DETERMINATION OF AFLATOXINS IN PEANUT (*Arachis hypogaea* L.) COLLECTED FROM KINSHASA, DEMOCRATIC REPUBLIC OF CONGO AND PRETORIA, SOUTH AFRICA: A COMPARATIVE STUDY

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I hereby declare that the dissertation 'Determination of aflatoxins in peanut (*Arachis hypogaea* L.) collected from Kinshasa, Democratic Republic of Congo and Pretoria, South Africa: a comparative study' 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.

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❖ Finally, a special word of appreciation to those not cited above, for their love, inspiration, prayers and for believing in me.

ABSTRACT

This study assessed the mycological and aflatoxin contamination of peanuts collected from Kinshasa, DRC and Pretoria, South Africa. Forty peanut samples were collected randomly at informal markets in the two cities and analysed for mycoflora and aflatoxins (B1, B2, G1 and G2) using standard methods. The results indicated that 95% and 100% of peanut samples collected from Kinshasa and Pretoria, respectively were contaminated with aflatoxigenic fungi with Kinshasa's samples being the most contaminated (up to 49, 000 CFU/g). Seventy percent (70 %) of Kinshasa-samples and 35% of Pretoria-samples exceeded the maximum allowable limit of aflatoxin B1 set by JECFA (5 ppb). Statistical evidence showed a significant positive correlation between mycoflora and aflatoxin level for Kinshasa-samples ($r = 0.4743$, $p < 0.005$) while Pretoria-samples showed no correlation. The study reveals that high level of contamination in Kinshasa-samples could be due to the tropical nature of the climate and poor storage conditions as compared to Pretoria which is sub-tropical and sanitary regulations are enforced.

Key terms: *Aspergillus*; aflatoxigenic fungi; mycotoxin; aflatoxin; peanut; Kinshasa; Pretoria; DRC; South Africa; HPLC; fluorometer

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LIST OF ABBREVIATIONS

AFB1:   aflatoxin B1

AFB2:   aflatoxin B2

AFG1:   aflatoxin G1

AFG2:   aflatoxin G2

AFs: total aflatoxins

ATA: alimentary toxic aleukia

aw   water activity unit

DNA: deoxyribonucleic acid

DRC: Democratic Republic of Congo

DON: deoxynivalenol

ELISA: enzyme-linked immunosorbent assay

EU: European Union

FAO: Food Agriculture Organization

FB1:   fumonisin B1

GDP: gross domestic product

GNP: gross national product

HBV: hepatitis B virus

HBsAg: hepatitis B anti-gene

HCC: hepatocellular carcinoma

HCV: hepatitis C virus

HIV/AIDS: human immunodeficiency virus/acquired immunodeficiency syndrome

HPLC: high-pressure liquid chromatography

IARC: International Agency for Research on Cancer

IAC: immunoaffinity column

IFAD: International Fund for Agricultural Development

JECFA: Joint FAO/WHO Expert Committee on Food Additives

RNA: ribonucleic acid

TLC: thin-layer chromatography

USA: United States of America

WHO: World Health Organization

ZEA: zearalenone

CHAPTER ONE: INTRODUCTION

1.1. INTRODUCTION

Developing countries, especially in Africa, face many socio-economic challenges of which poor food security and food safety are paramount (Schmidhuber and Tublello, 2007). The Food and Agriculture Organization (FAO) defines food security as a situation that exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food to meet their dietary needs and food preferences for an active and healthy life (Schmidhuber and Tublello, 2007). But in Africa, these challenges associated with ever-increasing human population and poverty lead more people to consume contaminated food (Devereux, 2009). As the world's second largest and second most populous continent after Asia, Africa counts the largest number of people dying of hunger (around 33%) (Wiesmann, 2006) and the issues of food safety are frequently subjugate to issues of food security (Shephard, 2003).

Mycotoxins, secondary metabolites produced by fungi, are toxic to both animals and humans and their occurrence in the food chain may have public health effects (Wu et al., 2011). The International Agency for Research on Cancer (IARC) (1993) has reported that aflatoxins, especially aflatoxin B1 (AFB1), are the most potent natural carcinogenic substances and are being linked to severe illnesses and also increase the risk of liver cancer in humans. Several food crops such as maize (*Zea mays* L.), peanut (*Arachis hypogaea*), sorghum (*Sorghum bicolour* L.), millets (*Pennisetum glaucum*) and tree nuts are susceptible to contamination by aflatoxigenic fungi (*Aspergillus flavus* and *A. parasiticus*) (Kamika and Takoy, 2011).

Aflatoxins are more prevalent in tropical and sub-tropical areas where environmental conditions such as high temperature and humidity prevail, which favour the growth of fungi and production of mycotoxins on the crops (Klich, 2007). More often discreet and only few perceptible, moulds form a part of many microorganisms which contaminate foods in storage or before when the conditions are favourable (Moss, 1996; D'mello, 2003). Concerning the above, many countries as well as multilateral agencies have established regulations to protect human beings from consuming highly contaminated food (Kamika and Takoy, 2011).

Democratic Republic of Congo (DRC) is located in central Africa and shares an extremely long border with nine neighbouring countries. As Africa's third largest country, the DRC boasts a land area of 2 344 872 km^2 including deep equatorial forests. It has an estimated population of 70 million and Kinshasa as its capital; this is the largest city in the country with an estimated population of 10 million inhabitants in 2010 (Romaniuk, 2011; Tuakuila et al., 2012). This country is home to over 200 different ethnic and linguistic groups with several customers (Fearon, 2003). It lies on the equator with a tropical hot and humid climate (Allison et al., 2009). In the low central basin average annual temperatures are around 25 ºC, while at the higher altitudes the temperatures hover around 20 ºC with an average annual rainfall in Kinshasa of 56 inches (1 422 mm) (Allison et al., 2009). From an economic perspective, after ten years of war and approximately 15 years of instability, the humanitarian crisis in the DRC remains among the most complex, deadly and prolonged ever documented since World War II (Coghlan et al., 2006). This situation has resulted in massive socio-economic hazards, food crises and malnutrition. For many in the DRC, food is not necessarily a part of daily life and when it is available, it

does not usually contain all the nutrients for a healthy life. The Congolese cuisine varies widely, but staple foods comprise cassava flour, maize, sweet potatoes and perch. In rural communities, meat is a delicacy reserved for special days while fish is seen as a primary food source (Allison et al., 2009). Peanut butter is commonly used in Congolese recipes (Tollens, 2002). According to the International Fund of Agriculture and Development (IFAD) (2001), the per capita gross national product (GNP) of the country is the world's third lowest and agriculture contributed nearly 58 % towards the GDP growth in 1996 compared to 26 % in 1977.

Due to the above, people are forced to find food by any means necessary and this compromises issues of food safety (Tollens, 2002). Good hygiene in food handling and basic hygiene practices are often neglected and there are no rigorous food-safety regulations and no strategy for the surveillance of food-borne diseases. Conditions such as cancer, kwashiorkor and malnutrition are amongst the major public health issues in the DRC (Ryder et al., 2000; Barclay et al., 2003; De Merode, et al., 2004; Longo-mbenza et al., 2007). Food contaminated by mycotoxins may be one of many possible contributing factors (Jiang et al., 2008). There are few or no control and preventative measures in various food-chain systems in place during processing and preservation of food products to prevent mycotoxin contamination (Wagacha and Muthomi, 2008). Coghlan et al. (2006) reported malnutrition as a primary factor contributing to 10.9 % of all deaths in the east of the country and 8.1 % in the west of the Congo (DRC). A study conducted by Longo-mbenza et al. (2007) amongst semi-urban schoolchildren from Kinshasa indicated high levels of malnutrition. The report of the FAO on the state of food insecurity in the world stated that the DRC had the highest number of undernourished people in the world during

the period of 1990-1992 to 1997-1999 (FAO, 2001). Consequently, people in the DRC experience food insecurity and concerns around food safety every day; this situation severely endangers the lives of the people and keeps them in crucial chronic food insecurity (FAO, 2001). Recently, the United Nations Development Programme (UNDP) reported that 59 % of Congolese people live on less than US$1.25/d and ranks the DRC bottom of 187 countries on its 2011 human development index (Canadian International Development Agency, 2011).

The republic of South Africa (RSA) is a country in the Southern African region located at the southern tip of Africa. The country covers a coastline stretches of 2 798 km from a desert border in the northwest along the Atlantic Ocean and then along the Indian Ocean to a border with subtropical Mozambique in the northeast. South Africa has an estimated population of approximately 47.39 million (2006 figure) divided into multi-ethnic groups. Pretoria is the legislative capital city (Statistics SA, 2006). The RSA has a generally temperate climate with a desert region in the northwest and a climate similar to the tropics along the eastern coastline (Partridge, 1997). The climatic zones in the Southern African region vary from the extremely arid environment in the Namib Desert in the farthest northwest to the lush subtropical climate in the east along Mozambique and the Indian Ocean (Partridge, 1997). South Africa has a mixed economy with rate of poverty and low GDP per capita which is ranked in the top 10 countries in the world for income inequality, seeing a wide income gap between its wealthiest and poorest citizens (Statistics SA, 2006). The Black majority still has a substantial number of rural inhabitants who lead largely impoverished lives and this is the result of many years of colonial and racist apartheid policies designed to create general conditions

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unfavourable to the well-being of Black people (Petersen et al., 2010). From an agricultural perspective, the South African agricultural industry is highly developed and the country is placed as one of the largest producers of chicory roots, cereals, maize, grapefruit, castor-oil seed, sisal, pears and fibre crops while South African cuisine is heavily meat-based (Machethe, 2004).

Commonly called 'the poor man's nut', peanut (*Arachis hypogaea* L.) is an important oilseed and food crop worldwide, serving as food for man and livestock (Nautiyal, 2002). It is also used as a source of nutrition for malnourished children (Nagai et al., 2009). Since it costs little and the crop is produced locally, peanuts are highly consumed raw, cooked (roasted or boiled) or mixed with other food substances (vegetables, meats, fishes, etc.) as a source of oil and is considered as an everyday meal by many Congolese families, rich or poor. According to the country rankings on peanut meal for domestic consumption, the DRC has been ranked 11th in the world with 46 000 Mt/yr while South Africa is ranked 28th in the world with 10 000 Mt/yr (Index Mundi, 2012). However, this figure might be higher since the DRC does not have the means to monitor peanut consumption in its entire territory due to war. Various traditional methods are employed in the processing and preservation of the peanut and some of these practices encourage fungal growth and mycotoxin production (Nautiyal, 2002). This becomes a serious health concern since it is very difficult to remove aflatoxins once in food (Moss, 1996). In addition, Kamika and Takoy (2011) reported that Kinshasa has favourable environmental conditions for fungal growth and production of aflatoxin in many of the Congolese staple foods, including maize and peanut.

Compared to the DRC, South Africa is a food-secure nation (Statistics SA, 2002) and has a well-developed agricultural industry which provides a modern food market with its requirements to satisfy consumers with a big variety of processed food products (Maenetje and Dutton, 2007). In addition, it is considered to be the biggest peanut exporter in Africa and produces between 80 000 t and 250 000 t of peanut per annum which is mostly used for plant protein and oil (Nautiyal, 2002; Kamburona, 2007). In spite of strict regulatory controls, fungal growth and mycotoxin production are still present and are found in most of the food commodities and foodstuffs all over the nation. Maize collected from Limpopo Province was found to have a high incidence of mycotoxin, especially aflatoxin (Mamphuli, 2007). Peanut butter given to schoolchildren from the Transkei region, Eastern Cape, was also found to have high levels of aflatoxin (Williams et al., 2004); and barley, wheat, etc., also contained high aflatoxin levels (Mashinini and Dutton, 2006). Although South Africa has the capacity to feed itself, a part of the population such as those from the rural areas still suffers from food insecurity and poor food safety (Koch, 2011). According to Statistics SA (2002), there are around 14.3 million people facing food insecurity and 43 % of families that experience a major food crisis (Hendriks, 2005; Maponya, 2008). As a result of this situation, some people, particularly those in food-insecure households in both urban and rural areas, are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. In 1991, Bressac and colleagues reported that humans and animals in South Africa are exposed to high levels of aflatoxins through their diets.

Despite the fact that several studies have been carried out and focused on aflatoxin (Ciegler and Bennett, 1980; Viljoen, 2003; Brera, 2008; Capriotti et al., 2011), poor

food safety in Africa is still a major concern, particularly with regard to the consumption of aflatoxin-contaminated food (Shephard, 2003; Wagacha and Muthomi, 2008). Furthermore, in the DRC very few studies have been reported on peanut and aflatoxin contamination (Kamika and Takoy, 2011) and given the current socio-economic status of this country, research in this area is urgently needed. In addition to that, the DRC and South Africa are presented as areas with a high prevalence of chronic infection with hepatitis viruses, therefore a high incidence of hepatocellular carcinoma has been reported (Sitas et al., 2008). Consequently, the levels of aflatoxin present in food crops such as peanuts for human consumption from the DRC and South Africa need further investigation.

1.2. PROBLEM STATEMENT

Considered as the most important mycotoxin, aflatoxin is a natural potent carcinogen known to affect both humans and animals. The evidence on a synergistic interaction between aflatoxin and hepatitis B virus contamination in the incidence of liver cancer with hepatocellular carcinoma as the most prevalent type has been reported (Moss, 1996). Sub-Saharan African countries such as the DRC as well as South Africa are seen to be hyperendemic areas for hepatitis B virus infection (Sitas et al., 2008). In addition, a correlation exists between socio-economic status and exposure to mycotoxins such as aflatoxins (Shephard, 2003). Due to the scarcity of food and lack of regulation due to war and food insecurity Congolese people are exposed to contaminated food and this is an alarming situation. Unfortunately, this problem is mostly neglected in many developing countries, since the aflatoxin contamination is often sporadic and hidden. In view of the fact that food consumption is one of the major factors affecting the health of both human, continuous surveillance of aflatoxin

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levels in peanut samples should be implemented in order to prevent the consumption of aflatoxin-contaminated food. This study will investigate the incidence of aflatoxigenic fungi and aflatoxins in peanut samples obtained from both Kinshasa and Pretoria.

1.3   GENERAL AIM AND OBJECTIVES

The main aim of this study is to determine the level of aflatoxin contamination in peanuts sold at informal markets in one city in Southern and Central Africa.

In this regard, the objectives of this study were to:

- identify and compare the fungal strains from both Kinshasa and Pretoria peanut samples using macroscopic characteristics
- quantify the level of aflatoxins in peanut samples collected from both Kinshasa and Pretoria
- validate and analyse aflatoxin contamination of peanut samples using fluorometry and HPLC compare fluorometry and HPLC methods for the determination of aflatoxin in peanut

1.4   SIGNIFICANCE OF THE STUDY

Although research on mycotoxins, especially aflatoxins, has received increasing attention worldwide, in the DRC, research on this subject is still very scarce (Kamika and Takoy, 2011). However, there is evidence to suggest that aflatoxin contamination is a major food-safety concern in the DRC where the environmental conditions and socio-economic problems are conducive to poor storage management and subsequent food spoilage and aflatoxin contamination. By

assessing the natural occurrence of aflatoxins in peanuts collected from Kinshasa and comparing these results to those obtained in samples collected from Pretoria, it is hoped that the data will provide base that could be used by policy markers especially from DRC. This survey will provide much-needed data from a country where conflict has largely prevented important research from being conducted. The results will be important in informing the crafting of food safety and possibly agricultural marketing policies in urban Kinshasa.

