxins became important in the 1960's after approximately 100 000 turkey poults died from eating a peanut meal contaminated with what became known as aflatoxins from *Aspergillus flavus* (Bennett and Klich, 2003; Forgacs, 1962; Blout, 1961). The five most important mycotoxins in terms of economic impact and risk to human health are aflatoxins, fumonisins, deoxynivalenol (DON), ochratoxin A (OTA), and zearalenone (ZON) (Fox and Hewlett, 2008; Bennett and Klich, 2003).

DON which is also known as vomitoxin is produced by Fusarium species and other fungi before harvest , and is one of about 150 related compounds known as trichothecenes (EMAN, 2011; Richard, 2007). It induces nausea and vomiting in animals, especially swine and its immunosuppressive effects may also cause kidney problems (Reddy et al., 2010; Richard, 2007). ZON is a phenolic resocyclic acid lactone that is oestrogenic when consumed by swine (Richard, 2007). It occurs in almost all the agricultural products including maize and maize products, breakfast cereals, beer, wheat flour, bread, walnuts and several animal feed products (EMAN, 2011). OTA is an innately fluorescent compound which is primarily a kidney toxin and carcinogenic and teratogenic effects have also been demonstrated in rats and mice (Richard, 2007). It is produced by *Aspergillus* species in tropical areas and *Penicillium* species in temperate areas (EMAN, 2011; Richard, 2007). However this study was primarily concerned with aflatoxins and fumonisins. These are discussed in greater detail below.

2.2 Aflatoxins

Aflatoxins are the most well known mycotoxins (WHO, 2006). There are four major aflatoxins, aflatoxin B1 (AFB1), AFB2, AFG1, and AFG2 (Reddy et al., 2010; Bennett and Klich, 2003). Classification is based on their relative chromatographic mobility during thin layer chromatography (TLC) and also on their fluorescence under UV light, blue (B) or green (G) (Bennett and Klich, 2003) while the numbers indicate major or minor compounds respectively (Sweeney and Dobson, 1999). Aflatoxins are difuranocoumarin derivatives with

closely related structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds (Figure 2.1).

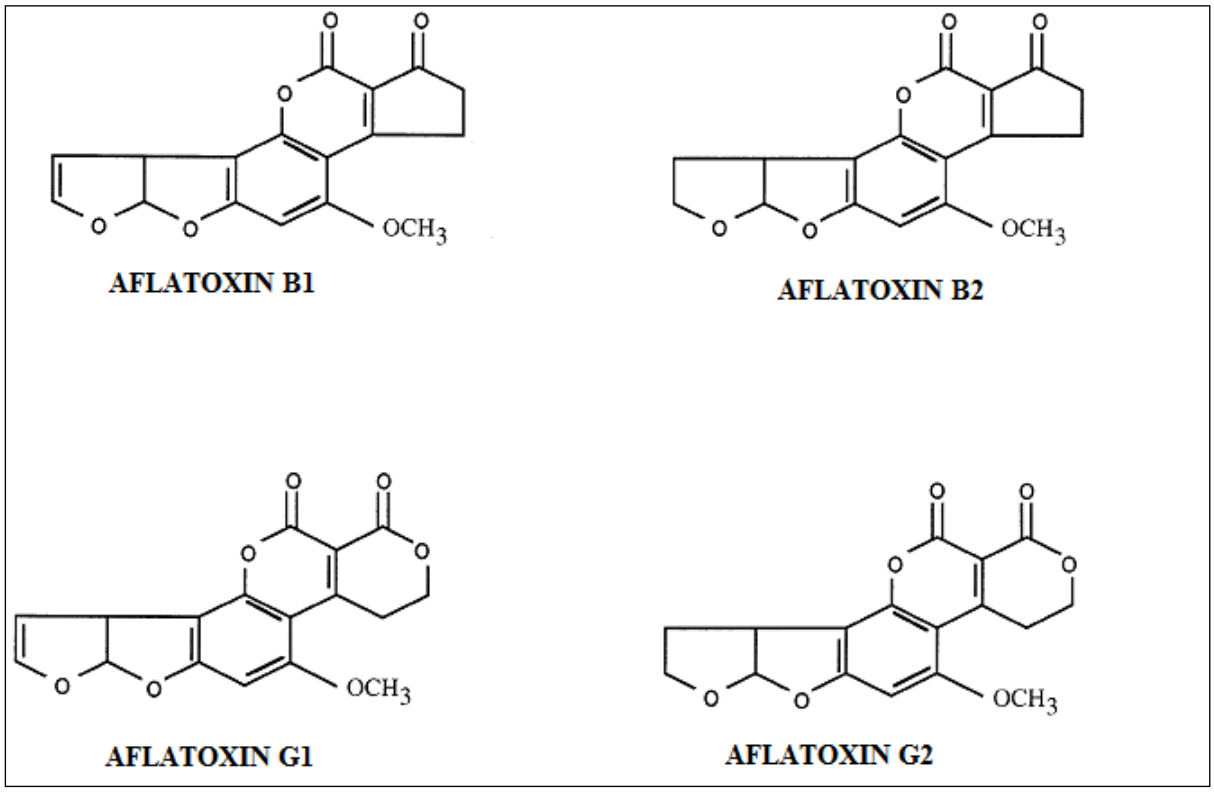

Figure 2.1: Structure of different aflatoxins (Sweeney and Dobson, 1999)

AFB1 is the most potent natural carcinogen known and has also been classified as a Group 1 human carcinogen by the IARC together with mixtures of AFB1, AFG1 and aflatoxin M1 (AFM1) (Wild and Montesano, 2009; Turner et al., 2009; IARC, 2002). It is usually the major aflatoxin produced by toxigenic strains and also the best studied (Bennett and Klich, 2003). AFB2 and AFG2 are classified as Group 2 probable human carcinogens (Reddy et al., 2010; IARC, 2002). AFM1 is a hydroxylated metabolite of AFB1 found primarily in animal tissues and body fluids like milk and urine (Reddy et al., 2010; Wild and Gong, 2010; Richard, 2007). AFM1 is thought to be both hepatotoxic and carcinogenic, but its toxicity and carcinogenic potential is estimated to be 8-10 times less than that of AFB1 (Wild and Gong, 2010; D'Mello and Macdonald, 1997). AFM1 is not a contaminant of grain products (Bennet and Klich, 2003).

2.2.1 Aflatoxin biosynthesis

Aflatoxins are produced via a complex polyketide pathway by many strains of *A. flavus* and the closely related *Aspergillus parasiticus* when the environmental temperatures are between 24 and 35°C and the moisture content exceeds 10% (Williams et al., 2004; Bennet and Klich, 2003; Bhat and Miller, 1991; Sweeney and Dobson, 1999). Biosynthesis involves 23 enzymatic reactions for conversion of acetyl coenzyme A (coA) to AFB1 (Rahimi et al., 2008). Acetate and malonyl CoA are converted to a hexanoyl starter unit by a fatty acid synthase and then to norsolorinic acid, the first stable precursor in the pathway (Sweeney and Dobson, 1999). The polyketide undergoes about 12-17 enzymatic conversions, eventually forming versicolorin B. The pathway then branches to form AFB1 and AFG1 which contain dihydrobisfuran rings and are produced from demethylsterigmatocystin. The other branch forms AFB2 and AFG2 which contain tetrabisfuran rings and are produced from dihydrodemthylsterigmatocystin.

The whole aflatoxin biosynthetic pathway is directed by 25 genes clustered in a 75-90 kb DNA region (Rahimi et al., 2008; Sweeney and Dobson, 1999). The physical order of the genes in the cluster appears to largely coincide with the sequential enzymatic steps of the pathway and both gene organisation and structure are conserved within *A. flavus* and *A. parasiticus*. The significance of this gene cluster is not known although the involvement of chromosome structure in gene regulation may be possible (Sweeney and Dobson, 1999). Also it is possible that the cluster may allow all of the pathway genes to be expressed rapidly upon onset of secondary metabolism since aflatoxins start to accumulate after 18-20 hours of mycelia growth (Rahimi et al., 2008). The conserved nature of this gene cluster may suggest that the function or regulation of aflatoxin biosynthesis may rely on an intact structural organisation (Sweeney and Dobson, 1999; Trail et al., 1995).

It has been previously noted that *A. flavus* produces only B aflatoxins and sometimes the mycotoxin cyclopiazonic acid (CPA), while *A. parasiticus* produces both B and G aflatoxins but never CPA (Shephard, 2009; IARC, 2002; Dorner et al., 1984). However, Gieser et al., (2000) noted that *A. flavus* (S strain *A. flavus*) isolates that produce abundant small sclerotia also produced both B and G aflatoxins, while those that produce fewer and larger sclerotia (L strain *A. flavus*) only produced B aflatoxins. In another study carried out on peanuts in Botswana by Mphande et al., (2004), 40.6% of all *A. flavus* isolates identified produced both B and G aflatoxins. However only one isolate produced sclerotia after 7 days incubation and

could be identified as an S strain in terms of the size of sclerotia (<400µm). Also some of the strains identified as *A. flavus* might have been the closely related *A. nomius* which produces all four aflatoxins (Mphande et al., 2004). Klich and Pitt (1988) also reported isolates of *A. flavus* producing G aflatoxins. The S strain *A. flavus* also produces higher levels of aflatoxins compared to the L strain (Mphande et al., 2004).

A. nomius is one of the recently identified aflatoxigenic fungi (Kurtzman et al., 1987). It is closely related to *A. flavus* but produces small bullet-shaped sclerotia, which are distinct from the large spherical sclerotia produced by many *A. flavus* isolates. However not all strains of either *A. nomius* or *A. flavus* produce sclerotia, so this distinction is not always practical. *A. nomius* is also distinguished from *A. flavus* by its ability to produce both B and G aflatoxins. The other new aflatoxigenic species recently identified include *A. bombycis* (Peterson et al., 2001), *A. pseudotamarii* (Ito et al., 2001), and *A. australis* (Geiser et al., 2000).

Currently available evidence indicates that *A. flavus* and *A. parasiticus* are responsible for the overwhelming proportion of aflatoxin contamination in food and foodstuffs throughout the world (IARC, 2002). Of the newer species only *A. australis*, which appears to be widespread in the southern hemisphere and is common in Australian peanut soils may also be an important source of aflatoxins in a few countries. The occurrence of aflatoxins is influenced by certain environmental factors; hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during preharvest, storage and/or processing periods (Park, 2002). Also during storage, humidity above 85% as well as the initial growth of fungi in kernels usually forms sufficient moisture from fungal metabolism to encourage further fungal growth and aflatoxin production (Richard 2007; Shephard, 2003). The optimal temperature for fungal growth is 36 to 38°C and maximum toxin production occurs at 25 to 27°C (Shephard, 2003; D'Mello and Macdonald, 1997).

2.2.2 Natural occurrence in food

A. flavus is the most widely reported food-borne fungus, sometimes with the proviso that *A. parasiticus* is sometimes not differentiated from it in general mycological profiling studies (IARC, 2002). Both *A. flavus* and *A. parasiticus* are abundant in the tropics and their occurrence in cooler temperate climates is uncommon except in foods and feeds imported from tropical countries. The major hosts of *A. flavus* among food commodities include maize,

peanuts, peanut butter, cotton seed and sorghum (Siame et al., 1998; Pitt and Hocking, 1997, Pitt et al., 1993). Food commodities associated with *A. parasiticus* include peanuts, peanut butter, and cotton seed but rarely in maize (Shephard, 2009; Pitt et al., 1993).

The crops that are associated with *A. flavus* (maize, peanuts, cotton seed, sorghum, wheat etc) have a potential of high contamination levels of aflatoxins because the fungus usually invades plants and developing seed before or immediately after harvest as well as in storage (Shephard, 2008a; Richard, 2007; IARC, 2002), even though some cereal crops like sorghum, wheat, and barley are not susceptible to pre-harvest aflatoxin contamination (Reddy et al., 2010). The fungi are disseminated via their conidia (asexual spores) which may be carried by wind or insects to growing crop (Reddy et al., 2010; Richard, 2007). Drought stress and insects damage (e.g. sap beetles, corn ear worms, and the European corn borer) usually provide a port of entry into the host plant tissue which leads to contamination of crops before harvest while improper drying and storage lead to contamination in storage facilities (Bennet and klich, 2003; Shephard, 2003,).

2.2.3 Health Risks

Human health effects of aflatoxicosis can be categorized as acute or chronic. Acute toxicity is generally caused by high toxic dose exposure and has a rapid onset while chronic toxicity is characterized by low-dose exposure over a long period of time, resulting in cancers and other generally irreversible effects (Bennett and Klich, 2003). Aflatoxins possess a high acute and chronic toxicity potential due to their capacity to bind DNA and cellular nucleoproteins resulting in deleterious effects on protein synthesis and cellular integrity (Covarelli et al., 2011). The dose and duration of exposure have a major effect on aflatoxin toxicology. Large doses lead to liver cirrhosis, acute illness and death while chronic sub-lethal doses have been shown to result in nutritional and immunologic consequences. All doses have a cumulative effect on the risk of cancer (Williams et al., 2004).

2.2.3.1 Acute effects

The symptoms of acute aflatoxicosis include hemorrhagic necrosis of the liver, bile duct proliferation, jaundice, oedema, fatty infiltration, toxic necrosis, gastro-intestinal haemorrhage and lethargy (Shephard, 2008b; Williams et al., 2004; Hendrickse, 1997). Adult humans usually have a high tolerance for aflatoxins and in acute cases it is usually the children who die (Williams et al., 2004; Cullen and Newberne, 1993). The numbers of acute

poisoning are rarely large compared to the population at risk, probably because humans are an aflatoxin tolerant species and people usually avoid visibly contaminated foods (Williams et al., 2004). In 2004, Kenya had the worst outbreak of acute aflatoxicosis. 317 cases manifested with acute hepatic failure, 125 of which were fatal (Azziz-Baumgartner et al., 2005; Lewis et al., 2005). High AFB1 levels were detected in maize collected from the affected areas with 35% and 7% of the samples having levels above 100µg/kg and 1000µg/kg respectively (Lewis et al., 2005). In general the levels of aflatoxins inducing acute toxicity are one or two orders above levels observed regularly in the staple foods of many populations worldwide hence the occurrence of cases of jaundice and acute liver failure unrecognized as aflatoxin poisoning is possible (Wild and Gong, 2010).

2.2.3.2 Chronic effects

The main human and veterinary health burden of aflatoxin exposure is related to chronic exposure which includes cancer induction, kidney toxicity, immune system suppression, and infectious disease modulation (Williams et al., 2004; Bennett and Klich, 2003). The data on aflatoxin as a human carcinogen is far more conclusive than the data implicating it in acute human toxicities (Bennett and Klich, 2003).

2.2.3.2.1 Hepatocellular Carcinoma

The liver has a central and critical biochemical role in the metabolism, anabolism and catabolism, detoxification and elimination of substances from the body (Crook, 2006; Dufour, 2006). Liver functions include glycogen storage, decomposition of red blood cells, immune factor synthesis, hormone production and bile production (Dufour, 2006). Due to its role in metabolism and detoxification the liver is also one of the most common sites for metastases with metastatic tumours accounting for 90-95% of all hepatic malignancies (Dufour, 2006). One of the most common and important primary liver tumours is hepatocellular carcinoma (HCC) (Kew, 2002).

HCC is among the most common cancers in the world (Groopman and Kensler, 2005), and is the second leading cancer in men and the fourth in women in sub-Saharan Africa (Sitas et al., 2006). Most patients survive less than a year after diagnosis (Wild and Hall, 2000). The incidence of HCC is unusually high in African and Asian regions (Reddy et al., 2010; Wild and Montesano, 2009; Kew, 2002). In 1987 HCC was the third most prevalent cancer in Zimbabwe accounting for 12% of all cancer cases and 2% of all deaths (Nyathi et al., 1987).

In the late 1990s a survey in Harare, Zimbabwe showed that HCC was affecting 11% and 5% of men and women (Chokunonga et al., 2000). A comparative estimated population risk of HCC between Kenya and France revealed a cancer risk of 11 versus 0.0015 cancers per year per 100 000 population respectively showing a heavy cancer burden in developing countries (Shephard, 2008a).

When people migrate from low HCC incidence countries to high incidence regions, for example Europeans moving to Africa or Asia, they always retain the low incidence of their country of origin (Kew, 2002). This may be due to the fact that these individuals usually retain their original behaviours and generally enjoy a higher standard of living than the locals. However migrants from high HCC risk areas to low risk regions show a decline in the incidence of tumours with successive generations (Kew, 2002), most probably because they adopt a higher standard of living and are no longer exposed to HCC risk factors in their home countries. These risk factors include infection with hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin exposure and chronic alcoholism (Herceg and Paliwal, 2011; Dufour, 2006; Wild and Hall, 2000), but the majority of the HCC cases in developing countries are associated with HBV, aflatoxins and HIV (Wild and Montesano, 2009; Kew, 2010). Figure 2.3 illustrates regional distribution of HCC attributable to aflatoxins.

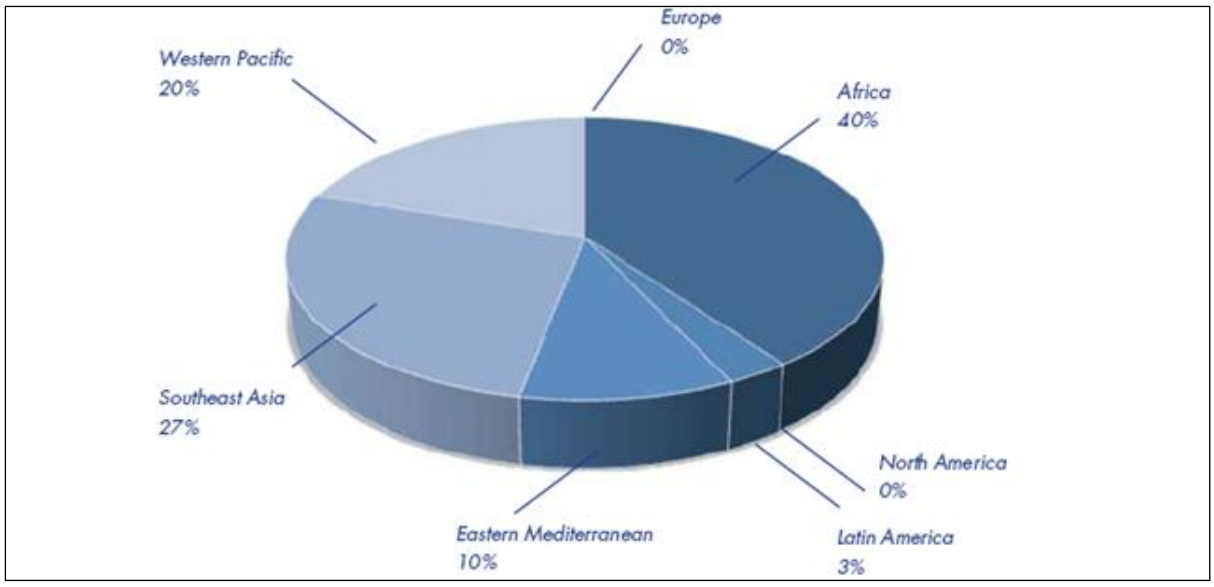

Figure 2.3: Distribution of HCC attributable to aflatoxins (Liu and Wu, 2010)

Aflatoxin exposure is etiologically associated with HCC (Wild and Gong 2010; Groopman and Kensler, 2005; Bennet and Klich, 2003). In the liver AFB1 is metabolised by the

cytochrome P450 enzyme system to its reactive intermediate aflatoxin B1-8,9-epoxide (AFBO), which readily binds to macromolecules to form adducts (Dufour, 2006; Bennet and Klich, 2003). It is the formation of DNA adducts such as AFB1-N7-guanine that leads to mutagenesis. If the adduct formed is not repaired, G to T transversion occurs at the third base of codon 249 of the p53 gene causing an inactivating mutation (Wild and Gong, 2010; Dufour, 2006) and this mutation is observed in HCC patients from regions of high aflatoxin exposure (Groopman and Kensler, 2005).

The AFB1-N7-guanine adduct is excreted in the urine of exposed individuals and can be used as a reliable biomarker for AFB1 exposure (Reddy et al., 2010; Wild and Gong, 2010), even though it only reflects recent exposure (Bennet and Klich, 2003). The AFB1 8,9-*exo*-epoxide intercalates more readily into the DNA yielding higher levels of adducts for a given dose making it more mutagenic and carcinogenic than AFG1 (Wild and Gong, 2010). AFB2 and AFG2 lack the 8,9 double bond hence do not form the 8,9-epoxide making them less toxicologically active than AFB1 and AFG1 (Wild and Gong, 2010).