1.3. LIMITATIONS OF THE STUDY

The high costs of sample collection especially from DRC and analysis limited the number of samples that could be collected and analysed from both cities. The sample size therefore might not be representative of the actual situation on the ground. However, as a preliminary study, this serves an important purpose as the findings presented here may be taken to the next level in subsequent studies.

1.5   CHAPTER LAYOUT

Chapter Two contains the review and critical analyses of the theory and previous research regarding mycotoxins (aflatoxins), especially AFB1, its incidence, economic and health effects, strategy for its prevention and legislation established to avoid aflatoxin contamination in human food.

Chapter Three is aligned with the methodology used to assess aflatoxins including AFB1, AFB1, AFG1 and AFG2 levels. This chapter describes the sampling method, and the aflatoxin extraction and determination method using fluorometry and high-pressure liquid chromatography (HPLC).

Chapter Four sets out the results as obtained in this study; a discussion of the results is also provided by comparing the findings with those previously reported in the literature.

Chapter Five provides the general discussion of the findings presented and interpreted in chapter four.

The conclusion is considered as Chapter Six and illustrates the research question answered and provides recommendations for further study

CHAPTER TWO: LITERATURE REVIEW

2.1. MYCOTOXINS

Mycotoxins are secondary metabolites produced by microscopic filamentous fungi, which can develop on food crops (maize, wheat, groundnut, etc.) and in some cases on commodities of animal origin (meat products, sausages) (Milicevic et al., 2010). Mycotoxins are harmful to vertebrates when they are absorbed through ingestion, inhalation, or dermal absorption. It has been shown that ingestion of contaminated food/feed is the main source of mycotoxin exposure to both humans and animals (Milicevic et al., 2010).

The word mycotoxin comes from the Greek word 'mykes', meaning mould, and 'toxicum' meaning poison, and the diseases caused by them are called mycotoxicoses (Viljoen, 2003; Brera et al., 2008). Historically, mycotoxins have probably been present in food and feed since early in the history of humanity and some of their effects have been recognised for centuries (Viljoen, 2003). The first documented mycotoxicosis, gangrenous ergotism or St. Anthony's fire, known since the Middle Ages, is a human disease resulting from consuming rye contaminated with *Claviceps purpurea.* There have been many mycotoxin-related outbreaks in the past century which have led to numerous deaths, e.g. 'yellow rice disease' in Japan and Alimentary Toxic Aleukia (ATA) which killed many Russian people. In the 1930s, another 'Russian' disease linked to mycotoxin exposure in the Ukraine led to the death of many horses (Ciegler and Bennett, 1980; Capriotti et al., 2011).

But the scientific interest in mycotoxins and their implications for human and animal health began in the 1960s when 100 000 young turkey poultry died in England from a seemingly new disease, '*Turkey "X" disease'*, followed suddenly by the death of as many as 5 000 partridge and pheasants poultry on a single farm, 14 000 ducklings on another farm and severe losses of ducklings, reported from as far as Kenya and Uganda; these events triggered renewed attention being paid to the mystery killer disease (Klich, 2007).

Presently more than 300 mycotoxins are known, but the scientific interest has thus far been concentrated simply on more or less 10 compounds presenting a known toxicological impact on human and animal health (Wu et al., 2011). Among them, aflatoxins, ochratoxins, fumonisins, trichothecenes, deoxynivalenol (DON), and zearalenone (ZEA) are the major groups of mycotoxins mostly studied. These mycotoxins have been shown to be associated with genotoxic effects, carcinogenic effects, immunotoxic effects, mutagenic effects, nephrotoxic effects and teratogenic effects in animal and human health (Moss, 1996; Brera et al., 2008).

Mycotoxins are mainly produced by fungal species belonging to the genera *Aspergillus, Penicillium* and *Fusarium* which are ubiquitous in the environment (Klich, 2007). Under varying conditions, also depending on the species and strain, specific fungi produce a particular mycotoxin, specific fungi produce many mycotoxins or again a specific mycotoxin is produced by a variety of fungi (Viljoen, 2003). Environmental conditions and the nature of substrate determine the type of fungi that dominate in particular food crops, and in some cases the type of mycotoxins produced (Marquardt, 1996). Environmental conditions, especially temperature and

humidity, influence fungal production of mycotoxins, thus the presence of fungi even at high infection rates does not necessarily imply that mycotoxins are present. In addition, different strains of a given fungal species differ in their ability to produce mycotoxins. In most instances, mycotoxins produced can remain within the infected material long after signs of fungal infection have disappeared (Viljoen, 2003).

The type and level of mycotoxin production result from the interaction between fungi, the host and the environment (Pitt, 2000). It has been estimated that 25 % of crops produced worldwide are contaminated each year with 'unacceptable' levels of mycotoxins during food production, processing, transport and storage (Kamika and Takoy, 2011). Of these, the economic, health and environmental impacts of these fungal toxins have pushed our understanding of food safety and food poisoning (Marquardt, 1996). Since aflatoxins represent the most widespread risk to food safety in tropical Africa and because of the interest and objectives of this study, aflatoxins will be discussed further in the following sections.

2.1.1. Aflatoxins

Aflatoxins are mycotoxins that have been well-known since the outbreak of *'Turkey "X" disease'* in England, first isolated and characterised from *Aspergillus flavus*; this mould is a common contaminant of poorly stored grains (Klich, 2007). Aflatoxins are now known to be mainly produced by *Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius* (Do and Choi, 2007). Aflatoxins have received most attention due to their significance in agricultural production loss, threats to human health and potential threats to food safety. There are roughly 20 known aflatoxins but only four of these (aflatoxins B1, B2, G1 and G2) are widely studied because of their toxic

effects. AFB1 is the most pernicious of these toxins (Moss, 1996; Henry et al., 1999; Lewis et al., 2005; Wangikar et al., 2005; Kamika and Takoy, 2011).

2.1.1.1. Chemistry of major aflatoxins

Major aflatoxins have been classified into B and G series due to their fluorescence being blue and green in UV, respectively (Pavao et al., 1995). The B series, AFB1 and AFB2 are chemically known as difurocoumarocyclopentenones and the G series (AFG1, AFG2) are difurocoumarolactone series (Fig. 2.1). Structurally the dihydrofuran moiety, containing a double bond, and the constituents linked to the coumarin moiety are of importance in producing biological effects. For the B series, cyclopentenone was reported to be responsible for the major toxicity observed (Fung and Clark, 2004).
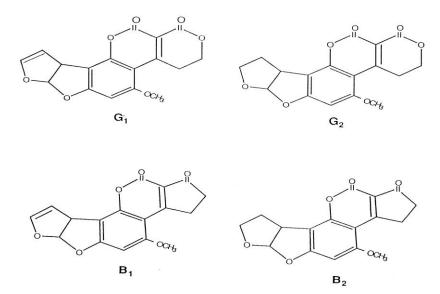
Figure 2.1: Chemical structures of main aflatoxins (Jaimez et al., 2000)

It has been demonstrated that aflatoxins are potent liver toxins and their effects vary with dose, length of exposure, species, breed and diet or nutritional status. Considerable doses generate acute toxicity and chronic exposure to low levels may result in cancer (Marquardt, 1996).

2.1.1.2. Aflatoxin-producing fungi

Fungi are spread worldwide, and constitute invisible inocula that can contaminate food, thereby producing effects that are life-threatening (Newberne, 1974). *Aspergillus* species, microscopic filamentous fungi, are widespread in nature as well and are regarded as soil fungi (Gourama and Bullerman, 1995). As a member of a large phylum of Ascomycota, the *Aspergillus* genus contains roughly 185 species within 18 groups with morphological, genetic and physiological similarity (Botton et al., 1990; Roquebert, 1998). In addition, around 20 species are human and animal pathogens (Sanglard, 2002; Barkai-Golan and Paster, 2008). Generally less exigent on the environmental conditions of the substrate, they have a wide geographical distribution, but are found mostly in the tropical and subtropical regions. They can develop on various substrates including food commodities of plant origin (peanut, maize, wheat, etc.) and in some cases also on commodities of animal origin (meat products, sausages, milk) (Castegnaro and Pfohl-Leszkowicz, 2002). Their growth on the substrate can lead to the alteration of nutritional and dietetic qualities of the products and also to the production of mycotoxins (Barkai-Golan and Paster, 2008).

Aspergillus species contain a large number of mycotoxigenic species such as *Aspergillus alliaceous*, *Aspergillus carbonarius*, *A. flavipes, A. flavus, A. parasiticus, A. fumigatus, A. nomius, A. tamari, A. versicolor, A. terreus, A. niger*, *A. bombycis, A. ochraceoroseus, A. pseudotamari,* etc., among them. Some species including *A. fumigatus* and *A. niger* can be directly pathogenic to humans and animals by being able to invade the living tissues and provoke illnesses such as aspergillosis (Badillet et al., 1987; Judson, 2004). Figure 2.2 illustrates an example of *A. flavus* and *A. parasiticus* spores.
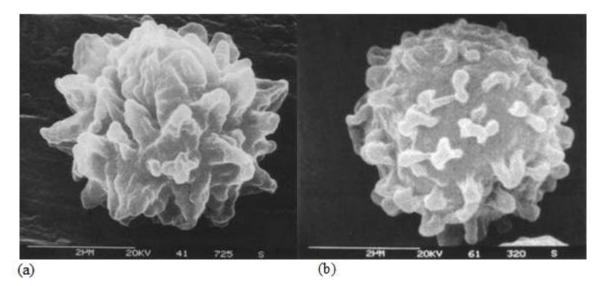
Figure 2.2: Scanning electron microscopy pictures of (a) *A. parasiticus*; and (b) *A. flavus* spores (Rodriguez et al., 2007)

Historically, only *A flavus, A. parasiticus* and *A. nomius* have been known as producers of aflatoxins (Ehrlich et al., 2007). However, Coppock and Christian (2007) have since reported that *A. bombycis, A. ochraceoroseus* and *A. pseudotamari* are also capable of producing aflatoxins.

a. Conditions of *Aspergillus* species and aflatoxin production

In the course of evolution, *Aspergillus* species as fungi have diversified to exploit a wide variety of habitats and different species therefore require different conditions for optimal growth (Klich, 2007). Classified as storage fungi, several *Aspergilus* species grow at 22-25 ºC; the thermophilic species (*A. fimigatus)* develop at 37-40 ºC and can also reach 57 ºC (Table 2.1) (Badillet et al., 1987; Klich, 2007). Moreover, the optimal temperature for fungal growth and aflatoxin production for *A. flavus* and *A. parasiticus* is 35º C with 0.95 *aw* and 33 ºC with 0.99 *aw*, respectively, and neither *Aspergillus* species produce aflatoxins when developed below 7.5 ºC or above 40 ºC (Pitt, 2000).

Table 2.1: Environmental factors for *Aspergillus* growth and aflatoxin production

			Aspergillus flavus			*Aspergillus parasiticus*		
			Minimum	Optimum	Maximum	Minimum	Optimum	Maximum
Growth		T(ºC)	10-12	33	43	12	32	42
		Water activity*	0.8	0.98	>0.99	0.80-0.83	0.99	>0.99
		pH	2	5-8	>11	2	5-8	>11
Aflatoxin production		T(ºC)	13	16-31	31-37	12	25	40
		Water activity*	0.82	0.92-0.99	>0.99	0.86-0.87	0.95	>0.99
		pH	-	-	-	2	6	>8

Source: International Commission on Microbiological Specifications for Foods (1996)
*water activity: *aw*

It was emphasised that the range for aflatoxin production was narrower than that for growth (Pitt, 2000). In spite of the nature of substrate, which includes a number of nutrients and pH values ranging from pH 4 to pH 8, and temperatures ranging between 10 ºC to 37 ºC, water activity (*aw*) was higher than 0.6 *aw*; the composition of the atmospheric gases (CO_2, O_2, etc.) around the toxigenic fungus represents another factor affecting toxin production (Ellis et al., 1993; 1994). According to Ellis et al. (1994), *A. flavus* growth and aflatoxin production occur in carbon dioxide-enriched atmospheres in the presence of oxygen. However, temperature (related to hot or warm conditions) and water activity (related to humidity or moisture) are major factors in the growth of fungi and mycotoxin production (Belli et al., 2004). This temperature and humidity range is found in most of the African countries where a tropical and subtropical climate prevails (Hell and Mutegi, 2011).

Cotty and Jaime-Gracia (2007) reported that heat or drought stress increases peanut susceptibility and favours fungal colonisation. Fungal colonisation can occur anytime, from pre-harvest, harvest and post-harvest time when the crop is exposed to warm,

moist conditions (Hell and Mutegi, 2011). However, *Aspergillus* species commonly produce aflatoxin during storage (Kamika and Takoy, 2011).

Aspergillus often forms powdery or granular colonies and the colour of the colonies allows for easy species identification, e.g. green-yellow for *Aspergillus flavus*, gray-green for *A. fumigatus*, yellow-rose for *A. versicolor*, etc. Indeed, *A. flavus* and *A. parasiticus* are broadly differentiated from each other primarily by the colour of their colonies (orange yellowish) and the morphology of their conidial structures (Steyn, 1980; Pitt, 2000).

The presence of aflatoxins in food crops can occur even in the absence of visible mould infestation due to a ceased vital cycle of the microorganism or by the effect of a removal of the mould due to food processing. Conversely, the presence of a visible mould on the surface of a food product does not represent a clear indication of the presence of a mycotoxin such as aflatoxins (Viljoen, 2003).

2.1.1.3. Biosynthesis of aflatoxin B1

The aflatoxins constitute a number of structurally related metabolites which differ considerably in their biological effects. However, all of them contain a coumarin ring combined to a bisdihydrofurano moiety and additionally either a cyclopentenone ring (B series) or a six-membered lactone ring (G series). Of all these toxins, AFB1 is the one with the greatest biological activity. Carcinogenic in several animal species, AFB1 reveals itself as the most potent hepatocarcinogen known in the rat and the rainbow trout (Yu and Cleveland, 2007). It has been reported that it is probable that the enzymes of aflatoxin biosynthesis and of other polyketides are similarly arranged

in discrete particles in the post-mitochondrial fraction (Wild and Turner, 2002). The aflatoxin biosynthesis is also characterised by 29 clustered aflatoxin pathway genes and can be described in two major stages: an early stage from acetate to Versicolorin A (VERA) (coloured pigment in brick-red, yellow, or orange) and a later stage from demethyl-sterigmatocystin (DMST) to AFB1 (colourless under normal light and fluorescent-blue under UV light) (Yu and Cleveland, 2007).

2.1.1.4. Bioactivation of aflatoxin B1

Like many other chemical carcinogens, AFB1 requires bioactivation to a reactive toxic metabolite-activation as an important stage in its toxicity expression (Donnelly, 1998). Aflatoxin B1 cannot itself be the toxic molecule but it is metabolised in the animal body in a complex network of reactions and it is the result of this metabolism which determines both acute and chronic toxicity (Figure 2.3). Many researchers have studied the relationship between the biological activity of AFB1 and its metabolism, and have shown the evidence that AFB1 needs metabolic activation to exert its carcinogenic and mutagenic effects (Gallagher et al., 1994; Kirby et al., 1996; Niu et al., 2008). After ingestion, AFB1 presents a short half-life; 65 % of the quantity absorbed after 90 min is removed from the blood and plasma and metabolised by the liver to a reactive epoxide intermediate. It has been estimated that in human liver monogenates, the half-life of AFB1 is 15 min (Fung and Clark, 2004; Bastaki et al., 2010).
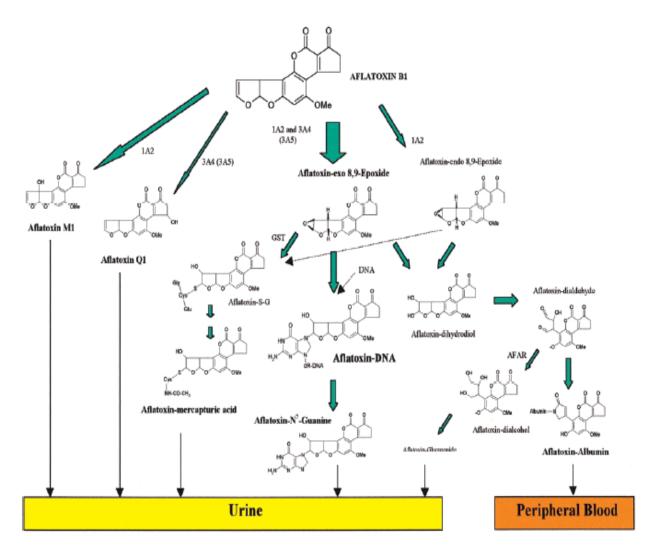
Figure 2.3: Metabolism of AFB1 leading to reactive metabolites and biomarkers (Wild and Turner, 2002)

In the metabolism, however, the first step of it takes place in the hepatocyte, with nonreversible detoxification which lea to the formation of hydroxylated metabolites (AFM1, AFQ1, AFP1, AFB2a), followed either by reversible detoxification through aflatoxicol formation, or by activation through the generation of AFB1-8-9-epoxide (Neal, 1998).

However, AFB1 is mainly bio-activated by cytochrome P450-dependent monooxygenase which results in the production of many metabolic products such as

aflatoxin Q1, aflatoxin P1, aflatoxin M1 and aflatoxin B1-8-9-epoxide. Aflatoxin B1-8-9-expoxide has been reported to be the most toxic metabolite (Do and Choi, 2007). Cytochrome P-450 monooxygenase has been demonstrated as a key factor in the metabolic activation of several chemical carcinogens such as AFB1, various heterocyclic and aromatic amines, and specific nitro-aromatic compounds (Viljoen, 2003).

Among these metabolic products, aflatoxin B1-8-9-epoxide has been shown as an important metabolite synthesised in the animal liver and can react with guanine residues in DNA and lead to depurination (Moss, 1996; Yu and Cleveland, 2007; Donnelly, 1998). The net result is gene mutation. The most regularly induced mutation is the GC→TA transversion, potentially leading to carcinogenesis (Bressac et al., 1991; Smela et al., 2001). In addition, the epoxide occurs in endo- and exo-forms. The exo-epoxide is highly electrophilic and reacts with several macro-molecular (Smela et al., 2001). The activated AFB1, aflatoxin B1-8-9-epoxide can bind to glutathione, cellular proteins, RNA and DNA. The binding of aflatoxin B1-8-9-epoxide to DNA has been investigated in rats and was found to take place at the critical nucleophilic sites of DNA and identified to form 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-aflatoxin B_1 (Fung and Clark, 2004), which is also associated with tumour development in animals (Dashwood et al., 1989). However, when bound to glutathione, aflatoxin B1-8-9-epoxide produces another metabolite which is less toxic Wild and Turner, 2002). Wild and Turner (2002) reported that there is evidence that the interaction of DNA with aflatoxin is not direct, but rather a process of activation through an epoxide metabolic activation pathway.

Many mineral elements including Zn^{2+}, Cu^{2+} and Fe^{2+} are also essential for this activation by contributing to the cyclisation of the polyketide precursors and also affecting the induction of the enzymes of secondary metabolism (Do and Choi, 2007). In light of this, AFB1 may be seen as a multiple menace by its carcinogenic, teratogenic, and mutagenic effects and also by its immunosuppressive effects (Do and Choi, 2007).

2.1.2. Aflatoxin B1 and its health effects

The evidence of mycotoxin effects on human and animal health has been demonstrated by many studies on laboratory animals and extrapolated to humans. After several studies on the correlation between mycotoxin-contaminated food and cancer in humans, the carcinogenic effects of mycotoxins, especially AFB1, have been established (Moss, 1996).

The impact of mycotoxins on human and animal health depends on the gender, age, length of exposure, dose, species (in the case of animals), etc. (Newberne, 1974; Moss, 1996). Human intoxication by mycotoxins may occur via dermal contact, ingestion and inhalation. Ingestion through consumption of contaminated food is the most likely and relevant route (Ciegler and Bennett, 1980; Brera et al., 2008). Aflatoxin B1 is shown to be the most toxic and therefore the most studied because of its effects on human and animal health. In terms of concentration and time of exposure, AFB1 can have an acute as well as chronic effect on humans and animals. The liver was found to be the principal target of AFB1 (Harrison et al., 1993) although other organs such as the kidney, stomach, lung, salivary and lachrymal glands, colon, and skin may be affected (Harrison et al., 1993; Henry et al., 1999).

The following section will explain in detail the acute and chronic effects of AFB1 on humans and animals.

2.1.2.1. Acute effects of aflatoxin B1

The acute toxicity of AFB1 is brought about by ingesting a considerable dose through aflatoxin-contaminated food. The ingestion of high-level aflatoxins which produces an acute hepatic failure (hepatic necrosis), is generally manifested by haemorrhage, oedema, alteration in digestion, changes to the absorption, mental changes and coma (Williams et al., 2004). In developing countries (Southeast Asia and sub-Saharan Africa) people are exposed to high levels of AFB1; there is high level of AFB1 exposure in humans and which may result in aflatoxicosis (Lewis et al., 2005).

An acute outbreak of aflatoxicosis in India associated with the consumption of aflatoxin-contaminated corn and hepatitis has been reported and over 100 deaths occurred and the ingested dose causative of the event was estimated at 2 mg/d to 6 mg/d over a month. Males were more susceptible and were affected twice as much as females (Khlangwiest, 2011). In China, aflatoxicosis caused the deaths of 13 children due to acute hepatic encephalopathy (Brera et al., 2008). More recently, one of the largest aflatoxicosis outbreaks occurred in rural Kenya in 2004, resulting in 317 cases with 125 deaths (Lewis et al., 2005). A case-control study of this acute aflatoxicosis outbreak was conducted by identifying the risk factors for contamination of implicated maize and quantified biomarkers. The outcomes of the study revealed that the aflatoxin concentrations in maize, the serum aflatoxin B1-lysine adduct concentrations, and positive hepatitis B surface antigen titres were all associated with the case status (Azziz-Baumgartner et al., 2005).

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In South Africa, isolated outbreaks of aflatoxicoses have been recorded on a farm where animals had been fed with inappropriately harvested and stored maize (Kellerman et al., 1988). It has been established that the consumption of contaminated food with 1.7 mg/Kg for a short time could result in severe liver damage, and a single dose of 75 mg/Kg may cause death (Stewart, 1997). Studies carried out on animals have demonstrated that some animals are remarkably more sensitive to AFB1 intoxication than others, e.g. day-old ducklings and the adult dog exhibited LD_{50} values of 0.35 mg/kg and 0.5 mg/kg body weight, respectively, while rats and mice had LD_{50} values of 9 mg/kg (Moss, 1996).

2.1.2.2. Chronic effects of aflatoxin B1

The chronic toxicity of AFB1 results from long-term exposure to low or moderate levels and does not lead to immediate symptoms as dramatic as acute aflatoxicosis (Moss, 1996). It is reported that chronic exposure to aflatoxin leads to a high risk of developing cancer, especially liver cancer, as well as stunted growth and delayed development in children. Developing countries have been reported as having the highest incidence of liver cancer as well as the highest risk of chronic aflatoxin exposure (Figure 2.4). It has been estimated that more than 5 billion people in the developing countries are at risk of chronic aflatoxin exposure through consumption of contaminated foods (Wu et al., 2011).

a. Role in cancer development

Aflatoxins, especially AFB1, are among the most potent naturally occurring carcinogens known and may induce tumours in many humans and animals (Moss, 1996; Shen and Ong, 1996; Moss, 2002). Classified as human carcinogen group A1

by the IARC (1993), it is predominantly perceived as an agent promoting liver cancer in humans, although it can also be involved in lung cancer (Williams et al., 2004). Aflatoxin may have a synergistic effect with other diseases such as kwashiorkor, HIV/AIDS, hepatitis B or C or with other mycotoxins, for example, fumonisin B1, T-2 toxin, zearalenone, etc. (Omer, 2001; Kew, 2002; Wangikar et al., 2005; Orsi et al., 2007). Aflatoxin B1 has also been shown to possess immunosuppressive effects (Creppy, 2002), teratogenic effects (Wangikar et al., 2005), and mutagenic effects (Harris, 1990; Shen and Ong, 1996) in several animal species.
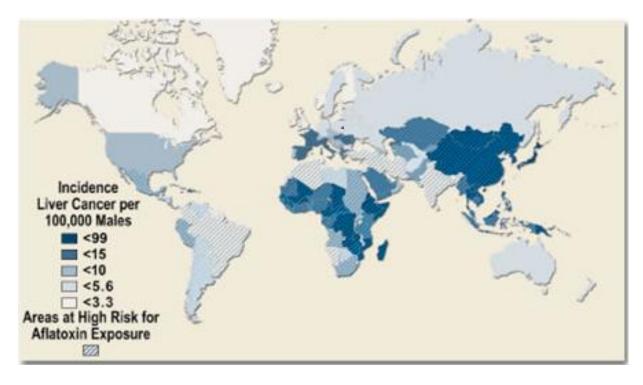
Figure 2.4: Correlation between populations with high liver-cancer rates and high risk of chronic aflatoxin exposure (National Institute of Environmental Health Sciences, 2011)

b. Liver cancer

Chronic aflatoxin exposure results mostly in primary liver cancer of which hepatocellular carcinoma (HCC) is by far the predominant type (Wild and Hall, 2000; Turner et al., 2005). In addition to aflatoxin exposure, it has been reported that

several factors such as hepatitis B/C viral infection, nitrosamines, alcohol, etc., may contribute towards the development of liver cancer in humans (Viljoen, 2003). A strong synergy has been reported between aflatoxin and hepatitis B (HB) and/or C (HC) with aflatoxin being approximately 30 times more potent in persons with hepatitis B than in those without the virus (Williams et al., 2004). A study investigating the induction of hepatocellular carcinoma in tree shrews exposed to human hepatitis B virus (HBV) and AFB1 showed that there is a synergistic role between AFB1 and HBV in the development of HCC (Yan et al., 1996). Wong et al. (1977) utilised *Salmonella typhimurium* T98 for testing the mutagenic effect of AFB1 and found that the compound has a relative mutagenic potency of 100 and suggested that the mutagenicity and carcinogenicity of this compound is associated with the bisdihydrofuran moiety. Miranda et al. (2007) analysed the DNA damage induced by AFB1 in Dunkin-Hartley guinea pigs and found that there is a relationship between the levels of DNA damage and the consumption of AFB1 in the liver cells. These authors also reported that exposure to this toxin increases the level of DNA damage in liver cells significantly, which is a key step in liver-cancer development.

Liver cancer has become a major public health problem being the third most frequent cancer accounting for 695 000 deaths in 2008 and 550 000 to 600 000 new cases each year, with an estimated 42 000 deaths occurring every year in sub-Saharan Africa (Wu et al., 2011). There is evidence that human exposure to high levels of AFB1 in food and hepatocellular carcinoma occurs more frequently in developing countries where the incidence of hepatitis B virus is also high (Henry et al., 1999; Smela et al., 2001; Lu, 2003; Turner et al., 2005).

Carcinogenic effects of AFB1 on humans and animals have been well documented. In particular, AFB1 was identified as carcinogenic in rainbow trout (Moss, 1996; Coppock and Christian, 2007). In the USA, some studies reported aflatoxins as the cause of epizootic hepatitis in dogs and as the cause of mouldy corn poisoning in pigs (Coppock and Christian, 2007). Aflatoxin B1 has been demonstrated to have a carcinogenic effect in rainbow trout, duck, rat, mouse, monkey and marmoset (Ciegler and Bennett, 1980). It is also reported as being a potent carcinogen in rats, causing liver cancer, but it is much less carcinogenic in mice (Moss, 1996). It has been reported that when fed with a diet containing 30 mg/Kg of AFB1, 11 ducks developed hepatocellular carcinoma (Lu, 2003). In their study, Svoboda et al. (1966) recorded that three out of six Fischer rats fed with 1.0 mg/Kg of AFB1 in the diet also developed hepatocellular carcinoma.

Although the carcinogenic effects of AFB1 on various animals have been demonstrated, some researchers remain unconvinced (Viljoen, 2003). This, despite the additional evidence on the role of AFB1 in human liver cancer provided by both Hsu et al. (1991) and Bressac et al. (1991), by demonstrating that 50% of human liver tumour tissues collected from Southern China and Southern Africa were associated with high dietary AFB1. Hepatocellular carcinoma incidence contained a single point mutation in the tumour suppressor gene *p53* and the majority of the detected mutations were GC→TA transversions at codon 249 (arginine to serine) and this mutation was isolated in people living with hepatocellular carcinoma (HCC) (Stewart, 1997).

Several epidemiological studies have been aimed at showing the evidence of the carcinogenic effects of AFB1 and the correlation between exposure to AFB1 in diet and the incidence of human liver cancer around the world (Ciegler and Bennett, 1980; Moss, 1996). In 1996, a cohort study combined with a molecular dosimetry approach to assess the effects of aflatoxin exposure was performed in the Penghu Islets where the HCC mortality rate was highest in Taiwan. The results implied that elevated risk of HCC among the residents may be attributable to their heavy exposure to aflatoxins and high hepatitis B surface antigen (HBsAg) carrier rate (Chen et al., 1996). High aflatoxin concentrations in the diet have also been linked to increased liver-cancer risk in mainland China (Wu, 2004).

Recently, some studies recorded the presence of liver tumours in the areas where there is high aflatoxin exposure and demonstrated that AFB1 has high mutagenic effects. Patients with liver cancer or cirrhosis were found to have this kind of mutation in their plasma (Montesano et al., 1997; Kirk et al., 2006). It has been reported that aflatoxin B1-associated mutagenesis represents a plausible cause for the higher chromosome instability observed in Chinese HCC (Pineau et al., 2008). This mutagenic effect associated with the carcinogenic effect was also demonstrated in the mouse and in *Drosophila* (Ciegler and Bennett, 1980).