Several epidemiological studies linking aflatoxins to HCC have been carried out. A study in Uganda by Alpert et al., (1971), indicated that the distribution of aflatoxins in foods stored for consumption correlated with the pattern of incidence of liver cancer in Uganda. In Mozambique, Van Rensburg et al., (1985) observed that mean aflatoxin dietary intake values were significantly related to HCC rates. They also noted variations in incidence rates between the regions studied and they attributed this to the variations in aflatoxin levels ingested. Li et al., (2001) compared aflatoxin contamination levels in maize from a high HCC incidence area with that from a low incidence area in Guangxi, China. They noted that aflatoxin levels were significantly higher in maize from the high incidence area compared to the low incidence area. They concluded that AFB1 appeared to be an important risk factor for primary HCC. In an area with endemic aflatoxin contamination of peanuts, Omer et al., (2001) noted a positive association between indicators of peanut butter consumption and hepatocelluar carcinoma in Sudan. However this was essentially limited to subjects with the glutathione-S-transferase M1 (GSTM1) null genotype who are unable to detoxify the aflatoxins properly. GSTM1 is an integral part in the body's defence system against reactive compounds.

It is also suspected that aflatoxins cause HCC by acting synergistically with HBV infection (Shephard, 2008; Groopman and Kensler, 2005). HBV DNA is thought to be integrated into the host genome, possibly caused by the action of the HBV X gene, which may block the

activity of p53, hence increasing the risk for HCC (Herceg and Paliwal, 2011; Dufour, 2006). Aflatoxins are thought to alter the pathogenicity of HBV by affecting susceptibility to infection and viral replication due to several factors including immune-suppression, DNA damage and mutations (Wild and Gong, 2010; Wild and Montesano, 2009). AFB1 has been shown to be immunosuppressive in animals and AFB1 exposure may affect susceptibility to chronic viral infection in vulnerable populations (Williams et al., 2004). HBV is also suspected to play a role in mutagenesis by causing preferential selection of cells harbouring this mutation while inflammation and oxidative stress associated with chronic HBV and aflatoxin exposure could result in DNA damage and mutations (Wild and Gong, 2010; Groopman and Kensler, 2005). Also increased cell proliferation and hyperplasia caused by HBV infection may lead to aflatoxin-induced DNA adducts being fixed as mutations due to chronic liver injury and regenerative hyperplasia which are critical in the development of liver cancer (Wild and Gong, 2010). Aflatoxin induced DNA damage is also suspected to increase viral DNA integration into the host genome (Wild and Gong, 2010). It is also hypothesized that aflatoxin induced DNA adducts are fixed as mutations due to HBV-related increase in cell proliferation and hyperplasia, thus promoting clonal expansion of mutant cells (Wild and Montesano, 2009). An increase in the rate of HCC has recently been reported in patients co-infected with HBV and HIV (Kew, 2010).

Aflatoxin and HBV infections commonly occur simultaneously in those regions with high HCC making aflatoxins a strong potentiating factor for human HCC (Reddy et al., 2010, Wild and Hall, 2000). A 7-year study in China revealed an association between markers of aflatoxin exposure and HBV infection and development of HCC (Qian et al., 1994; Ross et al., 1992). HBsAg positive individuals had a HCC relative risk of 7.3, but individuals that had both urinary aflatoxins and positive HBsAg status had a relative risk of 59 (Groopman and Kensler, 2005; Qian et al., 1994; Ross et al., 1992).

In an attempt to estimate the exact burden of aflatoxin-related HCC worldwide Lui and Wu, (2010) found that at the lower estimate aflatoxin may play a role in about 4.6% of total annual HCC cases and at the upper estimate, aflatoxin may play a role in roughly 28.2% of all HCC cases. Uncertainty and variability in data on cancer potency factors, hepatitis B virus (HBV) prevalence, aflatoxin exposure and other risk factors resulted in such a wide estimation range. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) also performed a quantitative risk assessment of AFB1 and concluded that in HBsAg positive

individuals, the potency was 0.3 cancers/year/100 000 population/nanogram (ng) AFB1/ kilogram (kg) body weight (b.w.)/ day and in HBsAg negative individuals the potency was 0.1 cancers/year/100 000 population/ng AFB1/kg b.w./day (WHO, 2002). Most cases of HCC (>80%) occur in either Eastern Asia or Sub-Saharan Africa (El-Serag and Rudolph, 2007; Liu and Wu, 2010), of which both Botswana and Zimbabwe are a part. These regions also have the highest levels of food contamination by aflatoxins as shown in figure 2.4 (Shephard, 2008a). It has also been noted that there is a geographic association between the contamination of food by aflatoxins and the incidence of HCC (Van Rensburg et al., 1985; Yeh et al., 1982; Bulatao-Jayme et al., 1982). However it is difficult to interpret data from human studies because of difficulties in properly assessing an individual's lifetime exposure to aflatoxins and the difficulties in disentangling the effects of aflatoxins from those of HBV infections (IARC, 2002).

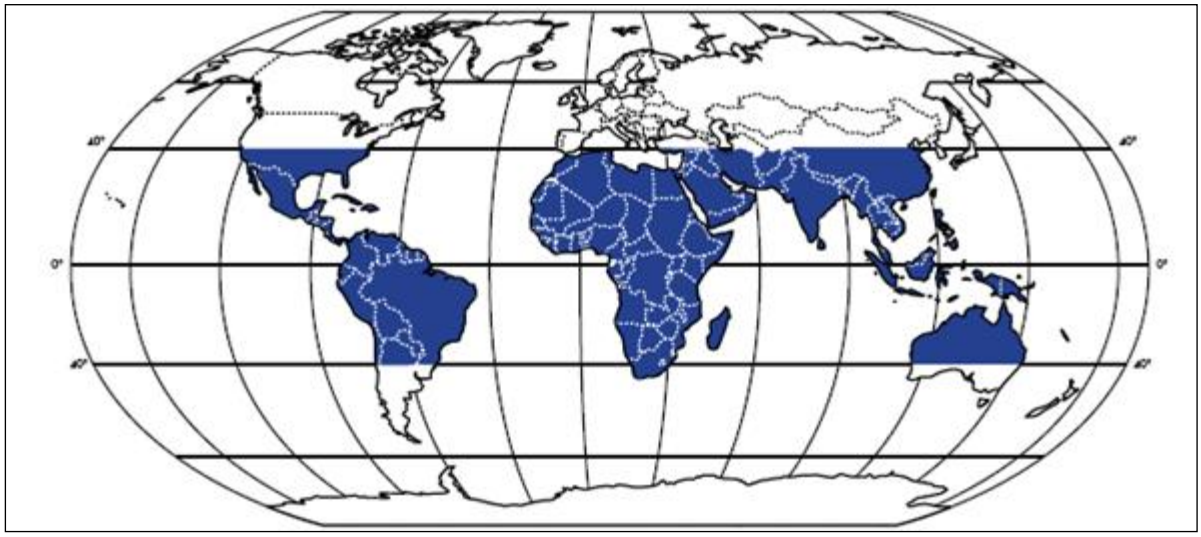

Figure 2.4: Areas at risk of chronic exposure to aflatoxins (Williams et al., 2004)

The overall body of evidence supports a role of aflatoxins in HCC etiology especially among HBsAg positive subjects (Williams et al., 2004; IARC, 2002), hence prevention of HCC can be achieved through HBV vaccination and reduction of aflatoxin exposure (Groopman and Kensler, 2005; Williams et al., 2004; Wild and Hall, 2000). However for the vaccine to be effective it must be delivered as early in life as possible since the majority of the people living in HCC high risk areas acquire HBV infection before age three (Groopman and Kensler, 2005; Wild and Hall, 2000). This vaccination will over time lessen the global carcinogenic impact of aflatoxin because removing the synergestic impact between HBV and aflatoxin exposure would significantly reduce HCC risk (Liu and Wu, 2010). It should

however be noted that HBV vaccination and aflatoxin reduction alone will not totally eliminate the incidence of HCC since there are many other factors (e.g. chronic alcoholism, diabetes, obesity and hereditary hemochromatosis) that lead to HCC development. Strategies to inhibit AFBO formation (through disruption of the cytochrome P450 system) and/or adduct formation are important in prevention of the damaging mutations that lead to HCC (Reddy et al., 2010).

2.2.3.2.2 Immunologic effects

A majority of the studies in poultry, pigs and rodents have shown that exposure to aflatoxins results in suppression of various aspects of the cell mediated immune response especially through altered cytokine expression and suppressed T-lymphocyte function (Wild and Gong, 2010; Reddy et al., 2010). Aflatoxin exposure results in increased susceptibility to bacterial and parasitic infections (Williams et al., 2004; IARC, 2002). Reduced humoral immunity has also been observed in aflatoxin exposed animals as well as increased susceptibility to infections and reduced response to vaccines (Williams et al., 2004). The amount of aflatoxin exposure is also influenced by HBV infection; this phenomenon has been extensively studied for its effect on cancer but has not been evaluated for other known toxicities of aflatoxins (Groopman and Kensler, 2005; Williams et al., 2004).

The threshold dose for aflatoxin immunotoxic effects in humans is not known (Williams et al., 2004), but is suspected to be above the threshold for malnutrition and growth stunting in children (Shephard, 2008b). Malnutrition and growth stunting might also influence immune response and consequently the disease burden as discussed in Section 2.2.3.2.3 below. In numerous animal studies, lymphocyte (T-cell, macrophage, natural killer cell, and B-cell) immune functions were suppressed or perturbed by AFB1 (Reddy et al., 2010). AFB1 may also decrease the lymphocytes' expression of the CD69 molecule which is essential for a normal immune response against an infectious agent or vaccine (Jiang et al., 2005).

Jiang et al., (2005) examined the cellular immune status of Ghanaians in relation to levels of AFB1-Albumin adducts in serum. They described the differential subset distributions and alterations of specific lymphocyte subsets between study participants with high and those with low levels of AFB1. The major changes were a decrease in activated T-cells and B-cells and significantly lower levels of perforin and granzyme A-expressing CD8+ cytotoxic cells in those with high AFB1 compared to those with low AFB1. This suggests that CD8+ T-cell

function in individuals with high AFB1 level is impaired hence affecting cellular immune function against infectious diseases (Jiang et al., 2005).

In Gambia, secretory Immunoglobulin A (sIgA) was found to be markedly lower in children with detectable serum aflatoxin-albumin compared with those with non-detectable levels (Turner et al., 2003), however the study failed to detect a dose-response effect when sIgA levels were separated by quintiles of aflatoxin-albumin biomarker levels (Shephard, 2008b; Turner et al., 2003). It is also known that sIgA is an important part of membrane barrier function that binds to bacterial and viral surface antigens (Turner et al., 2003) and that membrane barriers are an important component in fighting gastric diseases (Williams et al., 2004), hence aflatoxin exposed individuals are more prone to diarrheal diseases. This is compounded by the fact that the aflatoxin prone regions of the world are also plagued with lack of safe drinking water, sanitation and hygiene, the major factors implicated in about 88% of diarrheal diseases worldwide (Williams et al., 2004).

Williams et al., (2005) hypothesized that the decrease of sIgA levels as a function of aflatoxin exposure may modulate the infectivity of the HIV virus which is dependent on human sIgA levels and membrane integrity. When both the virus and aflatoxins suppress the body's immune system, a significant reduction in survival time is likely, particularly because these factors also compromise the nutritional status of the infected individuals (Williams et al., 2004). Aflatoxins have also been shown to suppress interleukin-2 (IL-2) production through the down regulation of transcription and it has the potential to effectively accelerate HIV progression by making CD4+ cells less effective (Jiang et al., 2005; Williams et al., 2004). In animals aflatoxicosis symptoms were shown to be similar to HIV infection symptoms and removal of aflatoxins from the diet reversed the symptoms (Williams et al., 2004). However most of the studies of immune-modulation in aflatoxin exposed populations have been cross-sectional, including relatively few subjects and have not been repeated in different populations hence are inconclusive (Wild and Gong, 2010).

2.2.3.2.3 Nutritional effects

Food conversion efficiency has been shown to be consistently less in animals that are exposed to aflatoxins than in those that are not exposed (Williams et al., 2004). A drop in food conversion efficiency (7-10% in pigs and poultry) and decreased growth rate are a consistent sign of aflatoxin exposure in animal studies. In children less than 5 years old in

Togo and Benin, chronic aflatoxicosis was also linked to both infant growth stunting (a reflection of chronic malnutrition) and being underweight (an indicator of acute malnutrition) (Gong et al., 2002). Children who were underweight or stunted had 30-40% higher mean aflatoxin-albumin levels (Gong et al., 2002). The growth stunting also coincides with weaning the children onto apparently aflatoxin contaminated solid foods (Gong et al., 2004). It is however not clear if the association between aflatoxin exposure and impaired growth is a direct result of aflatoxin toxicity or reflects consumption of fungus infected food of poor nutritional quality (Gong et al., 2002).

It should be noted that underweight children are prone to early mortality and acute morbidity due to diarrhoea, malaria, measles, pneumonia and other infectious diseases of childhood (Williams et al., 2004). However it is important to remember that malnutrition is influenced by several factors. These include micronutrient deficiency, poor maternal and child care, poor health services and environmental conditions, political and ideological factors, economic structure and potential resources (Kim et al., 2011; Frongillo et al., 1997). These factors are prevalent in the same geographical areas that are plagued with high aflatoxin exposure levels compounding the aflatoxin burden in vulnerable communities (Shephard, 2008a; Williams et al., 2004).

Aflatoxins are also closely associated with kwashiorkor, a nutritional disease of obscure pathogenesis prevalent in developing countries in the tropics (Katerere et al., 2008a; Hendrickse, 1997). Kwashiorkor has puzzling epidemiological features; it is a seasonal, tropical disease and goes hand in hand with overfeeding on starchy foods (Hendrickse, 1997). There are remarkable similarities in the geographical and climatic conditions that influence the prevalence of both kwashiorkor and aflatoxins (Reddy et al,. 2010; Hendrickse, 1997). Also the biochemical and metabolic derangements observed in kwashiorkor and those in controlled studies of animals exposed to aflatoxins are similar (Hendrickse, 1997). Aflatoxins have also been shown to impair the recovery rate from kwashiorkor (Katerere et al., 2008a; Hendrickse, 1997). However, it is still not clear whether the finding of aflatoxins in kwashiorkor is the cause or consequence of kwashiorkor (Peraica and Domijan, 2001).

Katerere et al., (2008a), reviewed available studies and data for a link between chronic aflatoxicosis and infant malnutrition in Southern Africa. They concluded that there is mounting evidence implicating aflatoxin contamination as an important factor in infant under-nutrition, increased morbidity and mortality due to negative impact on immune

function and micronutrient absorption. In animal studies aflatoxins have been shown to adversely affect absorption of vitamin A, vitamin D, selenium, and zinc but it is difficult to extrapolate animal studies data to humans due to a variety of factors including differences in species, patterns of exposure and metabolism (Willaims et al., 2004). Aflatoxins have also been shown to cross the human placenta (IARC, 2002) and the biochemical, immunological and metabolic derangements caused by aflatoxins in the foetus could lead to low birth weight and intrauterine growth retardation (Abdulrazzaq et al., 2002). In the United Arab Emirates, Abdulrazzaq et al., (2002), reported an extremely high rate of exposure of new born babies to significant levels of aflatoxins from their mothers and a negative correlation with birth weight.

2.2.4 Natural occurrence of aflatoxins in Africa

Extensive surveys of commercial maize crops performed in South Africa have consistently demonstrated a very low incidence of aflatoxin contamination (Shephard, 2003). However, analysis of samples of peanut butter being used in a school feeding scheme showed total aflatoxin contamination of up to 271 µg/kg (Shephard, 2003; PROMEC Unit, 2001) and AFM1 levels (range: 0.3-1.54 µg/L) above the South African legislation levels have been detected in raw milk (Dutton et al., 2011). Kamika (2012) reported total aflatoxin levels above 5 ppb in 60% (12/20, range: 2.1-73.83 ppb) and 85% (17/20, range: 4.02-825.67 ppb) of peanut samples collected from Pretoria, South Africa and Kinshasa, Democratic Republic of Congo respectively.

Total aflatoxin contamination levels above the acceptable limit set by the US Food and Drug Administration (FDA) were reported in peanut butter from Sudan with a range of 26.6-853 µg/kg, and a mean of 287 µg/kg (Elzupir et al., 2011). In Tanzania 18% maize samples were contaminated with total aflatoxins (range: 1-158 µg/kg) and 12% had AFB1 levels ranging from 5-90 µg/kg (Kimanya et al., 2008). AFB1 was also detected in beer samples (range: 10-50 µg/kg) in Dar es Salaam, Tanzania (Nikander et al., 1991). High levels of AFB1 were also detected in Ethiopia in red pepper (range: 250-525 µg/kg) and in a processed mixture of legumes (range: 100-500 µg/kg) (Shephard, 2003).In Nigeria AFB1 was detected in 63% of stored sorghum sample, 52% of marketed sorghum samples and 31% of field sorghum samples throughout the year (Hussaini et al., 2009). The highest incidence was during the rainy season.

2.2.4.1 Botswana

Peanut, peanut butter, sorghum, chicken feed and mophane worms (an edible larval stage of the emperor moth *Imbrasia belina* Westwood) samples from Botswana showed high levels of aflatoxin contamination (Siame et al., 1998). All mophane worms were purchased from harvesters in the field and from retail outlets while all the other samples were collected from storage depots or bought from retail outlets. Peanuts, peanut butter and mophane worms had aflatoxin levels ranging from 3.2-48 µg/kg, 1.6-64 µg/kg and 0.1-10 µg/kg respectively. Sorghum, sorghum meal and chicken feed showed minor aflatoxin contamination ranging from 0.1-0.7 µg/kg while maize showed no aflatoxin contamination (Siame et al., 1998). It should be noted that only 4 chicken feed samples were tested hence the results might not be statistically representative.

Mphande et al., (2004), reported 78% of raw peanuts tested in Botswana to be positive for aflatoxin contamination (range: 12-329 µg/kg, mean: 118 µg/kg). AFB1 was the most prevalent being found in 65% of the samples tested. The samples were bought from different retail outlets in the country. However, it should be noted that the investigators collected samples into plastic bags and this could have provided a perfect environment for aflatoxin proliferation post collection. The investigators also noted that most of the peanuts sold in Botswana are imported, yet they did not capture information on the original source of the samples as well as storage and shipping conditions.

No aflatoxins were detected in sorghum malt although *Aspergillus* species were isolated in 44% of the samples and *A. flavus* was isolated from 37% of the samples (Nkwe et al., 2005). This confirms the understanding that the presence of fungal isolates does not necessarily mean presence of toxin. There are no official aflatoxin regulatory levels in Botswana (Mphande et al., 2004; Siame et al., 1998), but the maximum allowable levels of total aflatoxins in food meant for human consumption in Codex Alimentarius countries is 15 µg/kg (Codex, 2001).

2.2.4.2 Zimbabwe

Routine monitoring of groundnuts by the Zimbabwe Government Analyst Laboratory noted seasonal variation in aflatoxin contamination. About 46% of the samples analysed during the 1995 season and 8% of the samples analysed during the 1996 season were contaminated with levels above 10 µg/kg (Shephard, 2003; Henry et al., 1998). Studies in the rural villages

indicated presence of AFM1 in human breast milk at levels up to 0.05 ng/mL, thus raising concerns about post-natal exposure to aflatoxins (Shephard, 2003; Wild et al., 1987). Recently Siwela et al., (2011), carried out a study to monitor aflatoxin carryover during large scale peanut butter production, their findings reveal that peanuts from local farmers have high levels of aflatoxin contamination (>80 ng/g in raw peanuts).

Nyathi et al., (1987), carried out a survey of urinary aflatoxin biomarkers in Zimbabwe and detected aflatoxins in 4.3% and 4.4% of samples from rural areas and urban areas respectively. There was no association between aflatoxin contamination and altitude or rainfall, but there was a significant reduction in aflatoxins in the low temperature province of Manicaland. Also the degree of mobility of the population in Zimbabwe between rural and urban areas is quite significant hence it was difficult for the investigators to draw conclusions about any differences based on whether donors belonged to the urban or rural community. However the highest percentages of contaminated samples for individual centres came from rural areas. The investigators also noted that since most of the samples came from hospitals and clinics, a selection bias towards the sick as opposed to a normal cross-section of the community could have risen.

Zimbabwe is one of the African countries that have aflatoxin regulations in place and 20µg/kg is the maximum allowable level of total aflatoxins in human food. There are no recent mycotoxin studies in Zimbabwe and to compound this situation, the food security situation has deteriorated extensively during the last decade due to a political and economic crisis (Mutisi, 2009). There is therefore an urgent need for continuing surveillance studies in the country.