In addition, Hosny et al. (2008) has reported that mutagenesis by aflatoxin may have played a role in high levels of human hepatocarcinogenesis in Egypt. Aflatoxin B1 can be inhaled and is implicated in lung cancer, but there is only weak evidence supporting this (Georggiett et al., 2000). In a study of agricultural workers exposed to

aflatoxins, a two- to three-fold increase in the risk of hepatocellular carcinoma and biliary tract cancer was found (Fung and Clark, 2004).

c. Lung cancer

In addition of being a potent hepatocarcinogen, AFB1 has been reported as a pulmonary carcinogen in experimental animals (Donnelly et al., 1996) and epidemiological studies have revealed the link in humans (Inoue et al., 2011). A study by Cusumano (1991) reported the presence of aflatoxins in sera from patients with lung cancer. However, there was no significant correlation found to provide evidence for a causal relationship between aflatoxin exposure and development of lung cancer in humans. Nevertheless, when van Vleet et al. (2002) compared aflatoxin B1 activation and cytotoxicity in human bronchial cells expressing cytochromes P450 1A2 and 3A4, they reported an evidence link between the inhalation of aflatoxin B1-contaminated dusts and increased lung-cancer risk. These authors stated that the link is possible under conditions in which appropriate human cytochromes P450 (CYPs) are expressed in the lung. A study conducted by Dvorackova et al. (1981) has demonstrated the evidence of the role played by the toxin in two cases of lung cancer in patients having a pulmonary *Aspergillus* mycosis. Another study evaluating the rate of mortality among peanut workers, showed increased rates of overall mortality and lung-cancer mortality (Cusumano, 1991). Additionally, in a study on the assessment of the human lung exposure risk to airborne AFB1 during farming activities, including swine feeding, Liao and Chen (2005) estimated that there is a potentially high risk for the bronchial region during swine-feeding activity.

d. Liver cirrhosis

Liver cirrhosis is generally irreversible and a consequence of chronic liver disease characterised by fibrous scarring and hepatocellular regeneration of the normal hepatic structure of regenerative nodules and fibrotic tissue (Kim et al., 2005). Cirrhosis is most commonly caused by alcoholism, hepatitis B and C, and fatty liver disease, but has many other possible causes. Some cases are idiopathic, i.e., of unknown cause. Little has been reported on the existent link between aflatoxin exposure and liver cirrhosis (Wild and Gong, 2010). An etiological study of liver cirrhosis conducted in Gambia reported the implications of aflatoxin in the rate of morbidity and mortality of Gambian people (Kuniholm et al., 2008).

e. Role in immunosuppression

Immunosuppressive agents are substances that inhibit or prevent activity of the immune system by suppressing the cell-mediated immunity, the humoral immunity, etc. (Williams et al., 2004). Previous studies have shown that AFB1 is an immunosuppressive substance in humans and in various animals (Bondy and Pestka, 2000; Meissonnier et al., 2008; Jiang et al., 2008). An immunological study conducted on chicks demonstrated that the progeny chicks from hens consuming an aflatoxin-spiked diet were increasingly susceptible to disease owing to suppression of humoral and cellular immunity (Qureshi et al., 1998). Other studies have shown that exposure to aflatoxins, especially AFB1, can affect the developing immune system during embryonic development (Todd and Bloom, 1980; Potchinsky and Bloom, 1993). When injected into 18-day-old chicken embryos, aflatoxin caused significant DNA damage in B and T lymphocytes (Qureshi et al., 1998). Theoretically, by impairing DNA-dependent RNA polymerase, therefore inhibiting RNA and protein

synthesis, they could damage the proliferation and differentiation of immune cells, immunoglobulin, and cytokines (Fung and Clark, 2004). Since both aflatoxin and HIV are immunosuppressive agents, Jiang et al. (2008) hypothesised that aflatoxin exposure may also influence the pattern of infection leading to an immune dysfunction of people living with HIV/AIDS. In addition, Sahoo and Mukherjee (2001) reported that AFB1 is an immunosuppressive in Indian major carp even at the lowest dose of 1.25 mg/kg of body weight of toxin treatment. On the contrary, Raisuddin et al. (1993) reported that at low levels, aflatoxin may enhance the susceptibility of the rats to infection and tumorigenesis.

f. Role in undernutrition

Mostly affecting children, undernutrition has been defined as the outcome of insufficient food intake and repeated infectious diseases. This included underweight and too short for one's age, dangerously thin, and deficient in vitamins and minerals (UNICEF, 2006). It has been reported that in sub-Saharan Africa, approximately 50 % of the 4.5 million deaths of children under the age of five are associated with undernutrition and growth impairment and aflatoxin contamination seemingly is the main contributor (Turner et al., 2007). Aflatoxin B1 has been also implicated to the aetiology of kwashiorkor and marasmic kwashiorkor in humans (Sibanda et al., 1997). This association has been reported from several African countries including Sudan, Nigeria, South Africa, Liberia, Rwanda, Ghana and the Philippines (Seres and Resurrection, 2003). Oyelami et al. (1997) reported the presence of aflatoxins in the lungs of children who died from kwashiorkor and miscellaneous diseases in Nigeria. In 1998, autopsy kidney specimens from Nigerian children with kwashiorkor and miscellaneous diseases were analysed for the presence of aflatoxin and

researchers found that aflatoxins can be detected in the kidneys of children exposed to aflatoxins (Oyelami et al., 1997) and this was related to the possible effects of AFB1 to kwashiorkor. In contrast, another study carried out on the effects of AFB1 on the development of kwashiorkor in Swiss albino mice concluded that AFB1 could not have contributed to the development of kwashiorkor (Kocabas et al., 2003).

g. Role in fertility

Aflatoxin was reported to affect the reproduction capacity and fertility of both animal and humans (IARC, 2002). A study on the determination of AFB1 effects on Ram epididymal and ejaculatory sperm viability and mortality demonstrated that AFB1 could also decrease epididymal and ejaculatory sperm viability and motility and could therefore affect male fertility (Tajik et al., 2007). Another study trying to discover the relationship between aflatoxin levels and infertility in human males, reported that when exposed to aflatoxin, albino rats produced deleterious effects on the spermatozoa that resembled those observed in the semen of infertile men exposed to aflatoxin (Ibeh et al., 1991).

h. Teratogenic effects

Previous studies have reported aflatoxin, especially AFB1, as a teratogenic agent to animals and humans due to its disturbance in the development of the embryo or foetus by stopping the pregnancy or producing a congenital malformation (Datta and Kulkani, 1994; Fung and Clark, 2004). In addition, AFB1 was incriminated to cross the placental barrier, and thus can adversely affect foetal systems, to increase still-births and neonatal mortality (Wild et al., 1991; Hendriks, 2005; Maxwell et al., 2000). Ciegler and Bennett (1980) reported the teratogenic effect of AFB1 in

hamster, guinea pig and rat. In contrast, Vismara and Caloni (2007) when evaluating the embryo toxicity of aflatoxin B1 using the frog embryo teratogenesis assay-*Xenopus* and the bio-activation with microsome activation systems, reported that AFB1 should be a strong teratogen in hamsters, but its effect in rats is equivocal and it is extremely limited in mice. These authors showed that AFB1 alone is not embryo-toxic but when bio-activated with MAS-rate or MAS-human the percentage of mortality and malformed larvae increased significantly. In human, *in utero* exposure to aflatoxin could be detected on assaying maternal venous peripheral blood and cord blood for AFB1-lysine adducts. In Gambia, there was a highly significant correlation between adduct levels in maternal venous and matched cord sera indicating maternal dietary intake to be an important determinant of the carcinogenic-induced damage in the foetus (Wild et al., 1991).

i. Synergistic effects with other mycotoxins

Another threat of AFB1 effects is its possible synergistic action with other mycotoxins which can lead to increased effects in humans and animals. A study conducted by Wangikar et al. (2005) on the teratogenic effects in pregnant New Zealand white female rabbits of simultaneous exposure to ochratoxin A and aflatoxin B, demonstrated that AFB1 was teratogenic with an antagonistic interaction with ochratoxin A1. An investigation into the comparative acute and combinative toxicity of AFB1 and fumonisin B1 (FB1) in animals and human cells found that these two toxins interacted to produce alterations in the toxic responses with a strong additive interaction noted in the cases of F344 rats and mosquito fish (Mckean et al., 2006). Furthermore, Orsi et al. (2007) also recorded the synergistic action of toxic effects of AFB1 and FB1 in the liver and kidney.

2.2. ECONOMIC IMPACT

Aflatoxin-producing mould is ubiquitous in the natural environment and can enter the food chain (Vesley, 1999). Due to the effects of aflatoxin on human and animal health, international trade bodies and health authorities have imposed limits of aflatoxins allowable in various crops. For example, in the European Union (EU), the presence of aflatoxins in peanuts is strictly monitored and regulated to guarantee their safety with a limit of 2 µg/kg for AFB1 and 4 µg/kg for AFs (van Egmond, 1995). These restrictions may cause major agricultural and economic problems since aflatoxins could occur in the field, during harvest, storage or during processing (Dorner and Cole, 2002). D'mello (2003) reported that aflatoxin is the most important problem regarding quality of peanuts worldwide.

Aflatoxins, especially AFB1, can also contaminate many other commodities such as Brazil nuts, pistachio nuts, cottonseed meal, and maize and grain sorghum during growth, harvesting, processing, storage and shipment, thereby causing serious economic losses due to production losses, loss of export markets and rejection of produce at import ports (Pitt, 2000; Kamika and Takoy, 2011). For example, in the United States of America, growers in Texas, Louisiana and Mississippi sustained losses estimated at $85 million to $100 million from maize that could not be utilised for human consumption because of high levels of aflatoxin (Henry et al., 1999). It has been estimated that both cattle farming and food packaging/processing industries in North America lose around $5 billion each year because of mycotoxin contamination (Olsen et al., 2008). In West African countries such as Senegal where peanut is an important export crop, the quantities of peanut exports declined substantially during the period of 1961 (269 436 t) to 2000 (1 792 t) because of increasing restrictions on

importation of contaminated produce into the EU. This has huge economic ramifications for African exporters and growers (Henry et al., 1999; Boakye-Yiadom, 2003). It has been reported that the biggest peanut-exporting regions which include USA, China, Argentina and Africa would experience economic losses of up to $450 million per year if the EU aflatoxin standard of 4 µg/kg were to be imposed worldwide (Wu, 2004). Africa alone, due to the more stringent mycotoxin limitations, has lost export opportunities estimated by the World Bank at US$ 650 million each year in exports of cereals, dried fruit and nuts due to the European Union regulations (Roberts and Unnevehr, 2003).

2.3.   NATURAL OCCURRENCE OF AFLATOXIN IN AFRICA

In Tunisia, Ghali et al. (2008) investigated the presence of aflatoxin in foods (spices, dried fruits, sorghum and rice) and reported that aflatoxins were detected in all analysed commodities with a contamination frequency of 50.5 % and AFB1 was found in 37 % of the samples.

In Morocco, Juan et al. (2008) found aflatoxins by using IAC clean-up with liquid chromatography and fluorescence detection in peanuts (5 % of total aflatoxins and 5 % of AFB1), dried raisins (20 % of aflatoxins and AFB1), dried figs (30 % of aflatoxins and 5 % of AFB1), walnuts (30 % of aflatoxins and AFB1) and pistachio nuts (45 % of aflatoxins and AFB1) with the highest contamination levels of AFB1 in walnut (2 500 µg/kg) and in pistachio nut (1 430 µg/kg).

Groundnut is an important crop in Senegal; however, it is subjected to many surveys due to environmental conditions that are favourable for fungal growth and aflatoxin production nationwide (Diop et al., 2000). Park and Njapau (1989) analysed 73

peanut samples for AFB1 and found that 100 % of the samples contained AFB1 ranging from 20 µg/kg to 200 µg/kg. Ndiaye et al. (1999) analysed peanut oil samples for aflatoxins by HPLC and found that 85 % of the samples contained AFB1 with a mean of 40 µg/kg. Diop et al. (2000) analysed artisanal and industrial peanut butter for aflatoxins by HPLC and found that 40 % of the samples contained AFB1 at a concentration of >5 µg/kg. This finding has had a great impact on the groundnut trade and therefore on the economy and confirmed that Senegalese peanuts are often contaminated with AFB1 at a level substantially higher than the allowable limit in terms of specifications (Ndiaye et al., 1999; Diop et al., 2000).

In Nigeria, one of the West African countries with climatic conditions very favourable for fungal growth and mycotoxin contamination, a number of studies have been conducted to investigate the presence of aflatoxins in a wide range of foods and foodstuffs. Ibeh et al. (1991) reported the presence of aflatoxins in various food samples collected from Nigeria; 15 % of yam flour, 40 % of cassava flour, 30 % of garri, 20 % of beans and melon and 10 % of rice samples were contaminated with aflatoxins. Akano and Atanda (1990) also investigated the presence of aflatoxin in peanut cake samples and found AFB1 levels ranging from 375 µg/kg to 455 µg/kg in 28 of the 32 samples. Bankole et al. (2005) analysed dry-roasted groundnuts for aflatoxins by TLC and reported that 64.2 % of the samples were found to contain AFB1 with a mean of 25.5 µg/Kg. Another survey reported the presence of AFB1 in maize with a mean of 22 µg/kg (Bankole et al., 2004). These reports reveal that AFB1 is present in Nigerian foodstuffs with concomitant economic and health problems.

In Botswana, a survey showed that Botswana peanuts are contaminated with aflatoxigenic fungi and aflatoxins (Mphande et al., 2004). Another survey reported the presence of aflatoxin ranging from 12 µg/kg to 329 µg/kg in raw peanut (Barro et al., 2002).

In order to estimate the relative exposure of Tanzanian people to aflatoxins, Kimanya et al. (2008) collected maize samples for analysis. Their results showed the presence of high levels of aflatoxin exceeding the Tanzanian limit (10 µg/kg) in maize samples.

In Uganda, several crops (480 samples) were investigated for aflatoxin and beans were found to be the most contaminated (72% of the samples) and 4% of the samples showed a concentration of up to 1 µg/Kg (Alpert et al., 1971). Later, Kaaya and Kyamuhangire (2006) reported the presence of *Aspergillus, Fusarium, Penicillium* and *Rhizopus* and high levels of AFB1 in maize.

Since aflatoxicosis outbreaks have occurred in Kenya, a large number of foods and foodstuffs have been investigated. Lewis et al. (2005) reported the presence of high levels of AFB1 ranging from 20 µg/kg up to 1 000 µg/kg in the offending maize. Another survey carried out by Mutegi et al. (2009) analysed the prevalence and factors associated with aflatoxin contamination of peanuts from western Kenya. Two regions were selected and it was found that peanut samples from both regions were contaminated with aflatoxins, in some cases >2 500 µg/Kg. Very few studies on aflatoxin occurrence have been conducted in the DRC. In 1977, Brudzynski et al. analysed peanuts, maize and dried cassava for AFB1 and found that the toxin was

present in almost all the samples and 33% of cassava contained AFB1 ranging from less than 12 µg/Kg to greater than 1 000 µg/Kg. Additionally, Masimango and Kalengayi (1982) analysed food and foodstuffs for aflatoxins by TLC with spectrophotometry for quantitation. They reported that sweet potatoes, groundnuts, dried cassava roots, powder of cassava roots, maize meal, banana powder, and sorghum contained aflatoxins with high levels in sweet potatoes and groundnuts. Recently, Kamika and Takoy (2011) reported high levels of AFB1 in peanuts collected from Kinshasa.

In South Africa, although there is well-developed commercial farming sector, mycotoxins, especially aflatoxins, are sporadically found mostly in maize (Shephard, 2003). Dutton and Kinsey (1995) investigated the presence of mycotoxins in cereals and animal feed in KwaZulu-Natal Province, South Africa and found aflatoxins in only 6% of 417 samples of agricultural commodities. According to several newspaper reports, peanut butter used to feed schoolchildren was contaminated with total aflatoxins and AFB1 at concentrations of up to 27 µg/Kg,163 µg/Kg and 16 505 µg/Kg, respectively (Williams et al., 2004).

2.4. LEGISLATION

The United Nations Food and Agricultural Organization (FAO) is charged with ensuring the security and safety of food for human and animal consumption. In so doing it has to ameliorate both microbiological (including algae, moulds, etc.) and chemical (mycotoxins, phycotoxins, etc.) hazards which may contaminate food and feed (WHO, 2002). Consequently, many international regulatory agencies and countries have established legislation for maximum limits of mycotoxins and monitor

mycotoxin levels especially aflatoxin levels in susceptible crops (Moss, 2002; Creppy, 2002). According to the FAO (2004) at least 100 countries have specific regulations to deal with mycotoxins or with at least regulatory limits on aflatoxins; 13 countries do not have specific regulations while 50 countries, of which 40 are from Africa, have no regulations/no data exist (Figure 2.5). Only eight African countries have existing regulations for aflatoxin, viz. Cote d'Ivoire, Egypt, Kenya, Malawi, Nigeria, Senegal, South Africa and Zimbabwe (van Egmond, 1989; FAO, 1997; van Egmond, 1999; Shephard, 2003). Figure 2.6 presents a map which highlights African countries with mycotoxin (aflatoxin) regulations.

Currently, the European Union enforces stringent maximum residue levels for total aflatoxin and AFB1 levels in human commodities of 4 µg/kg and 2 µg/kg, respectively (Moss, 2002), which enforcement has impacted on the export of food commodities from several African countries. The FAO and the WHO were faced with the dilemma of setting the limits of aflatoxin in human foods against high background levels of malnutrition and the danger that aflatoxin would produce liver cancer. They have established the upper aflatoxin limits of 30 µg/kg in foods for human consumption (Moss, 1996), while the Codex Alimentarius Commission and the JECFA have adopted the maximum limit for total aflatoxins as 15 µg/Kg in peanut, and 10 µg/Kg in peanut processed for human consumption, which means that half of those concentrations are applicable in terms of AFB1 (Freitas and Brigido, 1998; Codex, 2001).
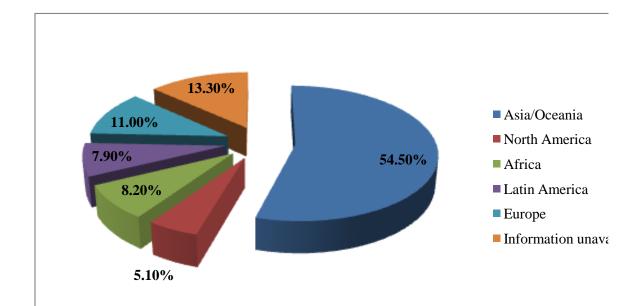
Figure 2.5: Percentage of global population covered by mycotoxin regulation in 2003 (FAO, 2004)
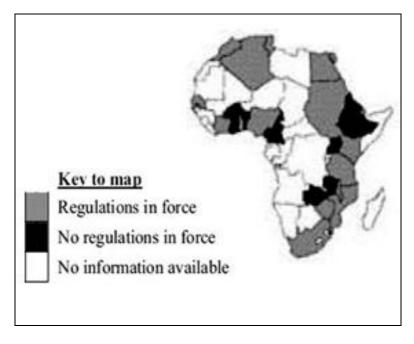
Figure 2.6: African map highlighting the presence or absence of mycotoxin regulation (in most case aflatoxins) (FAO, 2004)

Nevertheless, it should be mentioned that the WHO prescribed the maximum limit for AFB1 in various foodstuffs is 5 µg/kg (Papp et al., 2002). Table 2.2 illustrates the tolerance levels of AFB1 in foods in some developing countries.

Table 2.2: A selection of tolerated levels of aflatoxin B1 in food

Countries	Max level (µg/kg)	Products
Argentina	0	Groundnuts, maize
Brazil	15	products
India	30	All foodstuffs
Nigeria	20	All foods
South Africa	5	All foods
Zimbabwe	5	All foods
		Foods

Source: Boutrif and Canet (1998)

South Africa is one of the few African countries known to have a regulation for aflatoxin in foods and feeds; the maximum tolerable limit for aflatoxins and AFB1 has been in existence for years and is fixed at 10 µg/Kg and 5 µg/Kg, respectively (Viljoen, 2003). However, in the DRC the existing limit permissible is unknown and the country may be classified among those with no data available, therefore no specific regulation exists for mycotoxins (Kamika and Takoy, 2011). Although the mycotoxin regulations are the way to protect populations against consumption of contaminated foods, unfortunately, in developing countries, these regulations are very difficult to enforce because of poor socio-economic and humanitarian conditions and limited food supplies (van Egmond, 1999; Creppy, 2002; Shephard, 2003).

2.5.   COMMON ANALYTICAL METHODS FOR AFLATOXIN ANALYSIS

The need to meet the regulatory limit control of mycotoxins such as aflatoxins in foods and feeds for human or animal consumption has resulted in a plethora of methods from conventional analytical methods to rapid methods (Shephard, 2008). In addition, the accuracy and sensitivity of these methods in the determination of mycotoxin have become important requirements to meet food-safety concerns and

stringent legislated regulations (Shephard, 2009). Among those methods, conventional analytical methods such as thin-layer chromatography, liquid chromatography (LC) and gas chromatography (GC) have been widely used for decades and revealed high sensitivity for the determination of aflatoxins. In the history of mycotoxin analysis, TLC has been reported as one of the pioneering methods but it is often used as a mycotoxin-screening method due to several challenges in the quantification of toxins (Zheng et al., 2006). However, the LC methods such as HPLC, LC-MS, LC-MS/MS are seen as powerful methods for the screening and quantification of a number of mycotoxins when large numbers of samples are being assayed (Pascale, 2009).

The GC, indeed, is considered to be used in more technical laboratories for the analysis of particular mycotoxins which are not easily detected by HPLC. Unfortunately, due to the time consuming during the process and competition within the food and feed industry while using these conventional analytical methods, the need to develop rapid methods have been raised (Shephard, 2008). Most of the rapid screening methods rely on the reaction antigen-antibody to detect mycotoxins from the samples. Among those, enzyme-linked immunosorbent assays (ELISAs), flow-through membrane-based immunoassay, immunochromatographic assay, fluorometric assay with immunoaffinity clean-up column or solid-phase extraction clean-up column, fluorescence polarisation methods have been reported as the most used (Zheng et al., 2006). Besides having the disadvantages of being more a screening method rather than a quantitative method, rapid screening methods have demonstrated a very important advantage of being an easy-to-use and fast method during analysis when referring to reference methods (Shephard, 2008). Despite the

rapid development of analytical methods and sophisticated instrumentation, the basic key to laboratory performance of all methods lies in ensuring the accuracy and reliability of the analytical results which are important to food-safety programmes (Shephard, 2008).

CHAPTER THREE: MATERIALS AND METHODS

3.1. DESCRIPTION OF THE STUDY AREAS

Agro-ecological zones provide useful information on climate, including radiation, rainfall, temperature and humidity for the particular purpose (Quiroz et al., 2001). Since environmental factors such as humidity, temperature, etc., play a major role in fungal spoilage and aflatoxin production in food crops, the agro-ecological zone map gives a good indication of zones (humid, subhumid and semi-arid) with high risk (Figure 3.1).
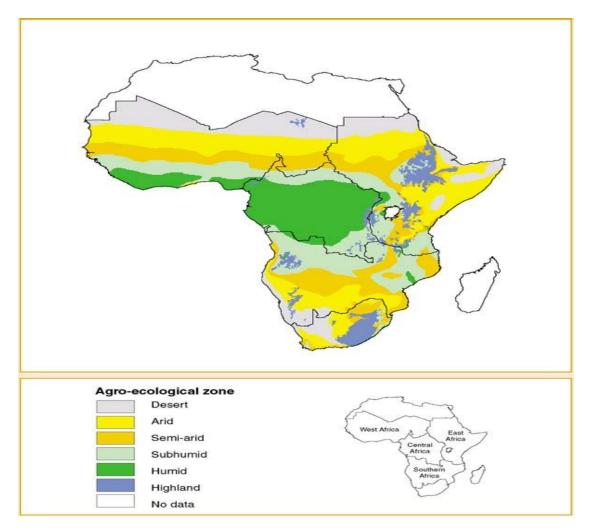
Figure 3.1: Agro-ecological zones in Africa highlighting zones with high/low risk of fungal spoilage and aflatoxin contamination

3.1.1. Kinshasa

Locality – Kinshasa is both a city and province in the Democratic Republic of Congo (DRC) and is located along the south bank of the Congo River (latitude: 4º 19' 47 S, longitude: 15º 18' 54 E). It is divided into four districts which are further divided into 24 municipalities (communes) (Wikipedia, 2011a).

Climate – Kinshasa is situated in a tropical wet and dry climate region which experiences a lengthy rainy season between October and April, with temperatures ranging between 29.4ºC and 37.8ºC, with high humidity, and a short dry season between May and August, with temperatures ranging from 18.3ºC to 26.7ºC, with low humidity (Hightower et al., 2009). Kinshasa's dry season is slightly cooler than the wet season with the average minimum and maximum temperatures for the study area described in Table 3.1.

Table 3.1: Average temperature and precipitation of Kinshasa, DRC

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Average high (ºC)	31	31	32	32	31	29	27	29	31	31	31	30
Average low (ºC)	21	22	22	22	22	19	18	18	20	21	22	21
Average rainfall (mm)	135	145	196	196	156	8	3	3	30	119	222	142
Average rainy days	11	11	12	16	12	1	0	1	5	11	16	15

Source: Wikipedia (2011a)

Sample collection – A total of 20 raw peanut samples (500 g each) were randomly collected from Kinshasa during the rainy season between November and December in 2010 which is considered to be a late sowing period. The samples were

purchased from street hawkers, local markets and retail shops in Matadimayo, Kinseso, Kimwenza, Ngaliema, Ndjili Brasserie and were estimated to be at least 1 month old. Figure 3.1 shows a typical informal market in Kinshasa. The samples were collected in nylon bags, shipped to the MRC Promec Unit Laboratory (South Africa) and stored at 4 °C until analysis.

Figure 3.2: Sampling point situated in Ngaliema, Kinshasa, DRC (photo by I. Kamika)

3.1.2. Pretoria

Locality – Pretoria is the legislative capital city of South Africa which is located at approximately 50 km north of Johannesburg in the northern part of the Gauteng Province (latitude: 25º44'46'S, longitude: 28º11'17'E). Divided into many suburbs

and townships, this city lies at an altitude of about 1 350 m above sea level, in a warm, sheltered, fertile valley, surrounded by the hills of the Magaliesberg range (Wikipedia, 2011b).

Climate – Pretoria has a moderately dry subtropical climate, specifically a humid subtropical climate with long hot and rainy summers and short cool and dry winters. Table 3.2 provides the average temperature as well as precipitation data for Pretoria.

Table 3.2: Average temperature and precipitation in Pretoria, South Africa

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Average high (ºC)	29	28	27	24	22	19	20	22	26	27	27	28
Average low (ºC)	18	17	12	8	5	5	8	12	14	16	14	17
Average rainfall (mm)	136	75	82	51	13	7	3	6	22	71	98	110
Average rainy days	14	11	10	7	3	1	1	2	3	9	12	15

Source: Wikipedia (2011b)

Sample collection – A total of 20 raw peanut samples (500 g each) were collected randomly from Pretoria city during the summer months between January and March in 2011. The samples were purchased from street hawkers, local markets and retail shops in Marabastad, Bosman Street and Sunnyside and were estimated to be at least 1 month old according to the retailer's comments (Figure 3.3). The samples were placed in nylon bags, shipped to the MRC, Promec Unit Laboratory (South Africa) and stored at 4 °C until analysis.

Figure 3.3: Sampling point situated at Marabastad, Pretoria, South Africa (photo by I. Kamika)

3.2. MYCOLOGICAL ANALYSIS

A total of 40 peanut samples were analysed for mycological contamination (isolation and identification of *Aspergillus* species). The isolation of *Aspergillus* section flavi was carried out using *Aspergillus flavus* and *parasiticus* agar (AFPA). The growth media were prepared by mixing 20 yeast extract, 10 g peptone, 0.5 g ferric ammonium, 0.002 g dichloran, 0.1 g chloramphenicol, and 1 mℓ ethanol (2%) and diluted in 1 000 mℓ of distilled water. Chloramphenicol and dichloran were added in the media to inhibit bacterial growth. The pH of the media was adjusted to pH 4.5 using 1.0 N HCl and 1.0 N NaOH and autoclaved for 15 min at 121°C, at a pressure of 15 psi and cooled down in a water-bath at approximately 50 °C.

The growth of fungal species was determined using the spread-plate method after dilution (APHA, 2001). Briefly, under aseptic conditions, peanut samples (500 g) were finely ground to obtain a homogeneous mixture using the commercial blender (VICAM) and 1 g from each sample was mixed with sterile distilled water (9 mℓ)

followed by a serial dilution. One millilitre from each dilution was transferred to the Petri-dishes and a cooled medium was spread on top and mixed gently for 1 min to 2 min. The mixture was allowed to solidify and then incubated at 30 °C for 3 d. The fungi were isolated from enumeration plates showing well-separated colonies and identified using macroscopic characteristics including colony colours, colony texture, reverse colour and soluble pigment (Pitt and Hocking, 1997; Elbashiti et al., 2010). During this experimental study, each samples were analysis five times for mycological biomass. Thereafter, the number of fungal colonies per gram of sample was calculated and expressed in colony-forming units per gram (CFU/g) which represent the biomass (Equation (3.1)):

$$CFU/g = \frac{\text{Number of colonies x reciprocal of the dilution factor}}{\text{Plate out volume (1ml)}} \qquad (3.1)$$

3.3. AFLATOXIN ANALYSIS IN PEANUT SAMPLES

Two methods were used for the analysis namely the fluorometric and HPLC methods. The experimental study was performed in triplicate for each sample.

3.3.1. Aflatoxin analysis using fluorometric method (VICAM)

Fluorometric analysis was performed at the laboratory of the Department of Agriculture, Ermelo, Mpumalanga, South Africa.

3.3.1.1. Chemicals and reagents

All solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). VICAM kits included the column AflaTest®, developers, microfibre filters

(1.5um, 11cm), VICAM fluted filter paper (24 cm), Tween-20 solution, culture tubes, disposable cuvettes, 15 x 85 mm test tubes, 12 x 75 mm cuvettes with cups, and plastic beakers. Non-iodised sodium chloride, methanol HPLC grade, phosphate buffer solution (PBS), and acetonitrile HPLC grade, were obtained from Sigma, Johannesburg, RSA. The VICAM kits where obtained from VICAM (Watertown, MA, USA).

3.3.1.2. Apparatus

A VICAM V1 Series 4 fluorometer purchased from VICAM (MO, USA) was used in this study, as well as a four-position pump stand, a blender with stainless-steel container, a calibrator bottle, a digital scale with AC adapter, and graduated cylinders (VICAM).