2.3 Fumonisins

Fumonisins were first discovered in South Africa by Gelderblom et al., (1988) and were chemically characterised by Bezuidenhout et al., (1988). They are a group of non-flourescent mycotoxins thought to be synthesized by condensation of the amino acid alanine into an acetate derived precursor (Bennet and Klich, 2003; Sweeney and Dobson, 1999). Branched chain methyl groups are added at C-12 and C-16 by an S-adenosyl methionine . Much of the biosynthetic pathway is yet unknown and none of the enzymes involved in the fumonisin biosynthetic pathway have been isolated (Sweeney and Dobson, 1999). The fumonisin chemical structure, which is a C-20 diester of propane-1,2,3-tricarboxylic acid and a

pentahydroxyicosane containing a primary amino acid, resembles sphingosine which forms the back bone of sphingolipids (IARC, 2002; Merrill et al., 2001; Sweeney and Dobson; 1999). Fig 2.5 shows the structural formulas of the major fumonisins.

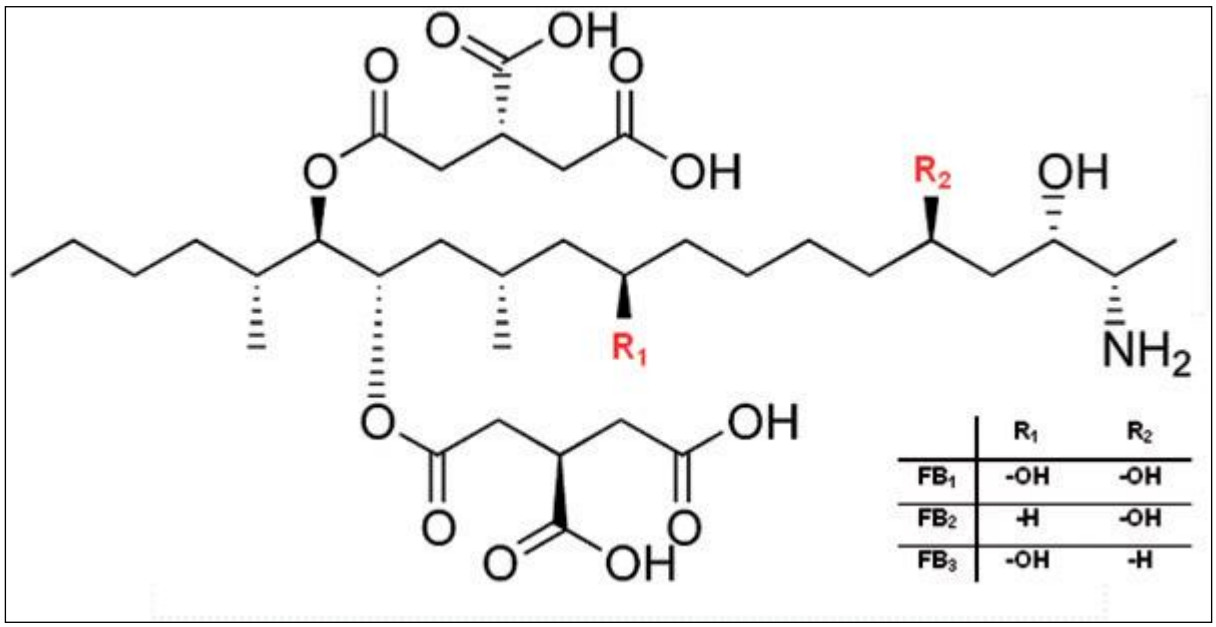

Figure 2.5: Structural formula of fumonisin B1- B3 (Scott, 2012)

More than 28 fumonisin analogues have now been described and classified as A, B, C, and P series (Rheeder et al., 2002). The major fumonisins are fumonisin B1 (FB1), FB2 and FB3 (Wild and Gong, 2010; Richard, 2007). FB1 is the most commonly found and the most toxic (Scott, 2011; Bennet and Klich, 2003). The other fumonisin analogues differ slightly from the FB series. The FA series is acetylated on the amino group at the C-2 position whereas FB series have a free amine, the FC series lack the methyl group at the C-1 position and the FP series have a 3-hydroxypyridinium functional group at the C-2 position (van der Westhuizen, 2011; Bezuidenhout et al., 1988). Fumonisins are difficult to study because they are hydrophilic, but they are usually extracted with aqueous methanol or aqueous acetonitrile and analysed by high performance liquid chromatography (HPLC) with fluorescence detection (IARC, 2002; Gelderblom et al., 1988).

2.3.1 Fumonisin Biosynthesis

Fumonisins are primarily produced by *Fusarium verticillioides* (formerly known as *F. moniliforme*), *F. proliferatum,* the principal causal agents of the "pink ear rot of maize" and other *Fusarium* species, during kernel colonization (Scott, 2011; Covarelli et al., 2011;

Richard, 2007). These fungi are taxonomically challenging, with a complex and rapidly changing nomenclature that has perplexed both mycologists and non-mycologists (Reddy et al., 2010; Bennett and Klich, 2003). *F. vertillioides* is the major species of importance; it grows as a corn endophyte in both the vegetative and reproductive tissues, often without causing disease symptoms in the plant (Reddy et al., 2010). However when conducive weather conditions, insect damage, and the appropriate fungal and plant genotype are present, it can cause seedling blight, stalk rot, and ear rot (Richard, 2007; Bennet and Klich, 2003).

2.3.2 Natural occurrence in food

Maize and maize based foods are the most affected by fumonisins even though occurrence has also been reported in sorghum, rice, soyabeans and cowpeas (Scott, 2011; Richard, 2007; IARC, 2002). In general much lower concentrations than are common in maize have been reported (EMAN, 2011). Fumonisins have also been detected in a number of finished food products including maize based cereals and beer but not in animal products like meat, eggs and milk (Richard, 2007). Drought stress followed by warm wet weather later in the growing season and insect damage seem to be important factors in fungal infection of the growing crop (Richard, 2007). However it should be noted that *F. verticillioides* is abundant in nature and is present in virtually every seed and is present in the plant throughout its growth and is therefore present in the ears and kernels (Richard, 2007; Bennet and Klich, 2003). Most fungal strains do not produce the toxin hence the presence of the fungus does not necessarily mean the presence of fumonisins (Bennett and Klich, 2003).

2.3.3 Health Risks

The two well-known conditions caused by acute fumonisin toxicity are porcine pulmonary oedema and leukoencephalomalacia in horses (Scott, 2011; IARC, 2002). Both these diseases involve disturbed sphingolipid metabolism and cardiovascular dysfunction. In humans fumonisins have been implicated in one incident of food borne poisoning in 27 villages in India (Bhat et al., 1997). The outbreak resulted from infection of crops by *F. verticillioides* due to unseasonal rain during harvest season. The main features of the disease were transient abdominal pain, borborygmus and diarrhoea which began about 30 minutes to an hour after consumption of unleavened bread prepared from mouldy sorghum or maize (Bhat et al., 1997). All the patients recovered fully when the offending food was withdrawn. It is also important to note that all the individuals affected were from the poorest social strata; who are

also at high risk of other infectious diseases due to food insufficiency, poor hygiene and sanitation, and lack of a comprehensive health care system (Williams et al., 2004; Shephard, 2003; Bennet and Klich, 2003).

Several studies have been conducted using experimental animals to check the carcinogenicity, toxicokinetics, and genotoxicity of fumonisins especially FB1 (Lemmer et al., 1999; Gelderblom et al., 1997; 1996; 1991; Wilson et al., 1985). FB1 was shown to be carcinogenic in rodents exhibiting both cancer initiating and promoting effects (Sun et al., 2011; Wild and Gong, 2010). Experimental animals fed with either pure culture of *F.verticillioides* or FB1 showed decreased weight gain and toxic liver injury and also developed papillomas and carcinomas in the forestomach, hepatocellular carcinomas, hepatic nodules, cholangiofibrosis and cholangiocarcinomas (Lemmer et al., 1999; Gelderbloem et al., 1997; 1996; 1991; Wilson et al., 1985; Marasas et al., 1984). FB1 has been shown to be hepatotoxic and nephrotoxic in experimental animals, causing increased apoptosis followed by regenerative cell proliferation (IARC, 2002). FB1 has also been shown to be acutely poisonous to rats, and high doses caused death within 3 days (Gelderblom et al., 1988). However fumonisins are poorly absorbed and rapidly excreted in unmetabolised form in animal systems hence there is no known risk of fumonisin carryover in animal products like milk, meat and eggs (Richard, 2007; Voss et al., 2001).

2.3.3.1 Interference with sphingolipid metabolism

Sphingolipids are a highly diverse class of lipids found in all eukaryotic cells especially in the plasma membrane and related cell membranes (Merrill et al., 1997). They serve as structural components critical for maintenance of membrane structure, as receptors for vitamins and toxins, as sites for cell-cell recognition and cell-cell and cell-substrate adhesion, as modulators of receptor function, while complex sphingolipids serve as lipid second messengers in signalling pathways responsible for cell growth, differentiation and death (IARC, 2002; Merrill et al., 1997).

Fumonisin B1 inhibits the co-enzyme A (CoA)-dependent acylation of sphinganine to sphingosine via interaction with the enzyme sphinganine/sphingosine N-acyltransferase (ceramide synthase) (Voss et al., 2009; Turner et al., 2009; IARC, 2002; Marasas et al., 2004; Merrill et al., 2001). Ceramide synthase is the enzyme in the *de novo* biosynthetic pathway of sphingolipids that adds a fatty acid to sphinganine to form dihydroceramide and also acts in

the reacylation of sphingosine derived from turnover of more complex sphingolipids (Merrill et al., 1997). This enzyme recognises both the amino group (sphingoid binding domain) and the tricarboxylic side chains (fatty acyl-CoA domain) of FB1 (IARC, 2002; Merrill et al., 2001). Its inhibition leads to an accumulation of the sphingoid bases and their 1-phosphate levels and decreases ceramide and more complex sphingolipids like sphingomyelin and gangliosides and their intermediates (Merrill et al., 1997). Voss et al., (2009) demonstrated that hydrolysed FB1 (HFB1) also disrupts sphingolipid metabolism even though it was a significantly less potent modulator of sphingolipid metabolism than FB1. Figure 2.6 illustrates the pathways of sphingolipid biosynthesis and turnover in mammalian cells. In boxes are the known biological activities affected by FB1 inhibition of ceramide synthase and associated with changes in the biosynthesis of various sphingolipid intermediates and products.

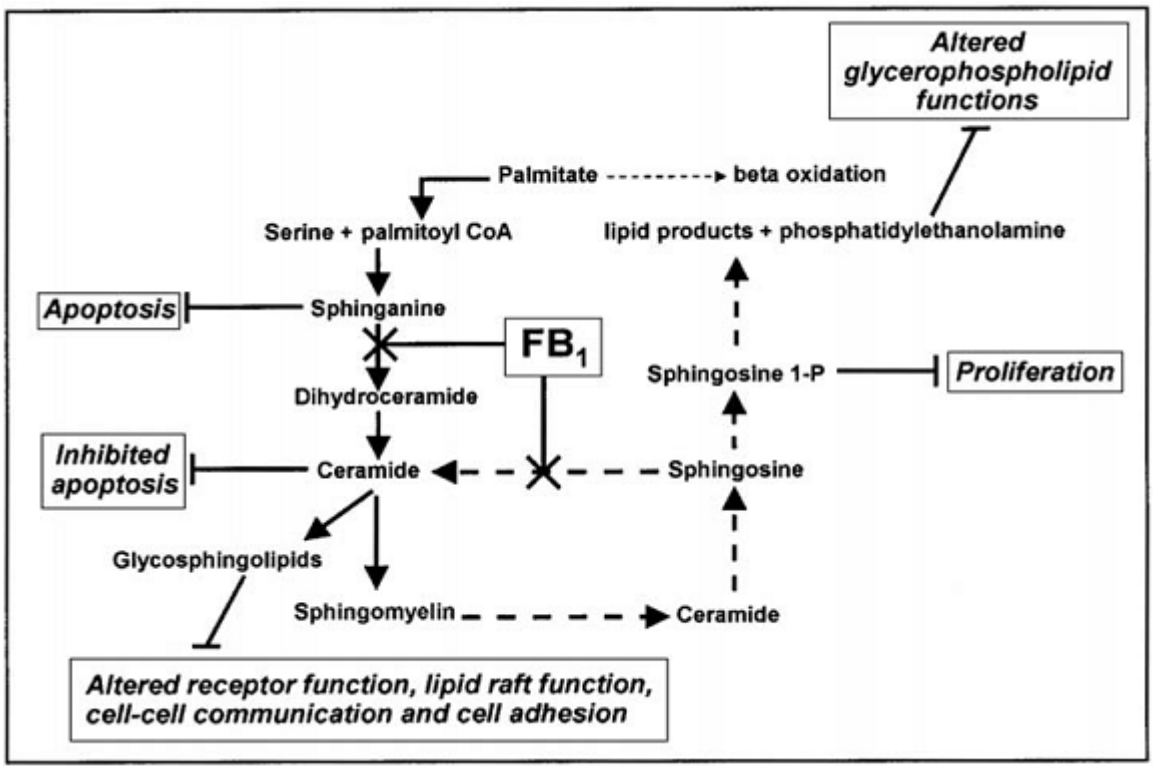

Figure 2.6 Pathways of sphingolipid biosynthesis and turnover in a mammalian cell (IARC, 2002).

This inhibition results in sphingoid base accumulation, imbalances of the sphingoid base metabolites, fatty acid and glycerophospholipids, disruption of sphingolipid metabolism and *in vivo* toxicity, depletion of complex sphingolipids, and increased dihydroceramide *in vivo*

(IARC, 2002*). In vivo* there is a close relationship between sphingoid base accumulation and the expression of fumonisin toxicity in the liver and kidney (IARC, 2002; Voss et al., 2001). The resulting increase in the sphinganine/sphingosine ratio occurs prior to changes in other biochemical markers of cellular injury and is used as a biomarker for fumonisin exposure in animals (Voss et al., 2009; Peraica and Domijan, 2001). Plasma sphinganine and sphingonine levels for male and female participants were reported to be higher in the high oesophageal cancer incident area than in the low incidence area in rural South Africa (van der Westhuizen et al., 2008).

Inhibition of ceramide synthase by FB1 can also result in the redirection of substrates and metabolites to other pathways, a good example is when accumulated sphinganine is metabolised to sphinganine-1-phosphate, whose breakdown results in production of aldehyde (a fatty acid) and ethanolamine phosphate (IARC, 2002). Disrupted sphingolipid metabolism leads to imbalances in phosphoglycerolipid, fatty acid metabolism and cholesterol metabolism via free sphingoid base- and sphingoid base 1-phosphate-induced alterations in phosphatidic acid phosphatise and monoacylglycerol acyltransferase (IARC, 2002).

2.3.3.2 Neural tube defects

Neural tube defects (NTDs) are common congenital malformations that occur when the embryonic neural tube, which forms the brain and spinal cord fail to close properly during the first few weeks of development (Waes et al., 2005; Marasas et al., 2004). It is assumed that through competitive inhibition of folate uptake, fumonisins induce NTDs in newborns (Hendricks, 1999). Depletion of cellular sphingolipids by FB1 has been shown to cause almost complete inhibition of uptake of 5-methyltetrahydrofolate by the folate receptor, and it is suggested that dietary exposure to FB1 could therefore adversely affect folate uptake and potentially compromise cellular responses dependent on this vitamin (Stevens and Tang, 1997). Figure 2.7 shows how fumonisins inhibit sphingolipid biosynthesis and affects the folate transporter. The scheme shows the step where fumonisins inhibit sphingolipid biosynthesis (the acylation of sphinganine by ceramide synthase in the endoplasmic reticulum) thereby reducing the formation of sphingomyelin which is a major component of the plasma membrane and is required for the proper function of Glycosylphosphatidylinisotol (GPI) – anchored proteins such as the folate transporter.

Voss et al., (2009) demonstrated that FB1 induces NTDs in mice but the hydrolysed FB1 proved to less potent and did not cause any NTDs even at 3.5 times the FB1 dose. *In vitro* HFB1 has also been shown to inhibit ceramide synthase in vitro less effectively than FB1 (Humpf and Voss, 2004). Waes et al., (2005) demonstrated that maternal exposure to FB1 during early pregnancy results in NTDs in the exposed foetus in mice and that ganglioside GM1 therapy was able to restore embryonic folate concentrations above some critical threshold level necessary for normal neural tube closure to take place. However they could be many confounding factors, like maternal nutritional status including folate and vitamin B12 deficiency as well as obesity (Missmer et al., 2006; Waes et al., 2005), that might have played a role in the development of NTDs in all these studies.

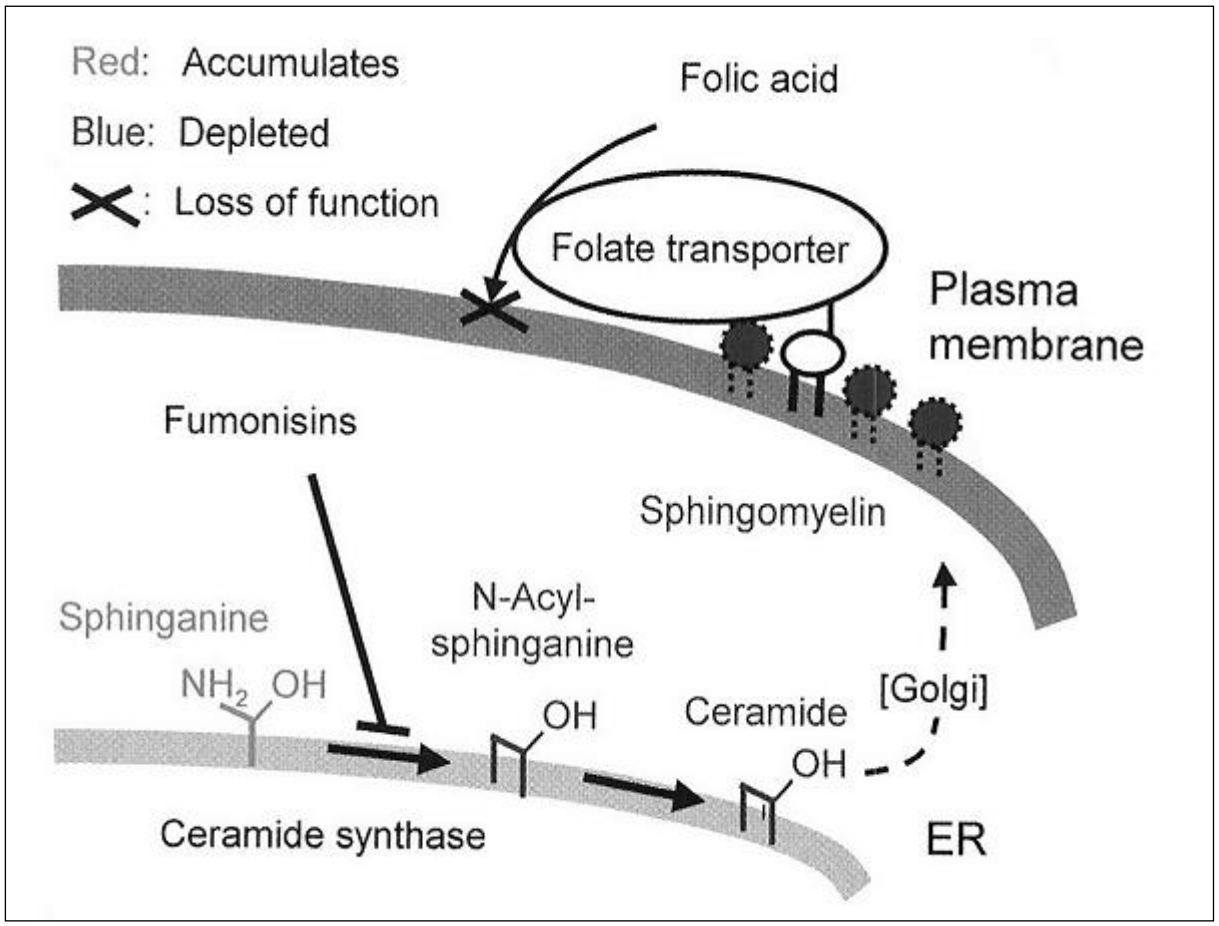

Figure 2.7: Disruption of sphingolipid metabolism and folate transport by fumonisins (Marasas et al., 2004).