3.3.1.3. Validation of VICAM analytical method

To determine the precision and recovery of the fluorometric analysis, peanut samples were purchased from a supermarket and spiked with AFB1, AFB2, AFG1 and AFG2 (Sigma-Aldrich, South Africa) as total aflatoxin at levels of 5 μg/Kg, 15 μg/Kg and 20 μg/Kg (see section 3.3.2.3) into 25 g raw peanut samples suspected to contain less than 2 μg/Kg of aflatoxin. The spiking was done in triplicate and extraction of the spiked peanuts was done as described below. The precision, linearity, accuracy and recovery tests were performed. The recovery of AFB1 from spiked peanut samples was determined as follows (Equation (3.2)):

$$Recovery\,(\%) = \frac{X}{Y}\,100 \qquad\qquad (3.2)$$

where:

      X is the recovered concentration of aflatoxin

      Y is the spiked concentration of aflatoxin

3.3.1.4. Sample preparation and aflatoxin determination using fluorometry

A total of 40 peanut samples (500 g each) were ground finely using the commercial blender (VICAM) until fine particles and homogeneity were obtained. A ground peanut sample aliquot of 25 g was weighed out together with 5 g of sodium chloride and placed in a blender jar. Methanol: water (125 mℓ) in the ratio 70:30 was added to the jar and the sample was blended at a high speed for 2 min. The blended sample was poured into a fluted filter paper, the filtrate was collected in a clean plastic beaker and 15 mℓ of the filtered extract was diluted with 30 mℓ distilled water. Two end caps from every affinity column to be used were removed, the tip of every column top cap was cut off to use as coupling columns and attached to the outlets of 15 mℓ glass syringe barrels on the pump stand.

The diluted extract was filtered through a microfibre filter in a plastic funnel placed in the top outlet of the glass-syringe barrel attached to a specific affinity column and 15 mℓ of the filtered extract was collected in the syringe. The extract was passed through the affinity column at the rate of about 1 to 2 drops/s with the aid of a pressure pump or manual pressure from a bigger syringe. Distilled water (10 mℓ) was passed through the AflaTest® affinity column at the rate of about 2 drops/s. This was repeated until air came through the column. The affinity column was eluted by passing 2 mℓ HPLC grade methanol through the column at the rate of 1 to 2 drops/s and collecting the entire sample elute in a glass cuvette. AflaTest® developer was

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added to the eluant, thereafter it was mixed well and the aflatoxin concentration was determined in the sample after 60 s by using a fluorometer (VICAM) previously calibrated.

3.3.2. Aflatoxin analysis using the HPLC system

Analysis using HPLC was performed at MRC, PROMEC Unit.

3.3.2.1. Chemicals and reagents

All solvents used to quantify aflatoxins in the samples were of HPLC grade unless otherwise specified and purchased from Sigma-Aldrich (Cape Town, South Africa). The solvents included methanol, acetonitrile, toluene and acetic acid. Non-iodised sodium chloride and phosphate buffer solution (PBS) were also purchased from Sigma (Cape Town, South Africa). Solvents and water were degassed for 20 min in an ultrasonic bath (Model EIA CP104, Italy). Individual aflatoxin (B1, B2, G1 and G2) standards were purchased from Sigma-Aldrich (Cape Town, South Africa).

3.3.2.2. Apparatus

An Agilent 1100 HPLC system consisting of a quaternary pump, auto-sampler from Agilent technologies (CA, USA), fluorescence detector from Agilent technologies (FLD, Darmstadt, Germany) was used for all separation. A photochemical reactor for enhanced detection (PHRED) (Aura, NY) was used to enhance the detection. The chromatographic column consisted of a Phenomenex Synergi 4 μm POLAR-RP 80A (150 X 4.6 mm id) column packed with C18 material (Phenomenex, Torrance, NY) at 25 °C).

3.3.2.3. Standard preparation

Aflatoxin standard solutions were prepared for the purpose of quantitative analysis by HPLC. Briefly, individual aflatoxin (AFB1, AFB2, AFG1, AFG2) (Sigma-Aldrich) samples at appropriate amounts were weighed out to prepare a stock solution of 14.3 mg/Kg, 5.7 mg/Kg, 10.9 mg/Kg and 15.4 mg/Kg for AFB1, AFB2, AFG1 and AFG2, respectively, in toluene-acetonitrile (9/1). The absorbance (A) read at 350 nm as well as the molar absorbance coefficient (ε) and the molecular weight (MW) for each aflatoxin were used to determine the concentration of each standard (see Equation (3.3)):

$$Aflatoxin - {}^{\mu g}/_{ml} = A \times MW \times 1000/\varepsilon \qquad (3.3)$$

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

While the molar absorbance (ε) was calculated with the following equation:

$$\varepsilon = \frac{Abs \times 100}{mM}$$

Where Abs is the absorbance , and mM the millimolar concentration.

To prepare the intermediate mixed standard solution (188 μg/Kg for AFB1, 68 μg/Kg for AFB2, 480 μg/Kg for AFG1 and 218 μg/Kg for AFG2); an appropriate amount of individual aflatoxin was transferred into a vial, evaporated to dryness at 60^{0}C and reconstituted in 2 mℓ of methanol for HPLC.

3.3.2.4. Validation of analytical method using the HPLC system

The aflatoxin analysis was carried out according to the method reported by Gnonlonfin et al. (2010) with slight modification. Briefly, to validate the method, 10 g

of ground peanut samples suspected to contain less than 2 µg/Kg of aflatoxin were spiked with a mixed aflatoxin standard solution at different concentrations (5 µg/Kg, 10 µg/Kg, 20 µg/Kg) and vortexed immediately for 20 min. The spiked peanut samples were allowed to stand and 1 g of sodium chloride along with 25 mℓ of 80 % methanol were mixed with each spiked peanut sample and shaken at 250 rpm for 30 min using the orbital shaker. After shaking, the spiked peanut samples were centrifuged at 4 000 rpm for 5 min at 5 °C and filtered using filter paper (Whatman No.1).

The filtrate (10 mℓ) was diluted with 40 mℓ of distilled water and 10 mℓ of the diluted filtrate was then passed through the AflaTest® immunoaffinity column at a flow rate of 1 to 2 drops/s. The column was washed with 15 mℓ of distilled water and then the aflatoxin was eluted with 3 mℓ of methanol. The eluent was dried under nitrogen gas at 60°C, reconstituted using 200 µℓ of methanol and stored at 4 °C until use. Aflatoxins (B1, B2, G1, G2) were analysed in HPLC system (Agilent Model 1100 HPLC system) equipped with a quaternary pump set at a flow rate 1.5 mℓ/min and connected to a fluorescence detector (FLD) set at 365 nm as an excitation wavelength and 435 nm as emission wavelength. A post-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) and the recovery of aflatoxins from spiked peanut samples was determined as previously described.

3.3.2.5. Determination of aflatoxin from peanut samples using HPLC

The method by Gnonlonfin et al. (2010) was used for aflatoxin analysis with slight modification. A total of 40 raw peanut samples (500 g each) were collected and ground finely using a commercial blender (VICAM, MO). An aliquot of 10 g of each sample was weighed out and mixed with 1 g of sodium chloride as well as 25 mℓ of methanol-water (80/20, v/v) used as an extraction solution.

The mixture was shaken at 250 rpm for 30 min using an orbital shaker (Stuart SSL1, Stone, Staffordshire, UK); thereafter centrifuged at 4 000 rpm for 5 min at 5 °C and filtered using filter paper (Whatman No.1). After filtration, the filtrate (10 mℓ) was diluted with 40 mℓ of distilled water and 10 mℓ of the diluted filtrate was then passed through the AflaTest® immunoaffinity column (VICAM, MO, USA) at a flow rate of 1 to 2 drops/s. The column was washed with 15 mℓ of distilled water and then Aflatoxins were eluted with 3 mℓ of methanol into an amber vial (4 mℓ). The eluate was dried under nitrogen gas at 60 °C, reconstituted using 200 µℓ of methanol and stored at 4 °C until use.

The purified extracts were analysed for aflatoxins (B1, B2, G1, G2) by a reversed-phase isocratic HPLC system (Agilent Model 1100 HPLC system) with a mobile phase 0.1 M KH_2PO_4:acetonitrile:methanol:acetic acid (690/150/75/20, v/v/v/v). The system was equipped with a quaternary pump set at a flow rate of 1.5 mℓ/min and connected to a fluorescence detector (FLD) set at 365 nm as an excitation wavelength and 435 nm as emission wavelength. The purified extracts (20 µℓ) were injected via a thermostatted Agilent 1100 Series auto-sampler. For enhanced detection, post-column derivatization was performed using an online photochemical

reactor for enhanced detection (PHRED) attachment. Data were collected and processed using HP ChemStation (Darmstadt, Germany) for LC software.

3.4. STATISTICAL ANALYSIS

The data were statistically analysed using Stata: Data Analysis and Statistical Software. An Independent-Samples T-Test was used to compare the means of the fluorometry and HPLC results. The tests for relationships were carried out using the Pearson Correlation Index and the interpretation was performed at two-sided 95% confidence limit. Regression analysis was also used to determine the linearity of the results for validation.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1. MYCOLOGICAL ANALYSIS

The results revealed that 95 % and 100 % of the samples collected from Kinshasa and Pretoria, respectively, were contaminated with either *A. flavus* or *A. parasiticus*. In general, the total colony counts ranged from 0 CFU/g to 49 000 CFU/g and 40 CFU/g to 21 000 CFU/g for peanut samples collected from Kinshasa and Pretoria, respectively (Figure 4.1). There was a significant difference between Kinshasa and Pretoria samples ($p < 0.001$).

Table 4.1: Biomass level of aflatoxigenic fungi in raw peanut samples collected from both Kinshasa and Pretoria (n=5)

Kinshasa		Pretoria	
ID	Biomass (CFU/g)	ID	Biomass (CFU/g)
K1	13 000±707.05	P1	390±70.71
K2	2 300±71.20	P2	240±65.25
K3	7 000±1354.05	P3	40±14.14
K4	140±21.00	P4	21 000±1414.21
K5	230±28.00	P5	410±7.07
K6	49 000±1344.00	P6	540±14.14
K7	2 400±141.00	P7	110±14.14
K8	40±3.5	P8	450±28.28
K9	47 000±6070.50	P9	160±42.43
K10	140±49.02	P10	400±56.57
K11	270±64.00	P11	160±28.28
K12	1 100±92.50	P12	190±28.28
K13	17 000±566.00	P13	190±14.14
K14	10 000±212.00	P14	350±25.21
K15	30±10.25	P15	150±21.20
K16	20±23.21	P16	460±49.50
K17	1 900±141.00	P17	340±56.57
K18	0±1.00	P18	700±21.21
K19	300±35.20	P19	40±3.54
K20	9 000±1450.20	P20	270±42.43

Figure 4.1: An example of Pretoria ground peanut samples plated on AFPA and showing a growth of aflatoxin-producing fungi

There was a high incidence of *Aspergillus* in the samples collected from Kinshasa; this may be due to both climatic and environmental factors since peanut samples were collected during a hot period of the year with heavy rain. This result corroborates those previously reported by several authors (Sanders et al., 1985; Sanders et al., 1993; Cotty and Jaime-Gracia, 2007). The presence of aflatoxigenic fungi (e.g. *A. flavus* and *A. parasiticus*) in peanut samples has been previously reported elsewhere and the results presented in this study are therefore comparable with those reported earlier (Umeh et al., 2000; Barro et al., 2002; Mphande et al., 2004; Bankole et al., 2005). The aflatoxigenic fungi were differentiated from other fungi by macroscopic characteristics such as colony colour, colony texture, reverse colour and soluble pigment (Figure 4.1).

4.2. AFLATOXIN ANALYSIS USING THE FLUOROMETRIC METHOD (VICAM)

4.2.1. Validation of the fluorometric method

4.2.1.1. Precision and recovery

In general, the method shows high sensitivity for the extraction of aflatoxins (B1, B2, G1 and G2) in raw peanut samples with recovery values obtained from the spiked samples in the range of 70.58 % to 101.60 % for the various aflatoxin concentrations combined. The RSD for precision was less than 15 % for all the samples spiked with aflatoxins at different concentrations.

Table 4.2: Recoveries and precision of total aflatoxin spiked at different concentrations in clean peanut samples

Aflatoxin	Spiked level (µg/Kg)	Mean amount recovered (µg/Kg)	SD	Recovery (%)	RSD (%)
Aflatoxin (B1, B2, G1, G2)	5	5.08	0.34	101.60	6.70
	10	7.93	1.92	79.30	4.23
	20	14.12	1.00	70.58	7.10

Similar results have been reported by Stroka et al. (2000) when validating a method combining an immuno-affinity column clean-up and thin-layer chromatography (TLC) for the determination of aflatoxins in various food matrices. These authors reported that peanut butter samples are some of the most difficult matrices for aflatoxin analysis, thus obtaining high recovery (as indicated in Table 4.2) is a good indication that the method is likely to give satisfactory performance.

4.2.1.2. Linearity validation

Linearity was determined by extracting and injecting samples fortified with aflatoxins in triplicate as described by Gnonlonfin et al. (2010). Calibration curves between the spiked concentrations vs. the response (concentration recovered) and correlation coefficient (R^2), y-intercept and slope of the regression line were used to evaluate the linearity of the relationship as reported by Rahmani et al. (2010). Figure 4.2 shows the results of linearity validation. The results showed good linearity (R^2 = 0.9996) in the range 5 µg/Kg to 15 µg/Kg.
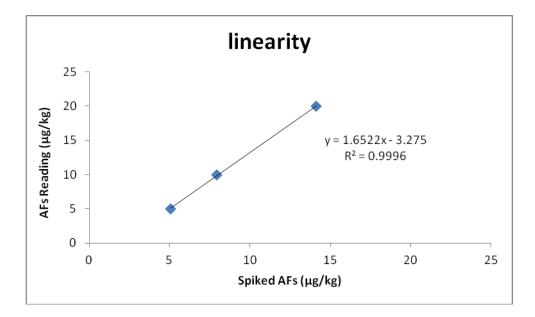
Figure 4.2: Standard curve highlighting the regression linear of fluorometric analysis

4.2.1. Determination of aflatoxin in the raw peanut samples

Table 4.3 summarises the total aflatoxin levels found in peanut samples using the fluorometric method. In general, peanut samples collected from both Kinshasa and Pretoria were contaminated with total aflatoxin ranging from ND to 825.67 µg/Kg and ND to 45.09 µg/Kg, respectively. Ninety-five per cent (19/20) of Kinshasa samples were contaminated with aflatoxins, and 60 % exceeding both the maximum limit of

15 µg/Kg and 10 µg/Kg for total aflatoxin as set by the Codex Committee on Food Additives and the WHO maximum limit (Otsuki et al., 2001). On the other hand, 80 % of samples collected from Pretoria were positive with 30 % and 15 % of the samples exceeding the maximum limit of 10 µg/Kg and 15 µg/Kg set by WHO and the Codex Committee on Food Additives, respectively. In the context of EU regulations 90 % and 50 % of Kinshasa and Pretoria samples, respectively, were found to be above the 4 µg/Kg concentration permissible. A statistical analysis carried out using the t-test showed a significant difference ($p < 0.001$) between samples from Kinshasa and Pretoria.

Table 4.3: Average aflatoxin levels in peanut samples collected from Kinshasa and Pretoria

Samples from Kinshasa		Samples from Pretoria	
ID	Aflatoxin (µg/Kg)	**ID**	Aflatoxin (µg/Kg)
K1	36.80	**P1**	2.73
K2	8.20	**P2**	3.24
K3	550.00	**P3**	7.90
K4	6.75	**P4**	7.13
K5	6.50	**P5**	13.27
K6	532.90	**P6**	9.37
K7	19.63	**P7**	7.43
K8	ND*	**P8**	2.67
K9	520.53	**P9**	12.37
K10	23.92	**P10**	ND
K11	4.83	**P11**	3.13
K12	825.67	**P12**	4.59
K13	32.24	**P13**	ND
K14	127.93	**P14**	2.18
K15	4.02	**P15**	ND
K16	7.97	**P16**	21.83
K17	209.91	**P17**	45.09
K18	32.56	**P18**	11.52
K19	41.22	**P19**	ND
K20	5.12	**P20**	41.80

*ND: Not detected

In the DRC, similar results have been reported by Kamika and Takoy (2011), who reported that 70 % of peanut samples (out of 60) collected in Kinshasa exceeded the recommended limit of 5 μg/Kg for AFB1. The researchers revealed that contamination may arise as a result of climatic conditions (high humidity, high temperature, etc.), poor transportation and storage practices and poor phytosanitary regulation. For samples collected from Pretoria, the results reported in the present study are similar to those reported by Lotter and Krohm (1988). These authors, when investigating the level of aflatoxins in several food crops collected from Johannesburg, reported very low levels of contamination. Compared to the findings in Kinshasa samples, the presence of aflatoxin in low concentrations in Pretoria samples might be due to the presence of regulation, as well as environmental factors which are not as favourable for fungal contamination and aflatoxin production as in Kinshasa.

4.3.   AFLATOXIN   ANALYSIS   BY   USING   HIGH-PRESSURE   LIQUID CHROMATOGRAPHY (HPLC)

4.3.1. Validation of analytical method using HPLC

4.3.1.1. Precision and recovery validation

Table 4.4 illustrates the recovery and precision of the analytical method using HPLC. The different recoveries of the B and G series of aflatoxins are shown in Table 4.4. Aflatoxin B1 was the aflatoxin analogue with high recoveries for the various concentrations followed by AFB2. The recoveries of AFB1 and AFB2 were found to be generally higher than those of AFG1 and AFG2 and were in line with those previously reported by Gnonlonfin et al. (2010) and Rahmani et al. (2010). These
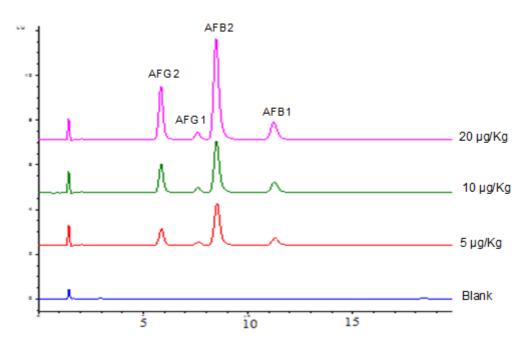
authors reported that to be validated, a method is required to have recovery in the range 70 % to 110 % or >70 %. Though low, the recoveries for AFG1 and AFG2 were still acceptable. This result also reveals high sensitivity of the analytical method due to the absence apparent of interferences in the chromatograms (Figure 4.3).
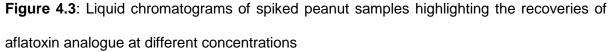
Table 4.4: Precision and recoveries of each aflatoxin analogue at different spiking levels

Aflatoxin analogue	Spiking level (µg/Kg)	Average recovery (µg/Kg)	SD	Recovery (%)	RSD (%)
AFB1	5	4.93	0.54	98.53	10.88
	10	8.73	0.59	87.33	6.73
	20	17.04	1.68	85.18	9.84
AFB2	5	4.02	0.25	80.47	6.22
	10	6.17	0.93	61.67	15.10
	20	13.97	3.37	69.87	24.14
AFG1	5	3.15	0.32	63.00	10.22
	10	5.75	0.89	57.47	15.52
	20	12.83	1.40	64.17	10.94
AFG2	5	2.92	0.50	58.47	17.19
	10	5.92	0.64	59.23	10.87
	20	13.75	1.90	68.77	13.85

The run time used was shorter than 15 min when compared to the method reported by Gnonlonfin et al. (2010). This might be due to the modification made in the current method in terms of mobile phase (0.1 M KH_2PO_4/ACN/MeOH/AA, 690/150/75/20, v/v/v/v), column temperature (35 °C), pump and flow rate used (quaternary pump set at 1.5 mℓ/min). For the precision test, these results revealed a relative standard deviation (RSD) in the range of 6.22 % to 24.14 % with AFB2 showing the lowest RSD in raw peanut samples spiked with 5 µg/Kg followed by AFB1 at 10 µg/Kg. This result meets the minimum requirement (RSD≤20 %) as reported by Rahmani et al. (2010). However, when compared to the requirement of ≤ 15 % as reported by

Gnonlonfin et al. (2010), the RSDs of AFB2, AFG1 and AFG2 at 20 µg/Kg, 10 µg/Kg and 5 µg/Kg, respectively, were higher than the recommended limit.

Figure 4.3: Liquid chromatograms of spiked peanut samples highlighting the recoveries of aflatoxin analogue at different concentrations

4.3.1.2. Linearity validation

Figure 4.4 gives a summary of the results of the linearity validation with regression coefficient value ranging from 0.984 to 0.9995 demonstrating good linearity in the range from 5 µg/Kg to 20 µg/Kg. This result is in agreement with the results reported by Braga et al. (2005), who, when validating a new method for the quantification of aflatoxins in *Maytenus ilicifolia* by HPLC-FLD, reported a very good linearity for all aflatoxins from spiked samples.
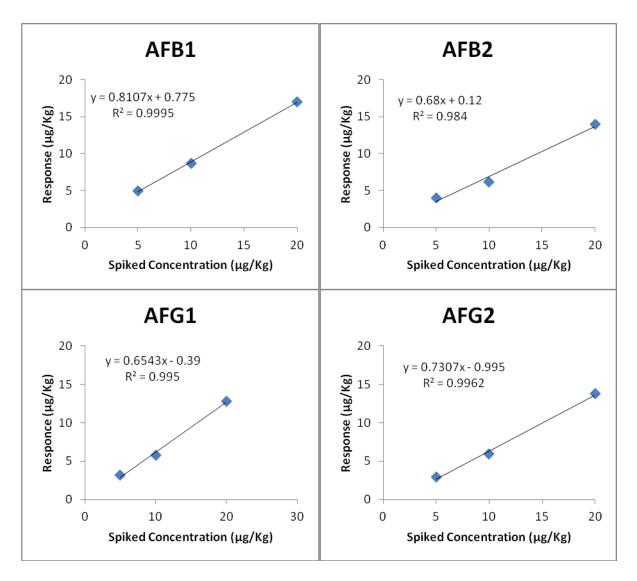
Figure 4.4: Standard curve highlighting the linear regression for the HPLC method

4.3.1.3. Specificity for HPLC method

The specificity is defined as the ability of the method to measure the analyte in the presence of its potential impurities (Rahmani et al., 2010). Figure 4.5 illustrates the specificity of the HPLC method. The results revealed a good chromatography with acceptable baseline and resolution of each aflatoxin highlighting the absence of interference with good separation of aflatoxin analogues. The results also showed a consistency for the retention time of each aflatoxin which was 6.176, 8.081, 9.082/5 and 12.103 for AFG2, AFG1, AFB2 and AFB1, respectively. However, the specificity

of the method was considered satisfactory and agreed with the findings reported by both Rahmani et al. (2010) and Gnonlonfin et al. (2010).
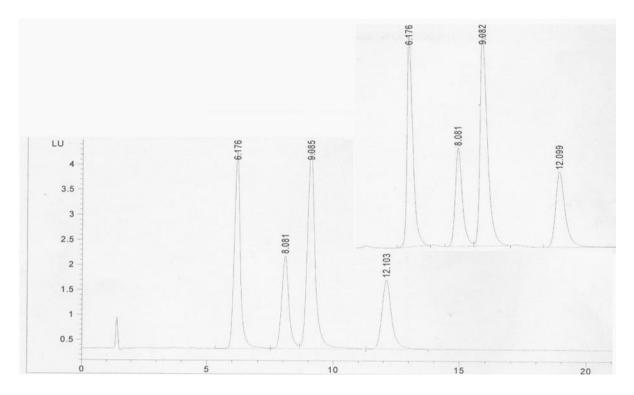
Figure 4.5: Liquid chromatograms highlighting the specificity of the HPLC method

4.3.2. Determination of aflatoxins in peanut samples using the HPLC system

After validation, the HPLC method was used to quantify aflatoxins in raw peanut samples. The results showed that all samples were contaminated with total aflatoxins with an exception of the Pretoria sample (P10) where no aflatoxin was detected (Table 4.5). In terms of individual aflatoxins (B1, B2, G1, G2) all samples except P10 were contaminated with AFB1 at levels ranging from 2.1 µg/Kg to 543.93 µg/Kg (98 %, 39/40), AFB2 ranging from 2 µg/Kg to 210.92 µg/Kg (80 %, 32/40), AFG1 ranging from 2.05 µg/Kg to 310.29 µg/Kg (58 %, 24/40) and AFG2 ranging from 2 µg/Kg to 192.93 µg/Kg (58 %, 24/40). Peanut samples collected from Kinshasa appeared to be more contaminated than those collected from Pretoria with AFB1 levels ranging from 2.19 µg/Kg to 543.93 µg/Kg while the highest for Pretoria

was 35.39 µg/Kg. Generally, AFB1 in all peanut samples was the most predominant of all aflatoxins. Aflatoxin G was not found in several of the peanut samples, especially those collected from Pretoria. This is in agreement with the findings of Alam et al. (2010) who reported that amongst all fractions, AFB1 is normally the most predominant in food and feed products. In addition, the absence of AFG in the same samples could be explained by the fact that several *A. flavus* isolates do not produce AFG (Alam et al., 2010). The present results clearly indicate that AFB1 and total aflatoxin levels exceeded the European Union and the World Health Organization (WHO) permissible limits of 2 µg/Kg and 4 µg/Kg and 5 µg/Kg and 10 µg/Kg, respectively (Codex Alimentarius Commission, 2001; Moss, 2002, Papp et al., 2002). Peanut samples collected from Kinshasa showed the most contamination than those from Pretoria ($p < 0.001$) revealing a 100 % (20/20) and 95 % (19/20) contamination with AFB1 and total aflatoxin more than the EU maximum limit. When compared to the WHO maximum limit, 70 % of Kinshasa's samples were contaminated with aflatoxins above the maximum limit, with 80 % being for AFB1. Kinshasa samples with the highest contamination (K12: 543.93 µg/Kg for AFB1 and 1 258.07 µg/Kg for total aflatoxins, see Table 4.5) appeared to be 110 times more contaminated than the WHO maximum limit for AFB1 and total aflatoxins, respectively.

Table 4.5: Aflatoxin levels (B1, B2, G1 and G2) in raw peanut samples collected from Kinshasa and Pretoria

Sample from Kinshasa						Sample from Pretoria					
Aflatoxin concentration (µg/Kg)											
ID	AFB1	AFB2	AFG1	AFG2	AFS	ID	AFB1	AFB2	AFG1	AFG2	AFS
k1	20	8.94	5.97	6.74	41.65	P1	3.29	2	ND	ND	5.29
k2	5.34	3.82	ND	ND	9.16	P2	2.91	2	ND	ND	4.91
k3	439.02	95.94	102.84	201.93	839.73	P3	4.28	2.01	2.27	ND	8.56
k4	4.95	ND	ND	3.19	8.14	P4	3.84	2.1	2.32	ND	8.26
k5	5.21	2.12	ND	ND	7.33	P5	6.71	2.31	2.18	2	13.2
k6	319	115.27	92.19	172.01	698.47	P6	6.29	4.93	ND	ND	11.22
k7	11.29	5.91	3.19	2.38	22.77	P7	2.19	ND	ND	ND	2.19
k8	2.19	ND	ND	ND	2.19	P8	4.27	2.93	ND	2.31	9.51
k9	281.92	78.93	102.93	110.2	573.98	P9	7.95	2.29	3.92	2.01	16.17
k10	14.83	4.29	9.21	6.21	34.54	P10	ND	ND	ND	ND	ND
k11	3.21	2.01	2.45	3.1	10.77	P11	2.38	2.1	ND	ND	4.48
k12	543.93	210.92	310.29	192.93	1258.07	P12	2.73	2.3	ND	ND	5.03
k13	18.29	7.38	11.92	5.06	42.65	P13	2.1	ND	ND	ND	2.1
k14	82.1	27.11	19.29	30.21	158.71	P14	3.29	ND	ND	ND	3.29
k15	3.2	2.17	ND	ND	5.37	P15	2.39	ND	ND	ND	2.39
k16	5.93	3.19	2.17	3.74	15.03	P16	14.49	3.93	5.94	7.92	32.28
k17	123.95	56.49	47.74	38.84	267.02	P17	35.39	16.3	9.48	12.31	73.48
k18	23.83	9.7	5.92	7.45	46.9	P18	7.59	3.94	2.05	2.01	15.59
k19	34.81	14.83	8.82	13.92	72.38	P19	ND	ND	ND	ND	ND
k20	4.73	2.12	2.32	ND	9.17	P20	26.01	12.93	9.77	5.32	54.03

Figure 4.6 illustrates the relationship between aflatoxin level and microbial biomass in peanut samples. In general, the results revealed that peanut samples collected from both Kinshasa and Pretoria contained both toxin-producing fungi and aflatoxin. Kinshasa's samples showed a direct relationship between fungal and aflatoxin concentration with an exception of sample K12 that appeared to have a very high aflatoxin level vs. low fungal concentration. On the other hand, Pretoria's samples showed a very weak relationship between both fungal and aflatoxin concentrations. When compared to Kinshasa samples, peanut samples collected from Pretoria showed low concentrations of both fungal spoilage and aflatoxin contamination. Statistical evidence showed no correlation (r = -0.0629, p > 0.05) between microbial biomass and aflatoxin level from the Pretoria samples. Peanut samples collected from Kinshasa showed a significant correlation (r = 0.4743, p < 0.001) between microbial biomass and aflatoxin level. This is in agreement with the results published by Kuhn and Ghannoum (2003) and by Blair (2008) who reported that the presence or absence of toxin-producing fungi is a poor indicator of the presence or absence of mycotoxins.
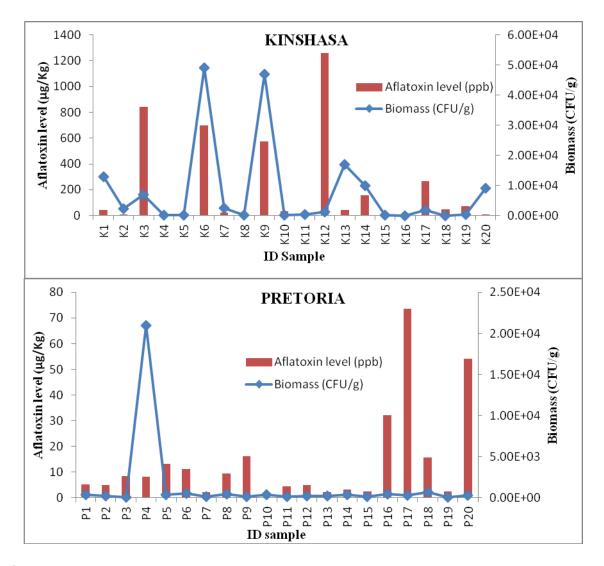
Figure 4.6: Comparison of aflatoxin and biomass level in peanut samples collected from Kinshasa and Pretoria

Colonisation of peanuts by aflatoxin-producing fungi (*Aspergillus flavus* and *A. parasiticus*) has often resulted in aflatoxin accumulation at harvest in Argentina (Passone et al., 2009). However, when comparing the aflatoxin results and the mycological results of this study, a disagreement was found since in peanut samples P10 and P19 no aflatoxin was found; on the other hand, sample K18 showed no growth of aflatoxin-producing fungi but revealed high levels of aflatoxin. The findings of this study revealed that the aflatoxins can occur in food crops, even in the absence of visible mould infestation, either due to a ceased vital cycle of the

microorganism or due to the effect of removal of the mould by the technological processing of the food commodity. Conversely, the presence of a visible mould on the surface of a food product does not provide a clear indication of the presence of a mycotoxin such as aflatoxins (Viljoen, 2003; Achar et al., 2009).