Epidemiologically it has been shown that fumonisins may be a risk factor for NTDs (Missmer et al., 2006; Hendricks, 1999). The rate for NTDs in black people in the Transkei area of South Africa was found to be about 10 times greater than that in black people in Cape Town,

South Africa (Kromberg and Jenkins, 1982). The Transkei area has significantly elevated levels of fumonisins in maize based foodstuffs as well as a high rate of oesophageal cancer (Marasas, 1996; 1993; Rheeder et al., 1992; Marasas et al., 1981). NTDs have been found to be 3 to 5 times higher along the Texas-Mexico border than anywhere else in the United States (Marasas et al., 2004; Hendrickse, 1999). The population along the border consume a high amount of maize based products and are therefore exposed to high daily intake of fumonisins which might explain the high incidence of NTDs (Missmer et al., 2006; Marasas et al., 2004; Hendricks, 1999).

2.3.3.3 Interference with fatty acid and glycerophospholipid metabolism

Essential fatty acids are major constituents of all cell membrane glycerophospholipids, sphingolipids and triglycerides (IARC, 2002). Their most important role is acting as structural components of all cell membranes; they also act as precursors of many bioactive lipids known to regulate cell growth, differentiation and cell death (IARC, 2002). Gelderblom et al., (1996) exposed rat liver and primary hepatocytes to FB1 and changes in the phospholipid profile and fatty acid composition of phospholipids indicate that FB1 interferes with fatty acid metabolism. The pattern of changes in specific polysaturated fatty acids suggests disruption of the Δ6 desaturase and cyclo-oxygenase metabolic pathways (IARC, 2002; Gelderblom et al., 1996). However, Gelderblom et al., (1997), showed that the pattern of changes *in vivo* is different from that observed *in vitro.* Major changes were associated with both the phosphatidyl-ethanolamine and the phosphatidylcholine phospholipid fractions while cholesterol levels in both the liver and serum are increased (IARC 2002; Gelderblom et al., 2001).

Numerous changes in the expression of proteins known to be involved in the regulation of cell growth, apoptosis and cell differentiation have been shown at FB1 doses that alter fatty acid and glycerophospholipid profiles in rat liver (WHO, 2002). Expression of hepatocyte growth factor (HGF), transforming growth factor ά (TGF ά), TGF β_1, c-*myc* oncogene were all increased during short term feeding of FB1 (Lemmer et al., 1999). Increased expression of the proto-oncogene c-*myc* could contribute to the enhanced cell proliferation that is required for the tumor progression observed in the livers of rats and mice exposed to hepatotoxic levels FB1 while overexpression of TGF β_1 could play a role in increasing apoptosis (IARC, 2002). Overexpression of TGF β_1 and c-*myc* (Lemmer et al., 1999) and oxidative damage

could further enhance apoptosis and alter cell growth in affected hepatocytes (WHO, 2002; Gelderblom et al., 2001).

FB1 disruption of sphingolipid metabolism and altered membrane phospholipids have been suggested to cause changes in several proteins (for example, cyclin D1 and retinoblastoma protein) that regulate cell cycle progression (IARC, 2002; Rmaljak, 2000). Accumulation of cyclin D1 is due to post-translational stabilisation of the protein (Ramljak et al., 2000). FB1-induced alterations in cellular glycerophospholipid content and the sphingomyelin cycle are thought to interact so as to modify a variety of cellular processes resulting in the increased apoptosis and altered hepatocyte proliferation (IARC, 2002). All these alterations are hypothesized to result in growth impairment of normal cells, selectively stimulating growth of hepatocytes that are resistant to FB1, hence increasing the likelihood of cancer development (WHO, 2002; IARC, 2002; Gelderbloem et al., 2001).

2.3.3.4 Oesophageal cancer

Fumonisins have been identified as an important risk factor in the occurrence of human oesophageal cancer (Marasas, 1996, 1993; Rheeder et al., 1992). In the late 1970s a statistically significant correlation between the incidence of *F. verticillioides* in maize and the rate oesophageal cancer was shown in the Transkei area of South Africa (Marasas, 1996; 1993; Rheeder et al., 1992; Marasas et al., 1981). In 1990 it was also shown that significantly higher levels of fumonisins occurred naturally in maize from the high oesophageal cancer rate area than in the low-rate area (Marasas, 1993). The maize samples collected by Marasas et al., (1986) were analysed for FB1 and FB2 by Rheeder et al., (1992), and both toxins were significantly higher in maize samples from the high risk area compared to the low risk area. FB1 and FB2 also occurred in more samples from the high risk area than from the low risk area.

Van der Westhuizen et al., (2003) also reported high levels of fumonisin contamination in maize intended for human consumption in the southern regions of the State of Santa Catarina, (Brazil). The mean FB1 level was 1.89 mg/kg which is similar to FB1 levels in other high oesophageal cancer regions like Centane, (South Africa), Mazandaran, (Iran) and Linxian County, (China). The southern regions of Brazil have the highest incidence of oesophageal cancer and these results seem to suggest possible association between the consumption of maize contaminated with fumonisins and oesophageal cancer. Recently Sun et al., (2011)

reported results from China which supports the hypothesis that FB1 may play an important role in the aetiology of human oesophageal cancer. The median FB1 levels were 2.6, 0.4, and 0.3 mg/kg in maize samples collected from Huaian (a high risk area for oesophageal cancer), Fusui (a high risk area for liver cancer) and Huantai (a low risk area for both oesophageal cancer and liver cancer) respectively. Another study in China compared fumonisin levels in corn from a high risk area for oesophageal cancer with samples from a low risk area. There was no significant difference in any of the measured fumonisin levels between the two areas (Yoshizawa and Gao, 1999; Yoshizawa et al., 1994).

There is no concrete evidence implicating fumonisins in the causation of oesophageal cancer (IARC, 2002; Voss et al., 2001). This lack of evidence to implicate fumonisins in oesophageal cancer led the IARC to conclude that, "there is inadequate evidence in humans for the carcinogenicity of fumonisins" and "there is sufficient evidence in experimental animals for the carcinogenity of fuminisin B1", hence it was concluded that "Fumonisin B1 is possibly carcinogenic to humans (Group 2B)" (IARC, 2002).

2.3.4 Natural occurrence of fumonisins in Africa

In Tanzania more than half of the samples (n=120) tested were contaminated with total fumonisins ranging from 61-11048µg/kg and FB1 with levels up to 6125µg/kg (Kimanya et al., 2008). A wide variety of Tunisian food samples were contaminated with fumonisins (range: 70-2130µg/kg) and FB1 contaminated the most samples (Ghali et al., 2009). Maize based foods were the most contaminated with FB1 in terms of quantity and frequency.

2.3.4.1 Botswana

In Botswana FB1 was detected in 85% (28/33) of maize samples, 15% (3/20) sorghum samples and 100% (4/4) chicken feed samples collected from storage depots or bought from retail outlets. No fumonisins were detected in peanuts, peanut butter, beans or mophane worm (an edible larval stage of the emperor moth) (Siame et al, 1998). The highest concentrations were detected in maize (up to 1270µg/kg) and chicken feed (up to 1050 µg/kg). The chicken feed results should however be treated with reserve since only 4 samples were tested making it difficult to establish statistical significance.

An analysis of sorghum based traditional malt, wort and beer for fungi, aflatoxins, fumonisin B1 and zearalenone in Botswana was carried out by Nkwe et al., (2005). *Fusarium* species

contaminated 63% of the malt samples and *F. verticillioides* was isolated from all *Fusarium* positive samples. FB1 was detected in 2% of the malt samples, but wort and beer made using the positive samples had no detectable FB1, implying that FB1 was not carried over during the beer making process, even though it has been shown before that FB1 can be carried over from raw materials to beer products (Turner et al., 2009; Torres et al., 1998). In another study Mpuchane et al., (1997) isolated *Fusarium* species from maize and sorghum that was stored in Botswana silos. *F. verticillioides* was the dominant species isolated from sorghum.

2.3.4.2 Zimbabwe

A study to ascertain the prevalence of *Fusarium* species of the Lesiola section on Zimbabwean corn and their ability to produce mycotoxins zearalenone, moniliformin and fumonisin B1 was done by Mubatanhema et al., (2000). The study showed a high incidence of *Fusarium* infection by the species of the Lesiola section on maize from different parts of Zimbabwe. Fumonisin B1 production was highly associated with *F.verticillioides*, with 97% of the 698 isolates producing FB1. In comparison only 31% of 67 *F. proliferatum* isolates produced FB1. *F. verticillioides* was also detected in 3.5% of maize samples and 3.7% of sorghum samples collected from Bulawayo by Gamanya and Sibanda, (2001). Peanuts and wheat did not show any *Fusarium* occurrence. The authors reported a direct link between FB1 levels and the incidence of *F. verticillioides* in the crops collected from different regions of Zimbabwe. FB1 was also detected in maize stover residues stored as cattle feed. It ranged from 11µg/kg to about 430µg/kg (Coker et al., 2003).

2.4 Legislation

The FDA maximum allowable limits for aflatoxins and fumonisins in human foods are 20 ppb (20 µg/kg) and 4 ppm (4000 µg/kg) respectively (Codex, 2001). The European Commission has stricter guidelines, 15µg/kg and 2000µg/kg for aflatoxins and fumonisins respectively (EC, 2006). In South Africa maximum tolerable limits for all foods are set at 10 µg/kg and 5 µg/kg for total aflatoxins and AFB1 respectively (Viljoen, 2003). In Zimbabwe the maximum allowable aflatoxin limits for all foods are set at 20 µg/kg and 5 µg/kg for total aflatoxins and AFB1 respectively. In Botswana there are currently no regulations in place. Botswana and Zimbabwe are members of the Codex Alimentarius that has set 15 µg/kg as the maximum allowable limits for aflatoxins in all food. None of the African Countries has regulations or guidelines for fumonisins.

However legislation works perfectly in the market economies of the developed world, but rural subsistence farming communities have a much more complicated situation which is difficult to address (Shephard, 2008b). In most developing countries the food consumed is usually from subsistence production, consumption of formally traded food items is minimal, and laboratories to test the food are inaccessible (Shephard, 2008b; Williams et al., 2004). When trade does occur, the least contaminated food is usually exported, leaving the highly contaminated food to be consumed by a population already at risk (Williams, 2004). In addition meeting these regulatory and advisory limits on individual foods does not translate into food safety, as significant aflatoxin and fumonisin exposures may result from high consumption of food with low mycotoxin levels (Shephard, 2008b). Hence mycotoxin risk reduction involves reduction of either contamination levels in food or reduction of consumption of contaminated food or both (Shephard, 2008b).

CHAPTER 3: METHODS AND MATERIALS

3.1 Study Area

The study was conducted on samples collected from Bulawayo (Zimbabwe) and Gaborone (Botswana), two large cities in the Southern Africa sub-region. Though they are only 700 km from each other, they are culturally, agriculturally, socio-economically and agro-ecologically different (Figure 3.1).

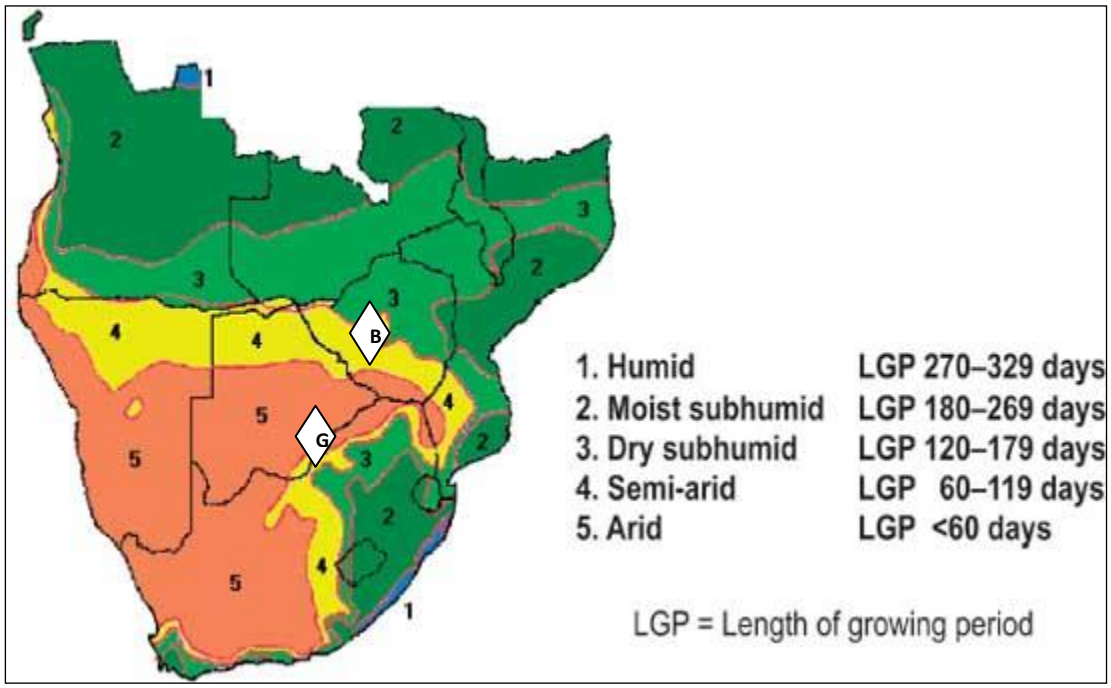

Figure 3.1: Map showing the location, agro-ecological patterns of Bulawayo (B) and Gaborone (G). (Source: FAO, 2003)

3.1.1 City of Bulawayo, Zimbabwe

Bulawayo (20°10′12″S 28°34′48″E), the second largest city in Zimbabwe is located in the Matabeleland region and is about 435 km from the capital, Harare. It covers a total area of 700 km^2 and the city's population was estimated to be 700, 000 people in 2009 (RUAF Foundation, 2012). Due to the economic crisis in the country many companies in Bulawayo have closed down or moved to Harare, leaving unemployment levels at 80% and poverty levels around 60%. The city is mostly urban and falls in Region IV of the agro-climatic zones of Zimbabwe, with an average annual rainfall of 450-650 mm (FAO, 2003). Natural region IV is a semi-extensive farming region with low rainfall, periodic seasonal droughts

and severe dry spells during the rainy season. Figure 3.2 shows the average monthly rainfall and figure 3.3 shows the average monthly minimum and maximum temperatures for the city. There is a lot of agricultural activity in the peri-urban and urban areas of Bulawayo (RUAF Foundation, 2012). Designated areas for agriculture include the peri-urban and low density areas of the city; however during the rainy season there is a lot of agricultural activity in non-designated areas mainly from the low income groups. Crop production includes maize, vegetables and fruits.

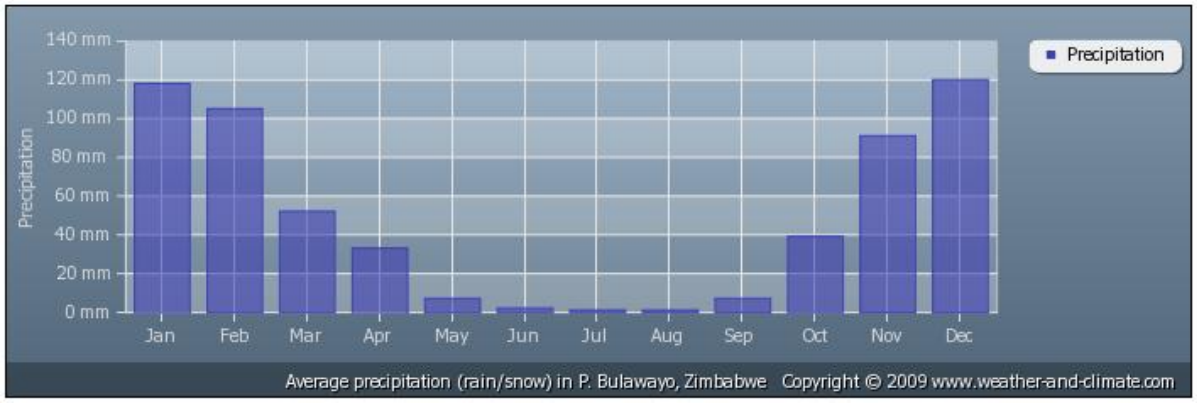

Figure 3.2: Average Monthly rainfall over the year for Bulawayo (source: Weather and Climate, 2012)

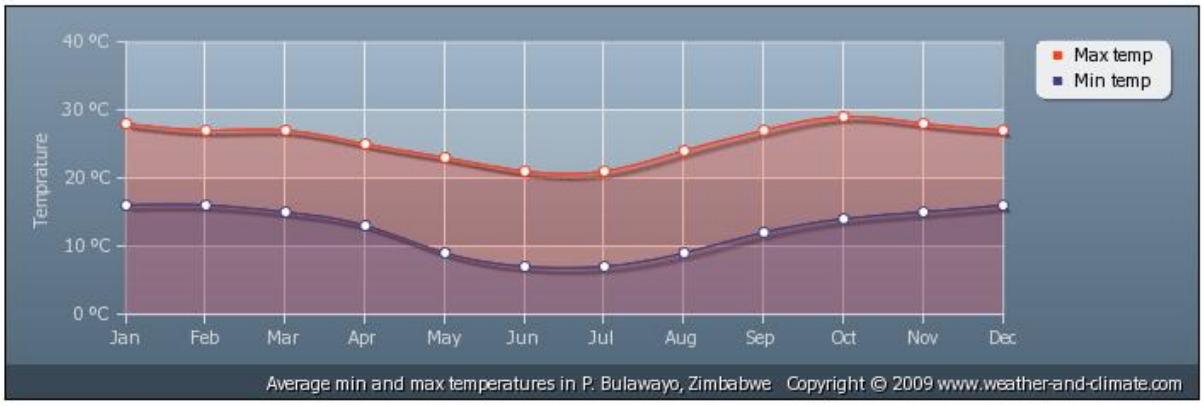

Figure 3.3: The average monthly minimum and maximum temperature over the year for Bulawayo (source: Weather and Climate, 2012)

3.1.2 City of Gaborone, Botswana

Gaborone (24°39′29″S 25°54′44″E) is the capital city of Botswana and is surrounded by several villages and the South African border on the south-east. The city covers a total area of approximately 169 km^2 (Maundeni, 2004). There are approximately 232,000 people living in the city according to the 2011 census (UNdata, 2012). Situated close to the subtropical high pressure belt of the southern-hemisphere Gaborone has an arid and semi-arid climate with mono-modal rainfall pattern. The rains fall during summer, between October and April, with a mean rainfall of 475mm per annum. Figure 3.4 shows the average monthly rainfall while Figure 3.5 shows the average minimum and maximum monthly temperatures in Gaborone.

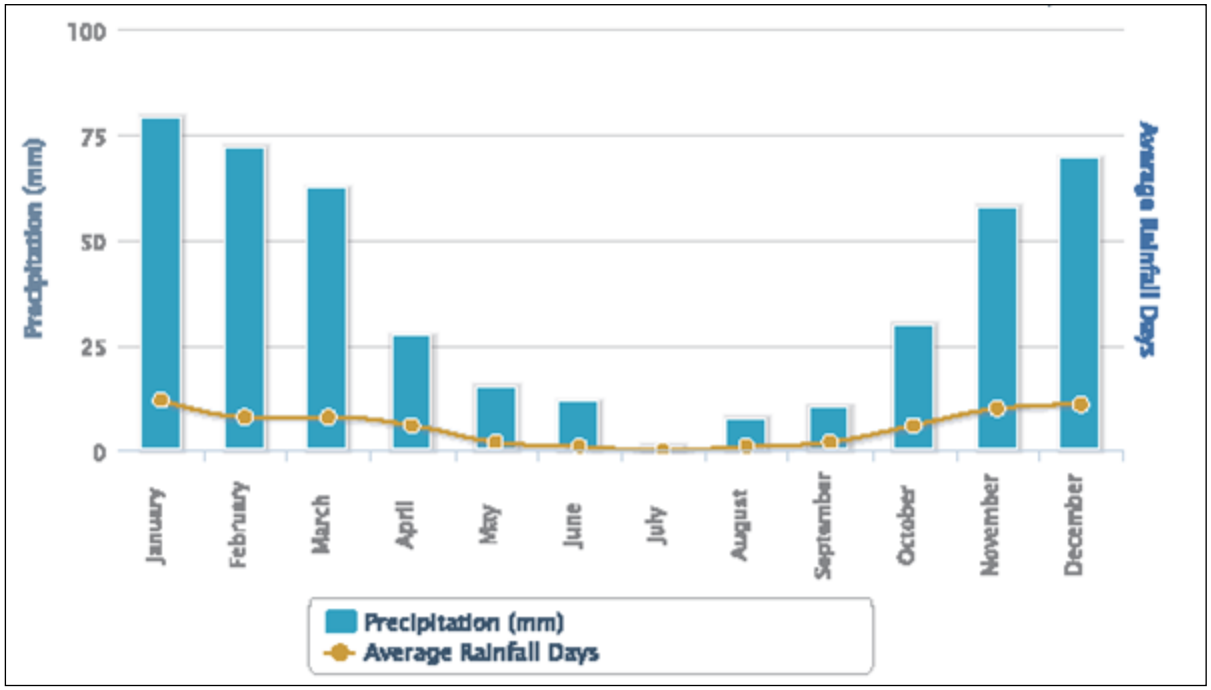

Figure 3.4: Average monthly rainfall over the year in Gaborone (source: World Weather Online, 2012)

The crop production sector is concentrated in the eastern part of the country and is mainly composed of the production of staple sorghum and maize crops together with peanuts, beans, cowpeas, millet and sunflower. Due to the semi-arid climate and erratic rainfall, arable land is limited to less than 1% of the total area of the country; hence, the country relies heavily on food imports (Botswana College of Agriculture, 2008).

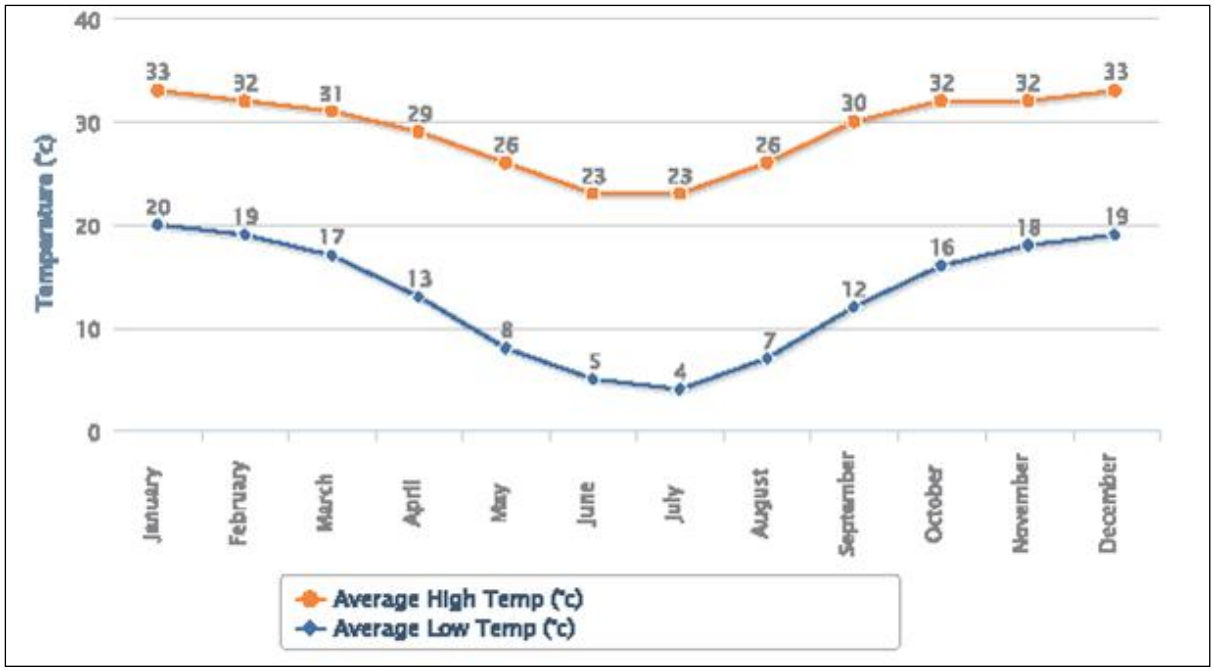

Figure 3.5: Average monthly minimum and maximum temperatures for Gaborone (source: World Weather Online, 2012)

3.2 Sample collection

Mycotoxin contamination does not occur in a homogenous way throughout solid matrix samples hence good sample selection, representative sample collection and sample preparation methods must be used to obtain accurate quantitative results (IARC, 2002; Krska et al., 2008; Shephard, 2009). Fungal infection generally occurs in isolated pockets and is not evenly distributed in stored commodities (Turner et al., 2009). Mycotoxins may be concentrated in the so called 'hot spots' (Krska et al., 2008), a scenario where the number of contaminated particles may be very low, but the contamination level in each particle is very high (Krska et al., 2008; Shephard, 2008b). Nearly 90% of the error associated with mycotoxin analysis can be attributed to how the original sample was collected (Turner et al., 2009). It was very important to ensure that the samples collected were representative of the whole consignment and this was achieved by sampling from different sections of the sack. The vendors allowed the researcher access to the sacks that were on display.

Ethics approval (Reference number: 2012/CAES/004) was obtained from the UNISA Ethics committee. Individuals providing samples and information for the questionnaire were asked to sign a consent agreement (see Appendix A). Sorghum, peanuts and peanut butter samples meant for human consumption were bought from retail shops and the informal market

between October and November 2011. The samples were immediately labelled with a unique identification number. Thirty four peanut samples (18 from Bulawayo and 16 from Gaborone), 34 sorghum samples (18 from Bulawayo and 16 from Gaborone) and 11 peanut butter samples from Bulawayo were collected making a total of 79 samples. At the time of sampling locally manufactured peanut butter could not be found in Gaborone. The only peanut butter on sale there was imported from South African multinational food manufacturing companies. Commercial peanut butter from South Africa is regularly monitored for mycotoxin levels (PROMEC Unit, 2001). As a result peanut butter samples were not collected from Gaborone. All the peanut butter samples from Bulawayo were manufactured in Zimbabwe (commercially and informally). All the vendors in Gaborone said they partially processed their sorghum by removing the pericarp before selling it on the market.

Information on the original source of samples, storage conditions and storage period was collected using a questionnaire administered to the suppliers (see Appendix B). Only one interviewer was used for data collection, this minimised differences due to personal bias as well as variations in depth and persistence of probing. For samples bought from the informal market, the vendors were interviewed, while for samples bought from retail shops, shop managers/supervisors were interviewed. Samples were collected only from those people who were willing to be interviewed and signed the consent agreement. Shops and vendors were randomly picked.

Sorghum and peanut samples were placed into cotton bags and properly sealed before being transported to the South African Medical Research Council (MRC) PROMEC Unit Laboratory in Cape Town for analysis. Peanut butter samples were kept in their original packaging which was 500mL pet jars with screw cap tops.

3.3 Laboratory Analysis

All the laboratory analysis was carried out at the PROMEC Unit and Indigenous Knowledge Systems Research Unit, Medical Research Council, in Cape Town, South Africa.

3.3.1 Mycology Analysis

Peanuts and peanut butter were cultured for *Aspergillus* species while sorghum was cultured for both *Aspergillus* and *Fusarium* species. Mycological analysis was conducted for

randomly selected peanut and sorghum samples from both Bulawayo and Gaborone and all the peanut butter samples from Bulawayo.

Aspergillus species were cultured onto *Aspergillus flavus* and *A. parasiticus* media (AFPA) while *Fusarium* species were cultured onto Malt Extract Agar media (MEA+). Sorghum and peanut samples were surface sterilized with 3.5 % Sodium Hypochlorite for one minute and rinsed twice with sterile water before plating to obtain percentage infection. This technique limits growth to those fungi which are present inside the kernels or particles at the time of plating hence the results will represent true fungal growth and not surface contamination (Pitt et al., 1993). However surface sterilization is sometimes not 100 % effective and cross-contamination between individual seeds might occur during sterilization and subsequent washing (Abbas et al., 2004). One hundred kernels per sub-sample of peanuts and sorghum were plated onto AFPA or MEA+ plates (5 kernels per plate).

The peanut butter samples were plated onto AFPA plates. The spread plate method was used for peanut butter samples. One gram of sample was mixed with 9mL of sterile distilled water and shaken until the mixture was homogeneous. This was followed by a 1 in 10 serial dilution. One millilitre from the primary mixture was mixed with 9 mL distilled water using a graduated pippette and shaken until the mixture was homogeneous. This was done for each dilution until a 10^{-6} dilution was reached. One millilitre from each dilution was transferred onto the petri dish and the cooled media was poured on top, and gently mixed for 1-2 minutes. The mixture was allowed to set before incubating at 30 °C for 3 days. The isolated fungi were enumerated from plates showing well separated colonies and identified using colony colour, colony texture, orange yellow reverse colour and soluble pigmentation (Pitt and Hocking, 1997). The number of fungal colonies per gram of food was calculated and expressed as colony forming units per gram of food (CFU/g). Commercial peanut butter is made by dry roasting shelled peanuts at 160°C, blanching/de-skinning, and grinding (Siwela et al., 2011). Salt, sugar and dehydrogenated fat (as stabilizer) are also added (Siwela et al., 2011, Peanut Institute, 2012). Homemade peanut butter is also made by roasting peanuts, blanching and grinding even though the roasting temperature is rarely monitored. The roasting is believed to destroy most of the vegetative fungi present. The preparation of both AFPA and MEA+ is described below.

3.3.1.1 *Aspergillus flavus* and *A. parasiticus* media (AFPA)

The AFPA was prepared according to the method developed by Pitt et al., (1983). Twenty grams of yeast extract was mixed with 10 g peptone, 0.5 g ferric ammonium citrate, 0.002 g dichloran, 0.1 g chloramphenicol (which was dissolved in 3-5 mL ethanol before adding to other ingredients) and 15 g agar. The mixture was dissolved in 1000 mL of distilled water, and sterilized by autoclaving at 121 °C for 15 minutes. The mixture was allowed to cool down to 55 °C before pouring into plates. The surface sterilized samples were plated and incubated in the dark at 30 °C for 48 - 72 hours. Samples infected with *Aspergillus* species were positive according to the yellow/orange pigmentation of the underside of colonies. The intense orange yellow reverse colouration is induced by ferric citrate (Pitt et al., 1983).

3.3.1.2 Malt Extract Agar (1.5 % MEA^{+})

The 1.5 % MEA^{+} was prepared according to the method describe by Rheeder et al., (1992). In summary, 17 g of malt extract were mixed with 17 g of bacto agar, and 0.15 g of sodium novobiocin. The mixture was dissolved in 1000 mL of distilled water and sterilized by autoclaving at 121 °C for 15 minutes. The mixture was allowed to cool down to 55 °C before pouring into plates. The surface sterilized samples were plated and incubated in the dark at 25 °C for 72 - 96 hours. All the suspected Fusarium species were sub-cultured onto ordinary Potato Dextrose Agar (PDA) for 48 - 72 hours before further processing. All the other mycoflora were reported as other fungi and as a percentage of the kernels plated.

3.3.2 Chemical Analysis

The different chemical and physicochemical properties of mycotoxins require specific extraction of the mycotoxin from the sample matrix using a suitable solvent, cleanup of the extract to reduce matrix effects, separation and detection methods (Shephard, 2009; Krska et al., 2008). A successful analytical method should be robust, sensitive and have a high degree of flexibility over a wide range of compounds and should also be specific when required (Turner et al., 2009). Mycotoxin analysis methods are usually based on labour-intensive sample preparation protocols (Shephard, 2009; Krska et al., 2008). Sample preparation is usually the main time factor in mycotoxin analysis, taking up approximately two thirds of the time (Turner et al., 2009).

The extraction method depends on the structure of the toxin. Polar toxins like fumonisins require the presence of water aided by the presence of polar organic solvents while aflatoxins are hydrophobic toxins and their extraction relies on the use of organic compounds (Turner et al., 2009). An ideal extraction solvent extracts the mycotoxin quantitatively and should be recoverable, stable, non-toxic and non-flammable. Extraction and clean-up procedures are usually long hence the extraction solvent should be stable for the duration of the entire process (EMAN, 2011).

Liquid-liquid extraction or liquid-solid extraction techniques can be used. Liquid-liquid extraction involves exploiting the relative solubility of the toxin in aqueous phase and in an immiscible organic phase, to extract the compound into one solvent leaving the rest of the matrix in the other (EMAN, 2011; Turner et al., 2009). It is widely used in the extraction of patulin in fruit juices and AFM1 in milk or urine. Liquid-solid extraction is used in solid samples and involves dissolving the toxin in the extraction solvent by either shaking or blending. Due to the presence of co-extracting impurities the extracts of most matrices are unsuitable for direct chromatographic analysis hence the clean up procedure is the most important step as the purity of the sample affects the sensitivity of the results (Turner et al., 2009; Shephard, 2009). This clean-up can be through several methods including liquid-liquid separation, solid phase extraction (SPE) and immunoaffinity columns (IAC). Liquid-liquid separation is less frequently used because it is labour intensive, requires large volumes of solvents which are sometimes chlorinated, and there is possible contamination and loss of toxin due to adsorption onto glassware (Turner et al., 2009).

In this study two sample cleanup techniques were used, IAC for aflatoxins and SPE using strong anion exchangers (SAX) for fumonisins (Figure 3.6). IAC and SPE cleanup techniques help to simplify protocols, improve selectivity and hence performance characteristics (Krska et al., 2008). IACs contain immobilised antibodies that exclusively retain a certain mycotoxin or mycotoxin class, use economic amounts of organic solvents and produce cleaner extracts with a minimum level of interfering matrix components (Shephard, 2009; Krska et al., 2008). Extracts of various matrices can be purified by essentially the same IAC protocol and IAC antibodies can tolerate high amounts of aqueous methanol hence IAC is the preferred clean-up method for aflatoxin analysis (Shephard, 2009).

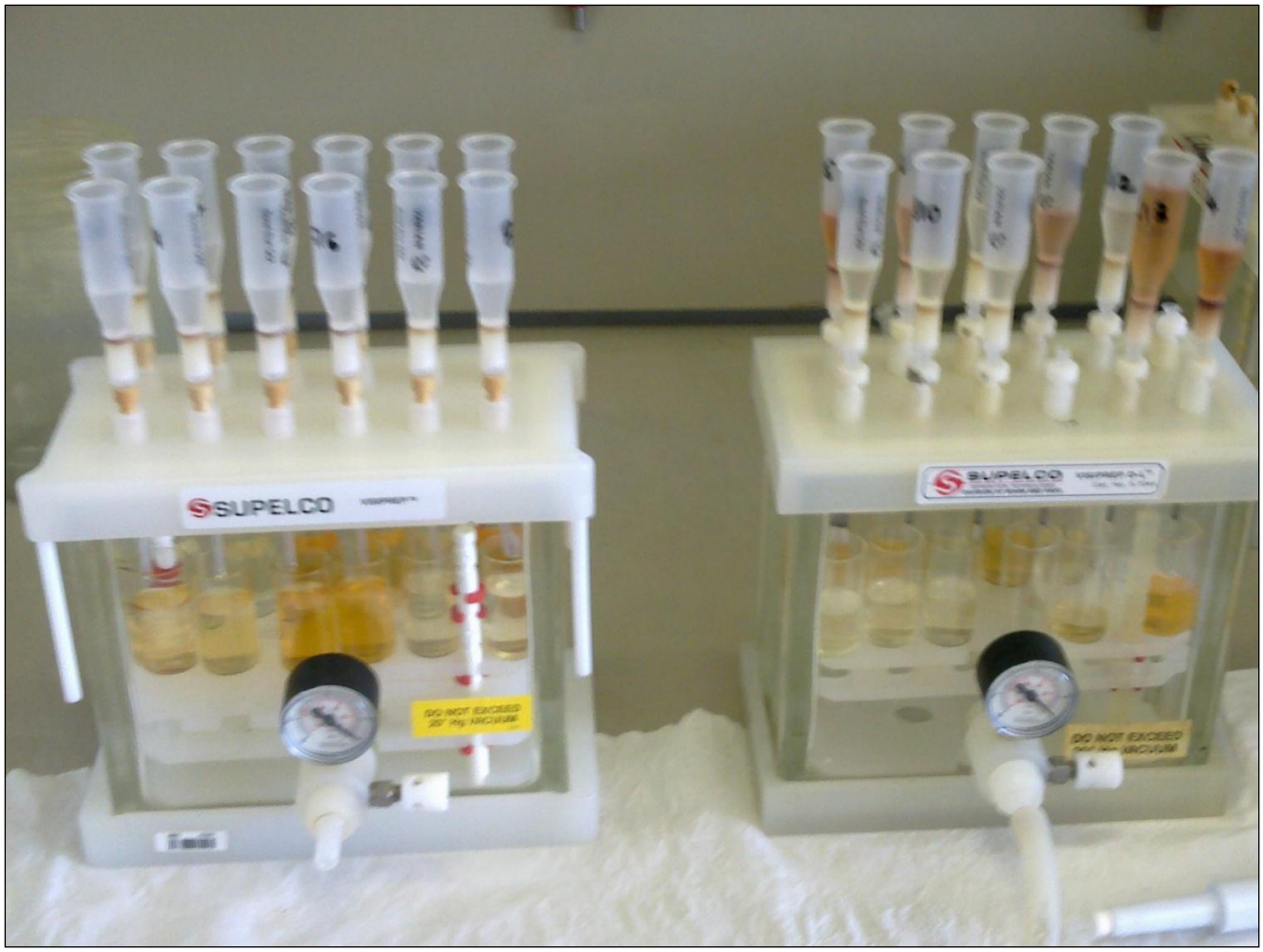

Figure 3.6: SPE SAX extraction for fumonisins

The SPE technology is based on small disposable cartridges packed with silica gel, or bonded phases which are in the stationary phase that have a high capacity for binding small molecules (Turner et al., 2009). SAX is the preferred material for fumonisin extraction, it utilises electrostatic interactions formed between the target molecule and charged groups bonded to the silica material (Turner et al., 2009). The sample is set to a pH where both groups are charged, loaded in one solvent, rinsed to remove most of the co-extracting contaminants and eluted by addition of a strong anionic solvent. However SPE performance can be affected by pH, solvent and ion concentration of the sample.

In previous times, the most popular separation method for mycotoxins was TLC (Shephard, 2009; Turner et al., 2009). It offers the ability to screen large numbers of samples economically with ease of identification, features that appeal to developing country laboratories (Turner et al., 2009). However, HPLC has superseded the use of TLC for the

separation of both aflatoxins and fumonisins because it offers high quality separation, low detection limits and can be automated (Turner et al., 2009; Shephard, 2009). Normal and reversed phase columns can be used for separation and purification of toxins depending on their polarity. Most of the separations are performed on reversed phase systems with mobile phases composed of water, methanol and acetonitrile mixtures (Shephard, 2009).

The most common detection methods include UV or fluorescence detectors that rely on the presence of a chromophore in the toxin molecules (Turner et al., 2009; Shephard, 2009; Krska et al., 2008). Fumonisins lack a suitable chromophore and require pre-column derivatization hence automated injection could not be used since the derivatization reagent, *o*-phthaldialdehyde (OPA) is unstable. Aflatoxins are naturally fluorescent making them ideal candidates for fluorescence detection; however the fluorescence of AFB1 and AFG1 is significantly quenched in the aqueous mixtures used for HPLC (Shephard, 2009). To overcome this quenching post-column derivatization is employed. The two analogues are derivatized at the reactive 8,9-double bond of the dihydrofuran moiety (Shephard, 2009). Photochemical derivatization is economic and was used in this study. The HPLC eluate was passed through a reactive coil wound around a UV light at ambient temperature which causes hydration of AFB1 and AFG1 to their respective hemiacetals.

The conventional detection methods described above are gradually being replaced by mass spectrometry. Most laboratories in the developed world have adopted liquid chromatography/mass spectrometry as the universal approach for mycotoxin analysis (Krska et al., 2008). Most potential mycotoxin analytes are compatible with the conditions applied during separation and detection. The advantages of mass spectrometry include increased selectivity and sensitivity, unambiguous confirmation of the molecular identity of the analyte and the option to use isotopically labelled substances as internal standards (Shephard, 2009; Krska et al., 2008). It is also possible to investigate the molecular structure of metabolites and sugar conjugates and to omit time consuming and error prone derivatization and clean-up steps (Shephard, 2009; Krska et al., 2008). However the reduction in sample preparation results in relatively poor method accuracy and precision due to the irreproducible and unpredictable influence of co-eluting matrix components on the signal intensity of the analytes (Krska et al., 2008). Also the high cost of the equipment, limitations in the type of solvents to be used and complex laboratory requirements are limiting factors in the use of mass spectrometry particularly in developing countries (Turner et al., 2009).

Peanuts and peanut butter samples were analysed for aflatoxin contamination, while sorghum samples were analysed for both aflatoxin and fumonisin contamination.

3.3.2.1 Equipment, Reagents and Chemicals

The following equipment and apparatus were used in this study:

(a) Agilent 1100 series HPLC system (Agilent, Santa Clara, USA)

(b) Waters 510 pump (Waters, Herdfordshire, UK)

(c) Phenomenex Luna C18 5µ particle size column (150 x 4.60mm) (Phenomenex, Torrance, CA, USA)

(d) Waters 470 scanning fluorescence detector (Waters, Herdfordshire, UK)

(e) UV lamp photochemical reactor for enhanced detection (PHRED) (Aura Industries, New York, USA)

(f) HP Chemstation software for liquid chromatography (Hewlett – Packard, Darmstadt, Germany)

(g) Aflatest® immunoaffinity columns (Vicam, Watertown, USA)

(h) Strong anion exchange (SAX) columns (Bond-Elut Varian, Palo Alto, USA)

(i) 12 port solid phase extraction (SPE) manifold (Supelco, Bellefonte, USA)

(j) Polytron PT 3100 homogenizer (Kinematica, Luzerne, Switzerland)

(k) EIA CP104 ultrasonic bath (Zona Industriale Viciomaggio, Arezzo, Italy)

(l) Stuart® Orbital Shaker (Bibby Scientific, Staffordshire, UK)

All the solvents used to quantify aflatoxins and fumonisins were of HPLC grade (unless otherwise stated). All the solvents were purchased from Merck (Darmstadt, Germany) unless otherwise stated. Below is a list of all the reagents and chemicals used:

(a) Aflatoxin standards (Sigma-Aldrich, St Louis, USA)

(b) Fumonisin standards (PROMEC Unit, Capetown, South Africa)

(c) Acetonitrile

(d) Methanol

(e) Toluene

(f) 1M HCL

(g) Mercaptoethanol

(h) Sodium tetraborate

(i) *o*-phthaldehyde

(j) Glacial acetic acid

(k) Pottasium dihydrogen phosphate

(l) Sodium chloride

(m) Distilled water

3.3.2.2 Aflatoxins

3.3.2.2.1 Preparation of the standard solutions

Aflatoxin standard solutions were prepared in accordance with AOAC Official Method 970.44, (1995). To a container of dry aflatoxins (AFB1, AFB2, AFG1, and AFG2) toluene: acetonitrile (9:1, v/v) was added to make separate solutions at the following concentrations 10.2 µg/mL, 9.99 µg/mL, 10.44 µg/mL and 6.14 µg/mL for AFB1, AFB2, AFG1 and AFG2 respectively. The absorbance (A) read at 350 nm as well as the molar absorptivity value (ε) and the molecular weight (MW) for each aflatoxin were used to determine the concentration of each standard (see Equation 3.1):

Aflatoxin concentration (µg/mL) = A x MW x 100/ ε

(MW of AFB1 = 312, AFB2 = 314, AFG1 = 328, AFG2 = 330)

Equation 3.1: Formula for calculating individual aflatoxin concentrations

20 µL of AFB1, 10 µL of AFB2, 50 µL of AFG1 and 20 µL of AFG2 were transferred into an amber vial and evaporated at 60°C under a stream of nitrogen. The sample was reconstituted in 2 mL of methanol and stored at 4 °C.

3.3.2.2.2 Method validation

The aflatoxin method was assessed for specificity, linearity, accuracy and precision. Specificity validation was done by injecting aflatoxin standards three times before injecting samples and comparing the retention times. This was done for every batch of samples. Linearity, accuracy and precision validations were done by spiking twenty seven blank samples (nine for peanuts, nine for peanut butter and nine for sorghum) at three levels (5 ng/g, 10 ng/g and 20 ng/g) with individual aflatoxins (AFB1 , AFB2, AFG1, and AFG2) in triplicate. A blank sample was run for all the samples (peanuts, peanut butter and sorghum). The spiking volumes were calculated using the formula in Equation 3.2 below. The standard curves generated by plotting area versus concentration were analysed by linear regression

Spiking volume = AB/C

Where A is the weight of the sample used in grams (g)

B is the concentration to be spiked in nanograms per gram (ng/g)

C is the concentration of the standard solution in nanograms per millilitre (ng/mL)

Equation 3.2: Formula for calculating spiking volumes for aflatoxins and fumonisins

3.3.2.2.3 Sample Extraction

The extraction method was adapted from a previously described method (Gnonlofin et al., 2012). In brief 10 g of sample were mixed with 1 g of sodium chloride (NaCl) extracted with MeOH: H_2O (80:20, v/v). The mixture was shaken using a Stuart® Orbital Shaker for 10 minutes at 250 rpm, then centrifuged for 5 minutes at 4000 rpm and 5 °C. The supernatant was filtered into a conical flask using Whatman number 4 filter paper and a 10 mL aliquot from the filtrate was diluted with 40 mL distilled water. 10 mL of the diluted sample were loaded onto AflaTest® IAC at a flow rate of 1-2 drops per second. The IAC was then washed with 15 mL distilled water and aflatoxins eluted with 3 mL methanol into an amber vial. The extract was dried at 60 °C under a stream of nitrogen gas. The residue was reconstituted in 200 µL of methanol and stored at 4 °C until injection time.

3.3.2.2.4 Chromatography

The reconstituted samples were analyzed using an Agilent 1100 series HPLC system equipped with a Quaternary pump set at flow rate 1.5 mL/minute, an auto-sampler set to inject 10 µL and connected to a fluorescence detector (FLD) set at excitation wavelength of 365 nm and emission wavelength of 435 nm. Post-column derivatization was performed using a photochemical reactor for enhanced detection (PHRED). The mobile phase used was 0.1 M KH_2PO_4: Acetonitrile: Methanol: Acetic acid (690:150:75:20, v/v/v/v) (Gnonlonfin et al., (2012). Data was collected and analysed by the Agilent ChemStation software and quantification was achieved by comparing area under the curve for samples with those of aflatoxin standards.

3.3.2.3 Fumonisins

Only sorghum samples were analysed for fumonisin contamination since peanuts and peanut products are not known substrates of the toxin (EMAN, 2011).

3.3.2.3.1 Method validation

The fumonisin method was assessed for specificity, linearity, accuracy and precision. Specificity validation was done by injecting fumonisin standards three times before injecting samples and comparing the retention times. This was done for every batch of samples. Linearity, accuracy and precision validation was carried out by spiking nine blank sorghum samples at three levels (500 ng/g, 5,000 ng/g and 10000 ng/g) with fumonisin standards (FB1, FB2, and FB3 prepared at PROMEC Unit, Medical Research Council, South Africa) in triplicate. The spiking volume was calculated using the formula shown in Equation 3.2. The standard curve generated by plotting area versus concentration was analysed by linear regression.

3.3.2.3.2 Sample Extraction

The extraction was based on the modified method of Sydenham et al (1996). In summary 20 g of milled sorghum was extracted by mixing with 100 mL methanol: water (3:1, v/v) and blending for 3 minutes in a Polytron PT 3100homogenizer followed by centrifugation at 4000 rpm for 10 minutes at 4 °C. The supernatant was filtered through Whatman 4 filter paper into a conical flask and the pH was adjusted to 6.00 with 1 M NaOH or 1 M HCL using a calibrated pH meter. SAX columns mounted onto the SPE manifold were pre-conditioned

with 5 mL methanol and 5 mL methanol: water (3:1, v/v) at a flow rate of <2 mL/min. The column was not allowed to dry throughout the clean-up process. A 10 mL sample aliquot was loaded onto SAX columns. The cartridge was then washed successively with 5 mL methanol: water (3:1, v/v) and 3mL methanol. The fumonisins were eluted with 10 mL methanol: acetic acid (99:1, v/v) under gravity. The eluate was transferred into 4 mL glass vials and dried at 60 °C under a stream of nitrogen. The samples were stored at 4 °C until analysis.

3.3.2.3.3 Derivatization

The derivatization procedure was based on the method by Shephard et al., (1990). OPA was prepared by dissolving 40 mg of OPA in 1mL of methanol and diluting in 5 mL of 0.1 M sodium tetraborate containing 50 µL of mercaptoethanol. The OPA reagent should not be kept for longer than a week.

3.3.2.3.4 Chromatography

The samples were re-dissolved in 200 µL of methanol and a 50 µL aliquot derivatized with 200 µL of OPA reagent. 20 µL of samples and 10 µL of standards were injected within 2 minutes after mixing. The derivatization product was analysed by reverse-phase isocratic HPLC system consisting of a Waters 510 pump at a flow rate of 1 mL/minute, a Luna C18 5 µ particle size column (150 x 4.60 mm) and a Waters 470 scanning fluorescence detector (FLD) set at excitation wavelength of 335 nm and emission wavelength of 440 nm. The mobile phase was made up of MeOH: 0.1 M NaH_2PO_4 (78:22, v/v) and adjusted to pH 3.35 with *o*-phosphoric acid. The mobile phase was filtered through a 0.45 µm Waters HV filter and degassed for 30 minutes using an ultrasonic bath. The standards and samples were injected at a flow rate of 1 mL per minute. Data was collected and analysed by the Agilent ChemStation software and quantification was achieved by comparing area under the curve for samples with those of fumonisin standards.

3.4 Health and Safety

Since mycotoxins are hazardous substances, safety precautions were adopted during handling, analysis and disposal of samples and standards. Personal protective equipment (PPE) including laboratory coat, gloves, goggles, sharps containers and a fume hood were used during sample preparation (Figure 3.7). Care was taken in handling and disposing liquid

waste and use of sharps containers. All spills were cleaned with bleach then water. All glassware was rinsed with bleach and washed thoroughly.

Figure 3.7: Working under a fume hood in the PROMEC Unit extraction laboratory

3.4 Statistical analysis

The data was summarized into a Microsoft Excel 2007 master sheet. Descriptive statistics were used to describe the source and storage conditions of the samples. Regression analysis was used to determine the linearity of validation results. Frequency tables, graphs and charts were also used for illustrative purposes.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Sample collection

Samples were collected from 30 respondents from Bulawayo and 24 respondents from Gaborone. Of the Bulawayo respondents 70% (21/30) were women and 30% (9/30) were men. Shop mangers/supervisors made up 23% (7/30) of the total respondents and they were all men. In Gaborone 81% (13/16) were women and 19% (3/16) were men.

The vendors in Bulawayo said they sourced most of their peanuts and sorghum commodities from Gokwe, a village about 400km away. The crops are then transported by buses and other public transport modes to the central market in Bulawayo. The vendors in Bulawayo then collect their orders from the central market and take to their respective selling points scattered around the city. The peanut butter samples from Bulawayo were a mixture of homemade (4/11) and commercial samples (7/11). All the commercial samples were manufactured by different Zimbabwean companies, the majority of which are small to medium scale companies. The peanuts on sale on the informal market were used to make homemade peanut butter and it was assumed the commercial peanut butter was also made from locally produced peanuts. All the homemade peanut butter samples were purchased from the market while all the commercial samples were purchased from retail shops around the city.

In Gaborone, 81% (13/16) of the respondents sourced their peanuts from the Botswana Agriculture Marketing Board and 56% (9/16) sourced their sorghum from the same source. 19% (3/16) of the respondents imported their peanuts from Zambia. The remaining sorghum samples came from the fields around Gaborone. The sorghum samples from Gaborone had the pericarp removed before being sold at the market. All the respondents in Bulawayo stored their peanuts and sorghum stored in sacks both at home and at the vending site. However in Gaborone 88% (14/16) stored their peanuts and sorghum in sacks while 12% (2/16) stored their commodities in plastic bags both at home and at the vending site. Sacks allow good air circulation within the sample that helps keep temperatures and moisture levels low hence prevent pockets of fungal growth.

4.2 Mycology Analysis

The mycology results for peanut butter, peanut and sorghum samples are presented below.

4.2.1 Peanut Butter

Only three samples showed fungal growth with the most contaminated sample yielding $1x10^2$ CFU/g of peanut butter. This is below the maximum tolerance limit (10^4 CFU/g) recommended by the International Commission on Microbiological Specification for Foods (Elliot, 1980; da Silva, 2000).

4.2.2 Peanuts

A. flavus/parasiticus isolates were obtained from all six randomly analysed peanut samples from Gaborone (Table 4.1). The contamination ranged from 2 - 15 % of the seeds examined. Similarly, Mphande et al., (2004) reported *Aspergillus* species from 94 % (113/120) of peanut samples from Gaborone and the *A. flavus/parasiticus* were detected on 66 % of the samples.

Table 4.1: Contamination of peanut samples from Bulawayo and Gaborone with *A. flavus/parasiticus* (as indicated by % kernel growth on AFPA media)

Gaborone Samples	(% kernels showing *Aspergillus* growth)	Bulawayo Samples	(% kernels showing *Aspergillus* growth)
1	5.0	**1**	20
2	3.0	**2**	4.0
3	2.0	**3**	NG
4	15.0	**4**	3.0
5	6.0	**5**	NG
6	10.0	**6**	5.0

NG – no growth

Of the Bulawayo samples only four were contaminated with *A. flavus/parasiticus* species, with a contamination range of 3 - 20 % of the seeds examined. In Thailand 100 % of the peanut samples showed some fungal infection and 84 % of all kernels examined were infected (Pitt et al., 1993). *A. flavus* isolates were the most dorminant followed by *A. niger*, being found in 95 % and 86 % of all the samples examined respectively. *A. parasiticus*

incidence was found to be very low. In this study differentiation of the *Aspergillus* species was not done.

4.2.3 Sorghum

Only the sorghum samples from Bulawayo showed fungal growth on both MEA+ media as shown in Tables 4.2.

Table 4.2: Contamination of sorghum samples from Bulawayo with *Fusarium* species and other fungi (as indicated by % growth on MEA+ media)

Bulawayo Samples	(% kernels showing *Fusarium* species growth)	(% kernels showing other fungi growth)
1	1.0	47.0
2	NG	32.0
3	3.0	58.0
4	1.0	64.0
5	NG	16.0
6	1.0	71.0

NG-No growth

On MEA+ 4/6 of the randomly analysed sorghum samples from Bulawayo showed contamination with *Fusarium* species ranging from 1 – 3 %. The following Fusarium species were isolated *F. scirpi*, *F. thapsinum*, *F. incarnatum* and *F. proliferatum*. All the sorghum samples from Bulawayo were also infected with fungi other than *Fusarium* species and this ranged from 16 – 71 % of the kernels. *Epicoccum* species were the most common genus identified under other fungi. The Fusarium species commonly associated with sorghum include *F. verticillioides*, *F. proliferatum*, and *F. thapsinum* (Nkwe et al., 2005). Gamanya and Sibanda (2001) reported *F. verticillioides* prevalence of 0.2 % and 3.5 % in sorghum and maize samples collected from Bulawayo respectively. They also noted a direct link between FB1 levels and *F. verticillioides* incidence. A high incidence of *Fusarium* infection by the species of the Liseola section on maize from different parts of Zimbabwe has also been reported, although there was no correlation between fungal contamination and mycotoxin production (Mubatanhema et al., 1999). The distribution of *F. verticillioides* has been shown

to be significantly higher in maize ($p < 0.05$) than in other crops (Gamanya and Sibanda, 2001) suggesting selective substrate preference with regards to fungal contamination and FB1 production (Gamanya and Sibanda, 2001, Doko et al., 1996).

There was no fungal growth in any of the randomly analysed sorghum samples from Gaborone. In contrast Nkwe et al., (2005) identified *Fusarium* species in 63 % of sorghum malt samples from villages around Gaborone. However the processing of sorghum into malt is suspected to encourage growth of *Fusarium* species (Nkwe et al., 2005). Also mouldy crops, specifically separated from visibly non-mouldy crop are usually used for beer processing (Shephard et al., 2005). In Brazil only 25 % of the samples yielded *Fusarium* species with *F. verticillioides* representing 25 % of the *Fusarium* identified (da Silva et al., 2000). In Thailand *F. verticillioides* was isolated from 42 % of the samples (Pitt et al., 1994). The mycoflora results of the present study differ quite significantly from the Brazil and Thailand results. This might be a reflection of the huge differences in sample numbers used.

Table 4.3: Contamination of sorghum samples from Bulawayo with *A. flavus/parasiticus* (as indicated by% kernels showing *Aspergillus* growth)

Bulawayo Samples	(% kernels showing *Aspergillus* growth)
1	3.0
2	NG
3	NG
4	2.0
5	2.0
6	22

NG - no growth

Only the sorghum samples from Bulawayo showed growth on AFPA media as shown in Table 4.3. 67 % (4/6) of the randomly analysed sorghum samples from Bulawayo showed contamination with *A. flavus/parasiticus*.

Samples infected with *Aspergillus* species were positive according to the yellow green pigmentation of the underside of colonies. Figure 4.1 shows a photo of *Aspergillus* species growth on one of the samples on AFPA media.

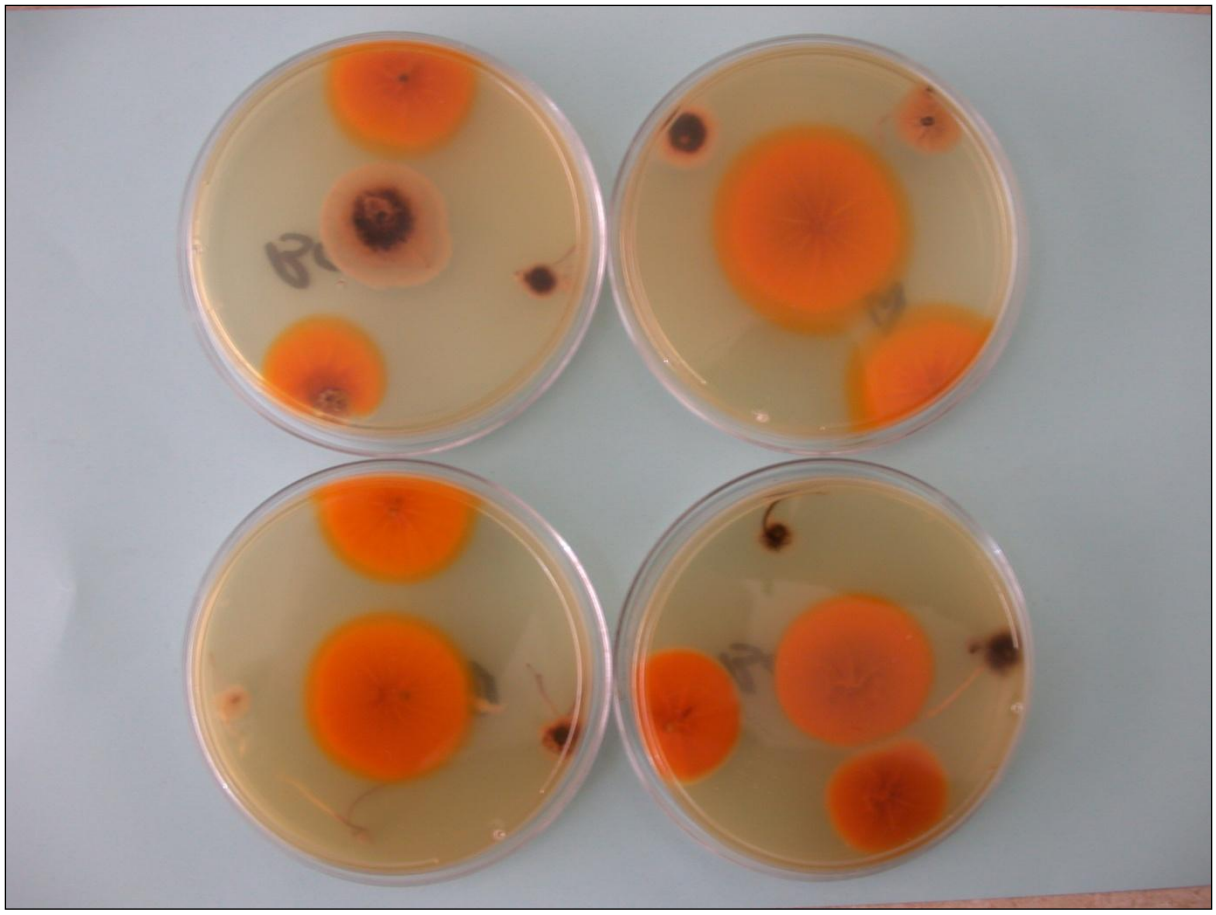

Figure 4.1: A photo of *Aspergillus* species growth in sorghum on AFPA media

None of the sorghum samples from Gaborone showed any fungal growth, this might be attributed to the fact that all the sorghum samples had the pericarp removed. Nkwe et al., (2005) reported *Aspergillus* species in 44 % of sorghum malt from villages around Gaborone. Maize and sorghum from grain storage silos in Botswana had *A. flavus* and *A. parasiticus* isolates in up to 41 % of the fungal isolates in maize and 25 % of those in sorghum (Mpuchane et al., 1997). Sorghum from Thailand had very high levels of *A. flavus*, about 86 % of the samples were infected (Pitt et al., 1994), but sorghum is used exclusively as animal feed in Thailand (Pitt et al., 1994). In Brazil, da Silva et al., (2000) observed *Aspergillus* presence in 42.7 % of sorghum samples and *A. flavus* was the most prevalent.

4.3 Chemical analysis

4.3.1 Validation of the aflatoxin method

The specificity and reproducibility validations for the HPLC method for aflatoxins were determined by measuring intra-day repeatability of the working standards. The retention times and percentage coefficient of variation (% CV) calculated from peak areas (Table 4.4) indicate that the reproducibility and specificity of the method with respect to aflatoxin standards is good.

Table 4.4: Peak areas and percentage CV's for aflatoxin standards

	AFB1	AFB2	AFG1	AFG2
Standard 2	39.4	96.8	44.5	53.1
Standard2	38.6	95.5	44.5	53.1
Standard 3	37.9	94.5	43.9	52.7
Mean	38.6	95.6	44.3	53.0
SD	0.8	1.2	0.3	0.2
% CV	2.0	1.0	1.0	0.0

Peanut and peanut butter samples bought from a shop in Cape Town and a pooled sorghum sample were spiked in triplicate with the aflatoxin standard at 5 ng/g, 10 ng/g and 20 ng/g. Recovery, precision and linearity validation were assessed by performing triplicate intra-day injections of the spiked samples. The recoveries, precision and linearity of the method are shown in Tables 4.5a, b, and c.

The average total aflatoxin recoveries for peanut butter were 95 %, 90 % and 75 % for samples spiked at 5 ng/g, 10 ng/g and 20 ng/g respectively. Yentür et al., (2006) reported an average recovery of 82 % for peanut butter analysed using HPLC. The recoveries in this study were considered to be acceptable. The method showed acceptable linearity in the range 5 ng/g – 20 ng/g with the regression coefficient value ranging from 0.9802 to 1. Elzupir et al., (2011) also reported regression coefficient values of >0.9.

Table 4.5a: A summary of the recovery validation results of the aflatoxin HPLC method for peanut butter

Aflatoxin	Spiking level	% Recovery (RSD %)	R^2	Equation
AFB1	5	107 (4)	0.9890	y = 1.1993x + 1.4035
	10	81 (0.9)		
	20	86 (2)		
AFB2	5	82 (5)	1.0000	y = 5.762x + 2.8195
	10	79 (0.6)		
	20	74 (8)		
AFG1	5	98 (3)	0.9802	y = 0.3648x + 1.2015
	10	65 (5.3)		
	20	72 (20)		
AFG2	5	95 (10)	0.9993	y = 1.8132x + 7.6115
	10	76 (0.9)		
	20	68 (15)		

The average total aflatoxin recoveries for peanuts were 65 %, 61 % and 84 % for samples spiked at 5 ng/g, 10 ng/g and 20 ng/g respectively. These recoveries are slightly lower than 82 % and 92 % reported by Siwela, (1996) for peanuts spiked at 5 ng/g and 20 ng/g respectively. The method showed acceptable linearity in the range 5 ng/g – 20 ng/g with the regression coefficient value ranging from 0.9310 to 1.

Table 4.5b: A summary of the recovery validation results of the aflatoxin HPLC method for peanuts

Aflatoxin	Spiking level	% Recovery (RSD %)	R^2	Equation
AFB1	5	66 (20)	1.0000	y = 1.5496x + 2.7456
	10	84 (1.3)		
	20	93 (18)		
AFB2	5	67 (17)	0.9975	y = 6.981x + 11.135
	10	73 (0.7)		
	20	85 (19)		
AFG1	5	55 (23)	0.9577	y = 0.6099x + 2.1102
	10	50 (4.7)		
	20	77 (18)		
AFG2	5	59 (17)	0.9310	y = 3.4652x + 13.501
	10	38 (3.3)		
	20	85 (9)		

The average total aflatoxin recoveries were 89 %, 75 % and 87 % for sorghum samples spiked at 5 ng/g, 10 ng/g and 20 ng/g respectively. These recoveries are similar to aflatoxin recoveries reported in spiked cassava samples (Gnonlonfin et al., 2010) and in spiked maize samples (Kimanya et al., 2008). Nkwe et al., (2005) used TLC and reported similar recoveries in wort and beer samples from Botswana. Sun et al., (2011) reported an average recovery of 92 % (RSD = 3.2 %) for maize samples spiked with AFB1. The current method showed acceptable linearity in the range 5 ng/g – 20 ng/g with the regression coefficient value ranging from 0.9956 to 1.

Table 4.5c: A summary of the recovery validation results of the aflatoxin HPLC method for sorghum

Aflatoxin	Spiking level	% Recovery (RSD %)	R^2	Equation
AFB1	5	87 (11)	0.9956	y = 1.3078x + 0.5475
	10	95 (0.1)		
	20	87 (9)		
AFB2	5	84 (12)	0.9999	y = 6.0687x + 1.2595
	10	80 (1)		
	20	80 (4)		
AFG1	5	97 (2)	1.0000	y = 0.634x + 0.114
	10	95 (0.9)		
	20	94 (11)		
AFG2	5	90 (7)	0.9992	y = 2.9558x + 1.1805
	10	91 (0.2)		
	20	87 (5)		

4.3.2 Aflatoxin contamination of peanut butter samples

Table 4.6 shows aflatoxin results for peanut butter samples from Bulawayo. All the peanut butter samples were contaminated with total aflatoxins (range: 6.8 – 250.9 ng/g). Two of the samples had total aflatoxin levels above 200 ng/g (250.9 ng/g and 244.9 ng/g) and one had total aflatoxin levels between 100 – 200 ng/g (105.1 ng/g). Figure 4.2 shows chromatograms of two highly contaminated peanut butter samples.

The mean aflatoxin concentration is about five times higher than the Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program limit of 15 µg/kg for total aflatoxins in food (Codex, 2001) and 18 times higher than the EU regulations. Zimbabwe is a member of the Codex Alimentarius (Codex, 2012). The EU has stricter regulations, with a maximum allowable limit for total aflatoxins of 4 µg/kg for groundnuts, nuts and processed products

Table 4.6: Aflatoxin results for peanut butter samples (ng/g).

Sample	AFB1	AFB2	AFG1	AFG2	Total AF
BCPB1	32.5	6.2	9.3	1.9	50.0
BCPB2	10.1	1.8	4.8	0.6	17.2
BCPB3	7.70	1.2	4.2	0.8	14.0
BCPB4	191.0	25.7	3.0	3.8	251.0
BCPB5	42.9	6.3	47.1	8.8	105.0
BCPB6	38.2	4.0	4.6	0.7	47.6
BCPB7	186.0	24.9	30.3	3.5	245
BCPB8	6.1	1.0	2.0	ND	9.1
BCPB9	19.0	1.8	10.4	1.4	32.6
BCPB10	23.8	2.3	3.8	0.4	30.2
BCPB11	3.7	0.9	1.9	0.3	6.8

ND – not detected

intended for direct human consumption or use as an ingredient in food stuff (EU, 2010). AFB1 was detected in all the samples and accounted for the highest toxin levels (mean: 51.02 ng/g, range: 3.7 - 191 ng/g). All the samples exceeded the maximum AFB1 level set by the EU of 2 µg/kg in peanut and peanut products for direct human consumption (EU, 2010) and 5 µg/kg the regulatory limit for AFB1 in food in Zimbabwe.

Similar results were reported by Yentür et al., (2006). Aflatoxins were detected in 100 % (20/20) of the peanut butter samples analysed (range: 8.16 – 75.74 ng/g). Elzupir et al., (2011) reported much higher levels (total aflatoxin concentration range: 26.6-853 µg/kg) in peanut butter from Sudan with 90 % of the samples exceeding the EU maximum level by a factor of >20. Only 27 % (3/11) of samples in the current study exceeded the EU level by a factor of >20. The differences might be due to the hot and humid storage systems for peanuts prevalent in Sudan (Omer et al., 2001; Elzupir et al., 2011). Also the Sudanese peanuts were crushed and stored in plastic bags at ambient temperatures before butter making which might have contributed to the higher aflatoxin levels reported in Sudan. Higher total aflatoxin

concentrations in peanut butter were also reported in Nepal with 42.5 % (43/101) of the samples contaminated and 19.8 % (20/101) of the samples having total aflatoxin concentration >30 ppb (>30 ng/g) (Koirala et al., 2005). The Indian subcontinent is a known hotspot for aflatoxins (Bhat and Vasanthi, 2003).

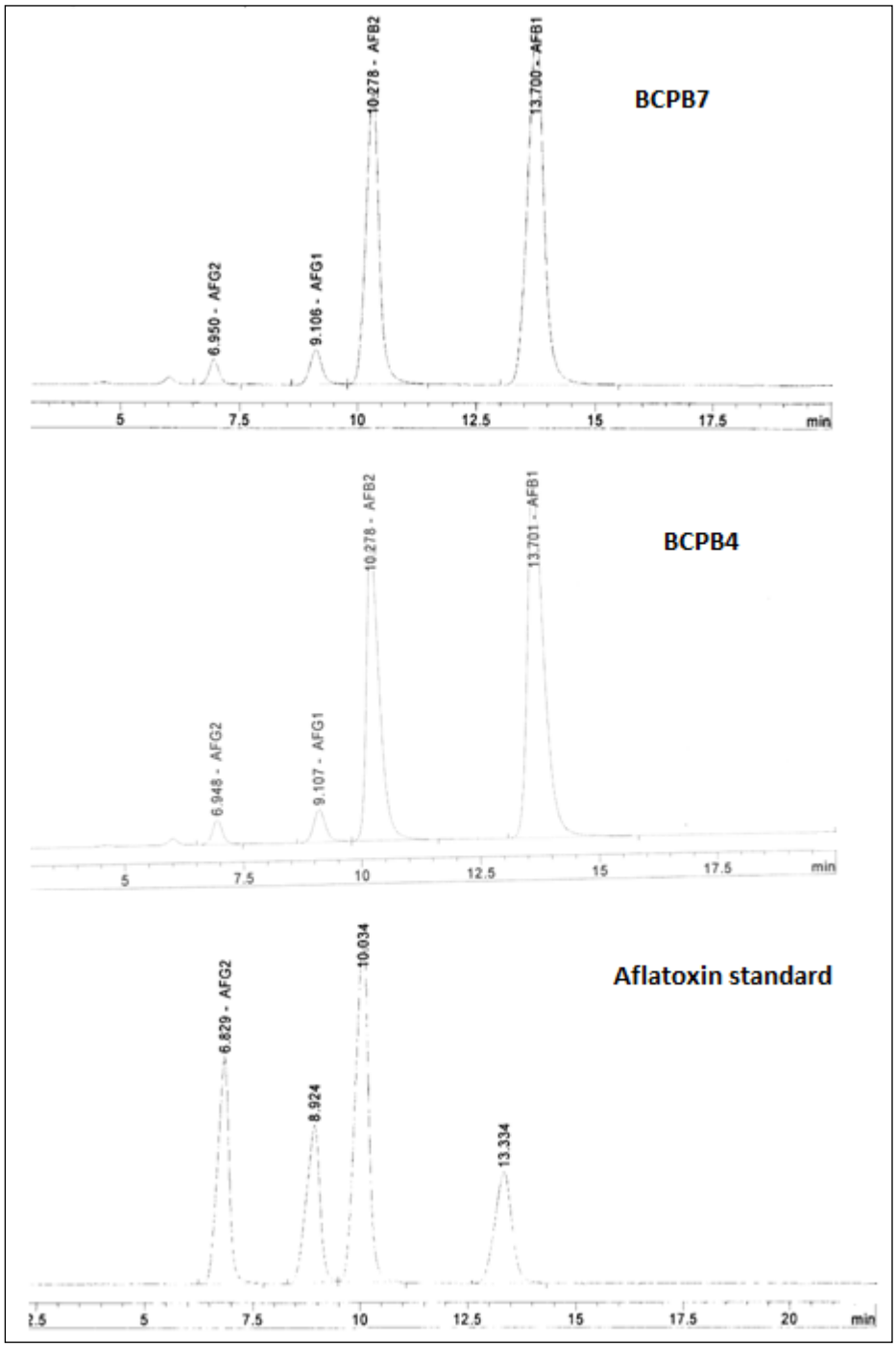

Figure 4.2: Chromatograms of contaminated peanut butter samples

Siwela et al., (2011) reported a 78 % reduction in total aflatoxins after roasting and blanching. During the roasting process, the melting point for aflatoxins (237 °C – 289 °C) is not reached, but the roasting temperature is high enough to cause change in the aflatoxin chemical structure in a significant number of peanuts hence reducing the toxin concentration (Siwela et al., 2011; Ogunsanwo et al., 2004). Roasting has also been shown to produce average aflatoxin reductions of between 45 % - 83 % in peanuts and coffee beans (Kabak, 2009). Using drastic conditions like roasting to attack any compound usually leads to indiscriminate bond breakage (Ogunsanwo et al., 2004). It has also been noted that the peanut skin contains a significant amount of aflatoxins (Siwela et al., 2011; Ogunswano et al., 2004) and blanching in the peanut butter processing removes about 27 % of the total aflatoxins (Siwela et al., 2011). However, peanut butter samples in this study were highly contaminated with aflatoxins despite the anticipated reduction due to roasting and blanching.

4.3.3 Aflatoxin contamination of peanut samples

Aflatoxin results for peanuts from Bulawayo showed that 17 % (3/18) of the samples were contaminated with total aflatoxins ranging from 6.6 – 622.1 ng/g. Two of the contaminated samples had detectable levels of both AFB1 (6.3 ng/g; 54.0 ng/g) and AFB2 (0.3 ng/g; 3.3 ng/g). All the contaminated samples were above 4 µg/kg set by the EU as the maximum allowable limit for peanuts destined for human consumption and the most contaminated sample exceeded that limit by a factor of 155. AFB1 was detected in all three samples (range 6.3 – 528 ng/g) and exceeded the Zimbabwean regulatory limit (5 µg/kg) for AFB1 in human food. Mphande et al., (2004) also reported AFB1 as the most commonly detected toxin. High aflatoxin contamination levels in Zimbabwean peanuts have been reported before (Siwela et al., 2011; Henry et al., 1998; Shephard, 2008b; Siwela et al., 2011; Wild et al., 1987). AFB1 is one of the most carcinogenic compounds known and is classified as a Group 1 carcinogen by IARC (IARC, 2002) hence any amount of AFB1 in food should be of concern to the consumers.

None of the peanut samples from Gaborone had any detectable levels of aflatoxins (LOD = 0.1 ng/g; LOQ = 5 ng/g) even though *A. flavus/parasiticus* isolates were identified from all the six samples analysed. In contrast, Mphande et al., (2004) reported detectable aflatoxins in 78 % (93/120) of peanut samples from Gaborone (total aflatoxin range: 12-329 µg/kg, mean: 118 µg/kg). About 49 % (59/120) of the samples had a total aflatoxin concentration above the Codex Alimentarius regulatory limit of 15 µg/kg, while all the positive samples were above

the stricter EU limits of 4 µg/kg. Siame et al., (1998) also reported aflatoxin contamination in 52 % (15/29) of peanut samples from Gaborone (range: 3.2-48 µg/kg, mean: 14 µg/kg). The lack of detectable aflatoxins in peanuts in this study might be due to several reasons. There is a possibility that the *A. flavus/parasiticus* strains isolated in the Gaborone peanuts were non-aflatoxigenic strains, or that the peanuts were properly dried with very low water activity or that the storage conditions were not conducive for aflatoxin production (Luque et al., 2011; Abbas et al., 2006, Mphande et al., 2004).

4.3.4 Aflatoxin contamination of sorghum samples

None of the sorghum samples from both Bulawayo and Gaborone contained any detectable levels of aflatoxins (LOD = 0.1 ng/g, LOQ = 5 ng/g). Nkwe et al., (2005) did not detect any aflatoxins in sorghum malt, wort, or beer samples from Botswana. Siame et al., (1998) detected low aflatoxin levels in 16 % of sorghum samples (range 0.1-0.5 µg/kg) from retail shops and storage depots in Gaborone. Mpuchane et al., (1997) reported aflatoxins ranging from 5-25 µg/kg and 5-50 µg/kg in sorghum and maize from storage silos in Botswana respectively. In Brazillian sorghum, da Silva et al., (2000) also detected low AFB1 levels (mean concentration range 7-33 µg/kg). In a Ugandan study, 38 % of sorghum samples were positive for aflatoxins ranging from 1 µg/kg to >1000 µ/kg (D'Mello and Macdonald, 1997). Kimanya et al., (2008) reported total aflatoxins in 18 % of maize samples (range: 1 – 158 µg/kg) analysed from Tanzania. AFB1 was the most prevalent toxin and had the highest levels.

4.3.5 Validation of the fumonisin method

The specificity and reproducibility validations for the HPLC method for fumonisins were determined by measuring intra-day repeatability of the working standards. The retention times and percentage CV's calculated from peak areas (Table 4.7) indicate that the reproducibility and specificity of the method with respect to the fumonisin standards is good. The percentage CV's were in agreement with those reported by Ndube et al., (2011).

Table 4.7: Peak areas and percentage CV's for the fumonisin standards

	FB1	FB2	FB3
Standard 1	19341	8313	6729
Standard 2	17848	7895	7926
Standard 3	18895	8052	8034
Mean	18428	8087	7563
SD	800	211	725
% CV	4.0	3.0	10.0

Recovery, linearity, accuracy and precision validations were assessed by performing triplicate intra-day injections of spiked samples. A pooled sorghum sample was spiked in triplicate with the fumonisin standard at 500 ng/g, 5000 ng/g and 1000 ng/g. The results of recovery, specificity and linearity validation of the method are shown in Table 4.8.

Table 4.8: A summary of the recovery validation of the fumonisin HPLC method for sorghum

Fumonisin	Spiking level	% Recovery	RSD (%)	R^2	Equation
FB1	500	80	0.65	0.9976	y = 9.1733x + 1870
	5000	78	9.44		
	10000	71	6.81		
FB2	500	76	6.98	0.9997	y = 9.062x + 679.86
	5000	74	9.57		
	10000	72	2.78		
FB3	500	71	6.13	0.9995	y = 9.1256x + 819.16
	5000	71	9.42		
	10000	68	0.94		

The average recoveries ranged from 71 - 80 %, 72 - 76 % and 68 – 71 % for FB1, FB2 and FB3 respectively. Similar recoveries (FB1 = 75 – 83 %, FB2 = 75 – 76 %) were reported for sorghum analysed using HPLC in Tunisia (Ghali et al., 2009). In Botswana, Nkwe et al., (2005) reported recoveries of 65 %, 57 % and 62 % from malt, wort and beer samples respectively. Ndube et al., (2011) also reported fumonisin recoveries in maize (58 – 75 %) that were similar to the recoveries in the present study. However, Kimanya et al., (2008) reported very high fumonisin recoveries in spiked maize samples. This might be due to the differences in extraction methods used. The recoveries in the current study were considered to be acceptable. The method showed good linearity in the range 500 ng/g – 10,000 ng/g and the regression coefficient value ranging from 0.9975 to 0.9997 which was considered acceptable.

4.3.6 Fumonisin contamination in sorghum samples

Only sorghum samples from Bulawayo were contaminated with fumonisins as shown in Table 4.9. Total fumonisins ranging from 8 – 187 ng/g were detected in 61 % (11/18) of the samples. FB1, FB2 and FB3 were detected in 61 % (11/18), 33 % (6/18), and 28 % (5/18) of the samples respectively. FB1 contamination ranged from 8 – 143 ng/g and only two samples had contamination levels above 100 ng/g. FB2 and FB3 contamination for all the contaminated samples was below 50 ng/g. The fumonisin levels in the current study were below the EU regulatory limit of 400 µg/kg for fumonisins in food meant for direct human consumption. FB1 was the most prevalent (50 %, 9/18) and had the highest levels of contamination (range 8-143 ng/g). These results are similar to 0.2 mg/kg reported by Gamanya and Sibanda (2001) for an aggregate sample of sorghum from Bulawayo. Similar results were also reported for FB1 by da Silva et al., (2000) in Brazil (mean concentration range 0.11-0.15 µg/g). However, high fumonisin contamination levels were reported in sorghum from Tunisia (Ghali et al., 2009). Doko et al., (1996) also reported very high FB1 levels (range 55-2735 ng/g) in maize from Zimbabwe, one of the staple foods for most Zimbabweans.

Fumonisins were not detected in any of the samples from Gaborone. This correlates with the absence of Mycoflora in this sample set as shown in section 4.1.3. FB1 contamination in 15 % (3/20) of sorghum samples (range 20-60 µg/kg) has been previously reported (Siame et al., 1998). Nkwe et al., (2005) reported FB1 in 2 % (3/46) of malt samples (range 47-1316

µg/kg), but there was no carryover to wort and beer samples. However, Shephard et al., (2005)

Table 4.9: Fumonisin results for sorghum samples from Bulawayo (ng/g)

Sample	FB1	FB2	FB3	Total
BCS1	ND	ND	ND	ND
BCS2	ND	ND	ND	ND
BCS3	98	25	9	132
BCS4	143	32	12	187
BCS5	13	ND	ND	13
BCS6	8	ND	ND	8
BCS7	ND	ND	ND	ND
BCS8	ND	ND	ND	ND
BCS9	137	31	8	176
BCS10	76	14	4	94
BCS11	11	ND	ND	11
BCS12	ND	ND	ND	ND
BCS13	ND	ND	ND	ND
BCS14	ND	ND	ND	ND
BCS15	67	13	3	83
BCS16	13	ND	ND	13
BCS17	9	ND	ND	9
BCS18	28	9	ND	37

ND – not detected

reported fumonisins contamination in traditional maize beer in the former Transkei area of South Africa between 1991 and 2003 (Range: 43 – 1329 ng/mL). Doko et al., (1996) also reported average FB1 concentration of 20 ng/g in sorghum meal and very high FB1 levels in maize from Botswana. In a recent study in Tunisia FB1 levels (mean: 223.8 µg/kg) were

detected in 10.2 % (5/49) of sorghum samples (Ghali et al., 2009). In Italy, fumonisin contamination in maize samples meant for animal feed ranged from 0.1 to 25.7 mg/kg, which is below the EU legal limit for fumonisins in animal feed of 60 mg/kg (Covarelli et al., 2011).

CHAPTER 5: GENERAL DISCUSSION

5.1 General Discussion

Mycotoxins are abundant in nature and they contaminate a variety of crops in the field as well as in storage and can be passed into the food chain (Bennett and Klich, 2003; D'Mello and Macdonald, 1997). Generally in developing countries rural dwellers are exposed to higher levels of mycotoxins than do urban dwellers mainly because urban dwellers usually consume a more varied diet than rural dwellers and also their food is better controlled for mycotoxins (Liu and WU, 2010; Wild and Hall, 2000). Nyathi et al., (1987) reported a significantly high level of aflatoxin contamination in urine samples collected from white middle class urban dwellers in Zimbabwe. This might be a reflection of significant aflatoxin contamination in processed commercial food commodities.

Tackling the problem of mycotoxins in developing countries is very difficult and complex (Wild and Gong, 2010, Liu and Wu, 2010). Policy makers and the general public in the regions with the highest contamination levels generally lack full knowledge of mycotoxins and the scale of adverse health effects they cause (Wild and Gong, 2010). A vast majority of the people most affected produce and consume their own food, rendering regulatory measures to control exposure ineffective (Shephard, 2003). In general, public health programmes like vaccination, malaria prevention and control, improved sanitation and clean drinking water supply are perceived to be of more value than mycotoxin control (Wild and Gong, 2010). Furthermore, the mycotoxin problem sits at the interface of agriculture, health and international trade and it is impossible to tackle it without first tackling the insurmountable problems affecting these sectors in the developing countries (Wild and Gong, 2010; Shephard, 2008a).

Poverty and consequently food security issues have plagued Africa for decades and are an impediment to improvements in food safety (Shephard, 2003). It is difficult to address food safety issues, especially mycotoxin contamination, in communities where poverty is rampant and food sufficiency has not been attained (Wild and Gong, 2010; Shephard, 2008a; 2003). These communities are more likely to consume mycotoxin contaminated food due to food insufficiency and lack of alternative diets (Shephard, 2003). The attendant malnutrition also exacerbates the detrimental effects of mycotoxins (Wild and Gong, 2010; Shephard, 2008a).

Fungal spores are ubiquitous and often impossible to eliminate in the environment and because of the microscopic nature of fungal mycelia and spores, contamination may not always be evident (Mphande et al., 2004). The fungi that produce mycotoxins proliferate in the tropics where climatic and crop storage conditions such as temperature, humidity, and water activity are conducive for fungal growth (Shephard, 2003; D'Mello and Macdonald, 1997). The incidence of fungal contamination has also been linked to high rainfall and high relative humidities (Gamanya and Sibanda, 2001). However it is important to remember that the presence of mycotoxigenic fungi in food does not necessarily mean the corresponding mycotoxin will be present, as many factors (e.g. conducive temperature and humidity) are involved in toxin production (Turner et al., 2009). Likewise the absence of any visible mould will not guarantee absence of toxin, since the toxin persists long after the fungus is dead (Turner et al., 2009; Hof, 2008,).

Aspergillus and *Fusarium* species are the fungi that produce aflatoxins and fumonisins respectively. *Fusarium* species are plant pathogens that contaminate crops in the field or immediately after harvest while *Aspergillus* species are predominantly storage contaminants (Sweeney and Dobson, 1999), however *A. flavus* invades some crops both in the field and in storage (Shephard, 2008a; Richard, 2007). Of the *Aspergillus* species, *A. flavus* is more abundant than *A. parasiticus* and is found in air, soil, food and water (Mphande et al., 2004; IARC, 2002). Data on *A. parasiticus* in food is rare because it is a less common contaminant of food and most researchers are rarely able to differentiate between *A. flavus* and *A. parasiticus*.

Traditional fungal detection methods that are based on fungal morphology were used in this study. These methods however are time consuming and require considerable expertise; hence molecular biology techniques, for example polymerase chain reaction (PCR), are becoming the preferred method for fungal identification (Luque et al., 2012). These techniques detect genes directly involved in the synthesis of mycotoxins for example the aflatoxin regulatory gene (*aflR*) and are also rapid, sensitive and specific for mycotoxigenic fungi however they require expensive technologies and specialised skills which are scarce in developing countries (Luque et al., 2012; Manonmani et al., 2005). To get precise and accurate information on the presence of fungal populations, mycological isolation followed by PCR identification is one of the most reliable tools (Covarelli et al., 2011).

In this study, peanut butter and peanut samples (from both Bulawayo and Gaborone) showed *A. flavus/parasiticus* contamination. Peanuts and peanut products are among the foodstuffs most susceptible to contamination by aflatoxigenic fungi in both pre- and post-harvest stages because they contain proteins, oils, fatty acids, carbohydrates and minerals providing a rich medium for fungal growth (Barberis et al., 2012; Mphande et al., 2004). Previous studies have also reported significant *A. flavus/parasiticus* contamination in peanuts (Mphande et al., 2004; Pitt et al., 1993). However, only the sorghum samples from Bulawayo were contaminated with *A. flavus/parasiticus*. The sorghum samples from Bulawayo also showed a low incidence of *Fusarium* contamination on MEA+ medium. This is in line with previous reports from Bulawayo (Gamanya and Sibanda, 2001). The sorghum samples from Gaborone had the pericarp removed and that processing might have destroyed any vegetative fungi present. The presence of *A. flavus/parasiticus* and *Fusarium* strains in food means there is always potential for aflatoxin and fumonisin production and changes in storage conditions like increases in water activity, humidity, and temperature may lead to fungal proliferation and toxin production (Mphande et al., 2004; Shephard, 2003; D'Mello and Macdonald, 1997).

The selection of sensitive methods is a pre-requisite to accurately quantify mycotoxins in stored food commodities (Shephard, 2009; 2008b). Conventional analytical methods including TLC, gas chromatography, and HPLC coupled with UV and/or fluorescence detectors are used to quantify mycotoxins in stored food (Turner et al., 2009; Shephard, 2009). In the last few years mass spectrometry has emerged as the most sensitive detection method replacing UV and fluorescence detectors (Krska et al., 2008). However due to capacity constraints in most developing countries (Shephard, 2008a), the application of simple, cheap, adaptable and effective solutions for detecting mycotoxins so as to meet regulatory requirements is increasingly being required (Turner et al., 2009). In this study the methods used for both aflatoxins and fumonisins in peanuts, peanut butter and sorghum were successful validated for precision, accuracy, linearity, reproducibility and specificity.

Peanut butter is widely promoted in children's food including soft porridge, sandwiches, and vegetables as a cheap protein source (Siwela et al., 2009). It has excellent nutritional benefits including proteins and micro-nutrients like phosphorous, potassium, zinc, folate, vitamin E and some phytochemicals (Peanut Institute, 2012). It is also high in mono- and poly-saturated fats hence consumption can contribute to improved cholesterol levels and improved health. In

this study all the peanut butter samples and a small number of the peanut samples from Bulawayo were contaminated with high levels of aflatoxins.

The commercial peanut butter was the most contaminated. This might be due to several factors including poor policing of set regulations by the authorities and lack of effective quality control systems (e.g. factory inspection for aflatoxin contamination, hand sorting to remove shrivelled nuts, and proper cleaning of equipment) in most of the manufacturing companies as a direct result of the decade long economic crisis in Zimbabwe. However Siwela et al., (2011) demonstrated that use of effective quality control systems in the manufacturing process can reduce aflatoxin contamination to levels that are acceptable in many countries. It is also imperative that effective systems for policing of set regulations be put in place. This will prevent manufacturers from churning out peanut butter that has aflatoxin levels above the maximum tolerable limits.

High levels of aflatoxin and fungal contamination result in a decrease in quality and nutritional value of the peanuts and peanut butter (Gong et al., 2002). Furthermore, available research suggests that children are most vulnerable to the detrimental effects of aflatoxins (Williams et al., 2004; Cullen and Newberne, 2003) hence the detection of high levels of aflatoxins in peanut butter is a cause for concern. Also the threshold dose for aflatoxin leading to malnutrition and growth stunting in children is very low (Shephard, 2008b; Cullen and Newberne, 2003). In Togo and Benin chronic aflatoxicosis was also linked to infant growth stunting and being underweight in infants under 5 years old (Gong et al., 2002). It should also be noted that underweight children are also prone to child mortality and acute morbidity due to diarrhoea, malaria, measles, pneumonia and other selected infectious diseases (Williams et al., 2004).

In Sudan, Elzupir et al., (2011) assumed a mean consumption of 10 g of peanut butter per child per day. Working with the same assumption and using the mean aflatoxin level of 73.48 ng/g, daily aflatoxin intake would be 734.8 ng. Using this consumption level, a 5 kg body weight child consumes approximately 146.1 ng/kg body weight (bw) per day. This is a very high aflatoxin intake which is approximately 365 times the estimated provisional maximum tolerable daily intake (TDI) of 0.4 ng/kg bw/day for adults with HBV infection and 146 times the maximum TDI for adults and children without HBV infection of 1.0 ng/kg bw/day (Kuiper-Goodman, 1998). This daily intake falls within the JECFA (2008) estimated daily intake for aflatoxins in Africa of 3.5-180 ng/kg bw/day confirming the high aflatoxin intake

levels in African children. In view of the carcinogenic, immunotoxicity, nutritional and biochemical derangements associated with aflatoxins, especially in young children, this is a very high aflatoxin intake that warrants intervention strategies.

Sorghum is an important part of the staple diet of many people in Africa and other countries like China and India (da Silva et al., 2000, Siame et al., 1998). It has been shown to have properties that inhibit tumor growth, protect against diabetes and insulin resistance and help in managing cholesterol (Whole Grains Council, 2012). Some of the respondents in Bulawayo pointed out that they sold most of their sorghum to people living with diabetes. The sorghum grains are also used as raw materials for poultry, swine and bovine feeds and also in the manufacture of a variety of products including beer (Nkwe et al., 2005; da Silva et al., 2000, Siame et al., 1998).

None of the sorghum samples in this study showed any aflatoxin contamination. The absence of aflatoxins sorghum in this study (although other studies have shown some level of contamination) could be due to several reasons. Contamination of samples takes place both in the field and in storage (Shephard, 2008a; Richard, 2007; IARC, 2002). In storage the major conditions favouring contamination include high moisture content and ambient temperatures (Reddy et al., 2010; Richard, 2007; Shephard, 2003; Bennet and klich, 2003). It was observed that the samples from both Bulawayo and Gaborone were stored in sacks that allow good air circulation (Kew, 2010). Most of vendors also said their crops were sold in a very short space of time, within a month on average. The seasonal and annual variations in aflatoxin contamination of food commodities may have come into play as well (Siame et al., 1998). Also the small sample size might not be representative of the full picture on the ground.

The sorghum samples in this preliminary study showed low fumonisin contamination levels compared to the levels reported in maize samples from different parts of Zimbabwe (Gamanya and Sibanda, 2001; Mubatanhema et al., 1999; Doko et al., 1996). Selective substrate preference by *F. verticillioides* may contribute to the differences in contamination levels noted between maize and sorghum (Gamanya and Sibanda, 2001). Significantly higher fumonisin concentrations have been reported in regions with high rainfall and moderate temperatures as compared to low rainfall regions. During high rainfall mature kernels experience prolonged periods of high water content that may favour fungal growth and mycotoxin production (Kimanya et al., 2008; Gamanya and Sibanda, 2001).

The aflatoxin and fumonisin burden may be reduced if the government encourages vulnerable communities to adopt sorghum as their staple diet replacing maize which is more susceptible to fungal infestation and fumonisin contamination. Sorghum is an indegenious crop and may have developed resistance to fungal infestation hence mycotoxin contamination. However the nutritive value of sorghum is lower than other cereals due to reduced content of some essential amino acids and the presence of condensed tannins which are anti-nutritional compounds (Anglani, 1998).

5.2 Conclusion and Recommendations

The specific objective of this study was to assess and compare natural occurrence of aflatoxins and fumonisins in selected food commodities in Botswana and Zimbabwe. All the peanut butter samples from Bulawayo were contaminated with aflatoxins. The peanut samples from Bulawayo also showed high levels of aflatoxin contamination while the samples from Gaborone had no detectable aflatoxin levels. Most of the peanut samples from Gaborone were from a commercial source while all the Bulawayo samples came from the fields. This might have contributed to the differences in aflatoxin contamination. Sorghum samples from both Bulawayo and Gaborone did not have any detectable aflatoxin levels. The differences were noted on fumonisin contamination, 61 % of the sorghum samples from Bulawayo had detectable fumonisin levels while fumonisins were not detected in any of the Gaborone samples. The partial processing of the Gaborone samples as well as the difference in humidity and average temperatures might have played a role.

The presence of fungal contaminants and mycotoxins in sorghum, peanuts and peanut butter from Bulawayo warrant intervention strategies for minimizing mycotoxin contamination. The traditional approach to preventing exposure to mycotoxins has been to ensure that foods consumed have the lowest possible concentrations (Williams et al., 2004). This has been achieved in developed countries mainly through regulations imposed on traded foodstuffs (Shephard, 2008a; Williams et al., 2004). However regulations are only partly feasible in reducing mycotoxins in developing countries (Turner et al., 2009; Shephard, 2008a; Abbas et al., 2004). Many developing countries are failing to enforce mycotoxin regulations due to capacity constraints and the informal channels of food distribution prevalent in these countries (Shephard, 2008a). However, manufacturers and government agencies should be encouraged to adopt rapid, cost effective and easy to use on-site methods for screening mycotoxins in food commodities (Krska et al., 2008).

Ways of reducing mycotoxin contamination of food as well as mycotoxin related disease can be divided into two, those applicable to the individual level and those applicable to the community level (Wild and Hall, 2000). At the community level primary intervention strategies applicable to subsistence farmers (like education, pre-harvest and post-harvest crop management) can be used (Williams et al., 2004; Wild and Hall, 2000). Pre-harvest intervention include use of crops resistant to fungal infection, reduction of crop stress in the field (through irrigation, and use of fertilizers, fungicides and insecticides), and biocontrol (for example use of non-aflatoxigenic fungal strains in the soil (Dorner, 2009; Kimanya et al., 2008; Wild and Hall, 2000; D'Mello and Macdonald, 1997). However in developing countries insect damage in the field is not controlled, drought is a common phenomenon, and crop production is usually done without the option of irrigation (Williams et al., 2004).

Post-harvest interventions will include improved drying methods (<10 % moisture content), sorting to remove kernels that are discoloured or visibly contaminated by fungi, using grain with very low mycotoxin levels to dilute heavily contaminated grain and controlled storage conditions (Kimanya et al., 2008; Williams et al., 2004; IARC, 2002; Wild and Hall, 2000). However, proper handling of stored products does not exclude fungal spores but will prevent germination of spores and fungal proliferation in the stored products (Mphande et al., 2004). Campaigns to sensitize the public on the health and economic impact of mycotoxins can also complement the abovementioned prevention strategies (Kimanya et al., 2008).

At the individual level dietary change and chemoprevention can be used to limit exposure as well as prevent mycotoxin related diseases (IARC, 2002; Wild and Hall, 2000). Ammoniation and certain adsorbents have been effective in reducing or eliminating the effects of aflatoxins in animals (Richard, 2007; IARC, 2002; D'Mello and Macdonald, 1997) but ammoniation was found to be ineffective in reducing fumonisin concentrations in maize (IARC, 2002). The Ministries of Health and Agriculture should educate the general public as well as Agricultural Extension Officers on mycotoxins, their impact on the economy and the range and scale of adverse health effects they cause. The general public also needs education on the dangers of consuming mouldy foods which might be contaminated with mycotoxins.

Policymakers should also encourage research translation that will lead to implementation of pre- and post-harvest mycotoxin control strategies that have been effective under research conditions. Van der Westhuizen et al., (2010) implemented a hand sorting and washing

procedure which successfully reduced fumonisin concentration by about 62 % in home grown maize. However, washing contaminated maize might present an effluent problem.

Further surveillance studies should be carried out on peanuts and peanut butter from Zimbabwe with a much larger sample size to confirm these results. Dietary exposure studies in children around Zimbabwe should also be carried out to ascertain the extent of the aflatoxin problem. Both Zimbabwe and Botswana are members of Codex Alimentarius (Codex, 2012) and frequent mycotoxin surveillance studies should be carried out to ensure food commodities in these countries meet the set regulations.

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Appendix A

Participant's informed consent

This is a Research project being carried out by Innocent Mupunga in fulfilment of University of South Africa's requirements for a Master of Science in Life Sciences

Title of research Project: A comparative study of natural contamination with aflatoxins and fumonisins of selected food commodities from Botswana and Zimbabwe.

The main purpose of this study is to compare natural contamination levels of peanuts, peanut butter and sorghum from Bulawayo, Zimbabwe and Gaborone, Botswana with aflatoxins and fumonisins.

Participation in this study is voluntary and will not affect your rights or your business. The information you are providing will be treated with strict confidentiality and your name and personal details will not be divulged to anyone else.

Your participation in this study will take less than 10 minutes of your time and it involves filling out a questionnaire.

Approved by Supervising Scientist: Dr L S Teffo

Name of Interviewer: .....

Sign: .....
Place: .....

Participant's Declaration: The purpose of the study has been explained to me. I am participating in this study out of my own will and being of the understanding that my name and details will be kept confidential at all times

Signed: .....

Date.....

Witness: .....

Date: .....

Appendix B

Questionnaire

This is a Research project being carried out by Innocent Mupunga in fulfilment of University of South Africa's requirements for a Master of Science in Life Sciences

Title of research Project: A comparative study of natural contamination of selected food crops from Zimbabwe and Botswana with aflatoxins and fumonisins.

Please answer all questions to the best of your ability

Date: .....

Sample:

Peanuts □ peanut butter □ sorghum □

Sample no: .....

Where did you get your peanuts/peanut butter/sorghum from?

Commercial □ Field □ Cross-border□ Specify.....

Other □ Specify.....

How long have you had this current stock?

<1 month □ 1-3 months □ 3-6 months □ >6 months □

How do you store your peanuts/peanut butter/sorghum? *(show them photos of the different storage conditions)*

Plastic bags □ sacks □ open containers □ other□ Specify.....

On average how much do you buy at a time?

..... Kg

How long does it take to sell everything?

<1 month □ 1-3 months □ 3-6 months □ >6 months □

Thank you for your time.....

Appendix C

Publications and Conference Presentations

Articles from the Thesis submitted for publication

1) **Mupunga, I., Mnqgwawa, P., Rheeder, J.P., Teffo, L.S., and Katerere, D.R., (2013).** Mycoflora and natural occurrence of aflatoxins in peanuts and peanut butter from Bulawayo, Zimbabwe. *Submitted*

Conference Presentations

1) **Mupunga, I., Teffo, L.S., Katerere, D.R., (2011).** A comparative study of mycotoxin contamination of selected food commodities from Botswana and Zimbabwe. (Poster) *UNISA CAES, School of Life Sciences Postgraduate Symposium on September 23rd 2011*

2) **Mupunga, I., Mnqgwawa, P., Rheeder, J.P., Teffo, L.S., and Katerere, D.R., (2013).** Mycoflora and natural occurrence of aflatoxins in peanuts, peanut butter and sorghum from Botswana and Zimbabwe. (Oral) *12th East and Southern Africa Laboratory Managers Association Conference, University of Botswana (9-13 December 2013)*