In this study, the efficiency and sensitivity of HPLC and fluorometric methods on aflatoxin determination were investigated and compared (Figure 4.7). In general, the presence of aflatoxin was detected in peanut samples by both methods. Although both analytical methods showed good recoveries and precision, the HPLC method appeared to be more sensitive than the fluorometric method by revealing high levels of total aflatoxins in most of the peanut samples. A large difference in terms of aflatoxin levels was observed in peanut samples with high aflatoxin levels. Although the quantitative fluorometric method developed by VICAM has been reported to be a quick, easy-to-perform and accurate method for the analysis of aflatoxins in several commodities (Pena, 2010), the present study revealed certain inaccuracies when samples contained high or very low levels of aflatoxins.
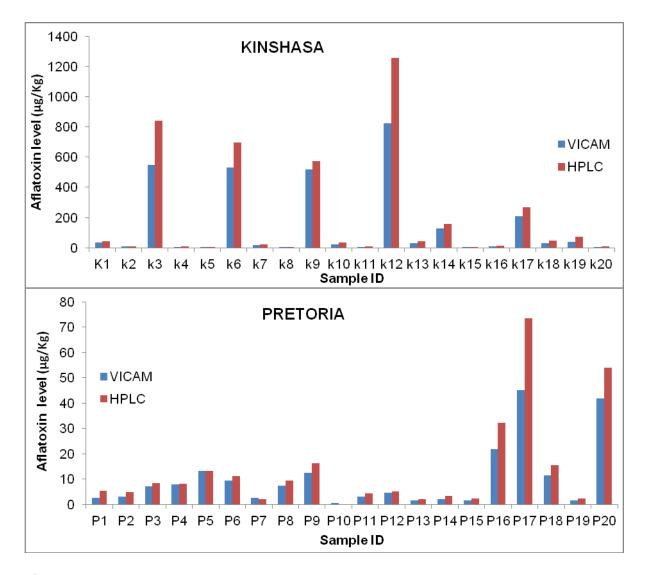
Figure 4.7: Comparison of aflatoxin level obtained using HPLC and VICAM method (fluorometric method) in peanut samples collected from Kinshasa and Pretoria

Though values of HPLC results obtained were found to be higher than VICAM results, the statistical evidence revealed a significant positive correlation ($r = 0.912$, $p < 0.05$). This evidence indicates that fluorometry is a good method to screen aflatoxin contamination in food crops such as peanuts. This result is in agreement with the finding reported by Pena (2010) when comparing the two methods for the analysis of aflatoxin M1 in cheese.

To detect and quantify aflatoxins in food and feedstuff, several methods rely on their fluorescence. But, aflatoxin B1 and aflatoxin G1 have poor natural fluorescence in

aqueous solutions and their enhancement has been reported elsewhere as a better option (Dall'asta et al., 2003). Analytical methods with merits such as high rapidity, high sensitivity and accuracy to determine aflatoxins are needed in order to properly assess both the relevant risk of exposure to humans and animals and to ensure that the regulatory levels set by the countries and other multilateral organisations are met and correct by minimising the false positive/negative (Pascale, 2009). Zheng et al. (2006) reported that the fluorometric analysis (VICAM) coupled with the clean-up step using immuno-affinity column has been reported to be an effective, quantitative method for the detection of mycotoxins and is regarded as a rapid method. But its accuracy is mostly affected by the interferences present in several samples which can lead to a false negative or a false positive (Abbas et al., 2004).

On the another hand, the HPLC method is an automated process with an improved accuracy, high sensitivity, reproducibility and precision but it is very expensive, its operation is complex and it is difficult to detect co-elution (Abbas et al., 2004). The difference between the fluorometry and HPLC methods in this study can be due to the high sensitivity, accuracy, and precision of the HPLC method over the fluorometry method as reported elsewhere (Abbas et al., 2004; Zheng et al., 2006; Pascale, 2009; Shephard, 2009).

CHAPTER FIVE: GENERAL DISCUSSION

For centuries, fungi have been part of human activities and thousands and thousands have been isolated and identified (Ndlovu, 2008). *Aspergillus* genera have been found to be the fungi of primary interest because of their ability to produce mycotoxins and their negative effect on both human and animal health. *A. flavus* and *A. parasiticus* have long been recognised as major contaminants of organic and nonorganic materials and also produce aflatoxins (Hassan and Lloyd, 1995). As common constituents of the microflora in air and soil, the presence of *A. flavus* and *A. parasiticus* in food such as peanut is obvious due to the suitability of the food crop to fungal spoilage and aflatoxin production (Moss, 2002; Kaaya and Kyamuhangire, 2006). According to Kaaya and Warren (2005), the mycotoxigenic fungi and aflatoxin contamination in peanuts starts at the farm level and contamination occurs in both pre- and postharvest phases. In the present study, the incidence of aflatoxin-producing fungi was reported in nearly all the samples analysed. Aflatoxin-producing fungi are ubiquitous and were also found in South African (Pretoria) as well as in DRC (Kinshasa) samples. Dutton and Westlake (1985), when investigating the occurrence of mycotoxins in 800 food/feed samples, revealed that *A. flavus* and *A. parasiticus* were the predominant species. In 1994, Dutton and Kinsey (1995) reported *Aspergillus* spp. at lower prevalence when compared to other fungi such as *Penicillium* spp. and *Fusarium* spp. in maize. Peanut samples from Kinshasa were found to be contaminated with more aflatoxin-producing fungi (*A. flavus* and *A. parasiticus*) than samples from Pretoria. Since peanut samples were collected during the period with the highest rainfall (Figure 3.2 and Table 3.1), this high contamination could be explained by the presence of high humidity, high temperature, crop

handling and storage in Kinshasa (Figure 3.2, Table 3.1) which appeared to be conducive to fungal growth and aflatoxin production.

Aflatoxin contamination is the most important quality problem in peanuts worldwide with serious commercial implications. To minimise the health and economic implications associated with the presence of aflatoxins in food and feedstuff, several guidelines have been developed and legislation has been adopted, both at international and national levels. In South Africa, aflatoxin contamination is regulated by Regulation No. R. 313 of 1990, promulgated under the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972). According to the Act as well as the WHO, all foodstuffs containing more than 10 µg/kg aflatoxin, of which AFB1 should not be more that 5 µg/kg, are deemed contaminated and not fit for human consumption. In addition, the Codex Alimentarius Commission on Food Additives and Contaminants set the limit for total aflatoxin at15 µg/Kg, half of this limit being for AFB1(Henry et al., 1999). A stringent regulation has been reported by the European Commission which set the maximum limit for aflatoxins at 4 µg/Kg and for AFB1 at 2 µg/Kg (Wu, 2004).

The selection of sensitive methods is a prerequisite to accurately meet these regulations. Conventional analytical methods such as thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used to determine aflatoxin contamination in commodities (Zheng et al., 2006). Most of these methods are seen as laborious due to the time consumed during analysis, expertise required, cost, etc. Competition among food- and feed-supply industries compelled manufactures to develop cost-effective methods which

employ cheap labour, are easy to use and rapid in delivering results; among those are enzyme-linked immuno-sorbent assay (ELISA), membrane-based immunoassay, fluorometric assay, etc. (Zheng et al., 2006). Since these methods are classified as fully quantitative, semi-quantitative, and screening methods, their use depends on the reason for the analysis (Pena, 2010). Prior to be used to assess aflatoxin levels in raw peanut samples, the methods were validated in terms of precision, recoveries, linearity and specificity. However, both methods (fluorometry and HPLC) were found to have good linearity, high precision and sensitivity to detect a significant amount of aflatoxins in peanut samples. The HPLC method was also found to be precise and highly accurate. This result is in agreement with the findings of both Gnonlonfin et al. (2010) and Rahmani et al. (2010) who reported that in terms of recovery, a method can be validated if the percentage recovery ranges between 70 % and 100 %. In terms of precision, the methods were validated to meet the minimum requirement as reported by Rahmani et al. (2010).

Peanuts are among the most common nuts included in many diets worldwide and play an important role in the diets of several African populations, especially children, because of their high content of protein (approximately 25 %), fat and carbohydrate. Peanut is, however, also a suitable substrate for fungal spoilage and aflatoxin production (Kamika and Takoy, 2011). Several studies have been carried out in Africa on the nutritional values of peanut and its importance for undernourished children. However, very little has been done in the DRC even though it is reported to have a high prevalence of HBV in comparison to South Africa (Figure 2.4) (Williams et al., 2004). In the analysis of field samples for aflatoxin contamination, using the fluorometric method, the results revealed that approximately 60 % and 30 % of the

samples collected from Kinshasa and Pretoria, respectively, contained aflatoxin levels above the recommended JECFA and Codex Alimentarius Commission limits. Although aflatoxin levels were found to be higher when using HPLC, the results revealed that approximately 65 % and 25 % of samples from Kinshasa and Pretoria contained total aflatoxins above the recommended limits. Considering AFB1 alone, the results revealed that 85 % of Kinshasa's and 35 % of Pretoria's samples exceeded the limit of 5 µg/Kg (JECFA, 1998; Codex, 2001; Papp et al., 2002). Brudzynski et al. (1977) carried out a study on the occurrence of AFB1 in peanuts, maize and dried cassava sold at the local market in Kinshasa, DRC, and found that AFB1 levels ranged from 12 µg/Kg to 1 000 µg/Kg. In a previous study conducted by Masimango and Kalengayi (1982) on Congolese staple foods such as peanut, dried cassava roots, maize meal, sorghum, and sweet potatoes, the results revealed the presence of high levels of aflatoxins, especially AFB1. On the other hand, Kamika and Takoy (2011) reported high AFB1 levels of up to 937 µg/Kg in peanut samples collected from Kinshasa. Peanut samples from tropical regions appeared to be more contaminated. In 1991, 1 044 peanut samples collected from Brazil were analysed for aflatoxins; 940 samples contained aflatoxins with concentrations ranging from 30 µg/kg to 5 000 µg/kg (Ellis et al., 1991). Oliveira et al. (2009) found that 44.2 % of 240 peanut samples analysed were positive for aflatoxin at levels of between 0.5 µg/kg and 103.8 µg/kg and nine of those samples had total aflatoxin levels higher than the Brazilian permissible limit (20 µg/kg). Still, in Brazil, in the state of Sao Paulo, peanuts have been recorded to contain high levels of AFB1 (from 51 µg/kg to 420 µg/kg) (Nakai et al., 2008).

Omer et al. (2001), in a study on peanut-butter intake, demonstrated that peanut-butter consumption has been identified as a strong risk factor of hepatocellular carcinoma (HCC) in a region with endemic aflatoxin contamination in Sudan. In several African countries, the incidence of aflatoxin in crops is correlated to the incidence of liver cancer. In Mozambique and Swaziland the incidence of liver cancer was found to be very high and a significant correlation between the level of aflatoxin consumption and the incidence of primary liver cancer was also reported, especially in adult males (Sibanda et al., 1997). However, since previous study reported that the DRC has high and an increasing incidence of liver cancer (Henry et al., 1999; Ryder et al., 2000), the presence of aflatoxin at high concentrations (100 times the maximum limit) is an alarming situation.

In South Africa, a few isolated research conducted across the country reported the presence of aflatoxin in food for both human and animal consumption. Dutton and Kinsey (1995) further investigated the presence of mycotoxins in cereals and animal feedstuffs in Natal, South Africa and found aflatoxins in only 6 % of 417 samples of agricultural commodities. Recently, the study conducted by Ncube et al. (2010) reported the presence of aflatoxins at levels of up to 131 µg/Kg, 160 µg/Kg and 2 µg/Kg in groundnut kernels collected from subsistence farmers in three South African provinces, namely KwaZulu-Natal, Mpumalanga and Limpopo, respectively.

When comparing the HPLC results and those of fluorometry, aflatoxin levels from the same samples appeared to be higher when using HPLC ($r = 0.912$, $p < 0.05$) (Figure 4.7). This revealed the sensitivity of the HPLC analytical method over the fluorometric methods developed by VICAM and classified the latter as a screening

method rather than a quantitative analytical method for aflatoxin detection. However, these two analytical methods were found to be effective due to their high recoveries and low relative standard deviations (RSD) when validating. The present result on the comparison between fluorometry and HPLC methods corroborates the results reported by Pena (2010).

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

There is evidence that the sub-Saharan African population is highly exposed to food-borne aflatoxins, due to the tropical climate which is present in most of the African countries and provides optimal conditions for toxigenic fungal growth. Aflatoxins have been shown to cause a variety of toxic and adverse health effects in humans and these lead to reduced life expectancy in Africa. However, where quality control is absent, unsafe levels of aflatoxin are present. Since peanut is a suitable substrate for aflatoxin production as well as an important food crop and oilseed in the DRC and South Africa, the present study assessed and compared aflatoxin levels in raw peanut collected from Kinshasa and Pretoria. The results revealed that the methods (fluorometry and HPLC methods) used to assess aflatoxin levels in raw peanut were validated and found to be sensitive for aflatoxin (B1, B2, G1 and G2) detection. However, the HPLC method was more sensitive for aflatoxin detection than the fluorometry method. Peanut samples collected from both Kinshasa and Pretoria were contaminated with aflatoxins as well as AFB1. When using the fluorometry method, aflatoxins were found in the proportion of 65 % and 30 % for Kinshasa and Pretoria, respectively, which is above the WHO limit. Based on the HPLC method, the percentages of aflatoxin B1 recorded in peanut samples collected from Kinshasa (70 %) and Pretoria (35 %) were found to be higher than the percentages recorded using the fluorometry method.

Furthermore, microbial biomass was also investigated in this study. The results revealed that peanut samples were contaminated with aflatoxin-producing fungi in the range of less than 1 CFU/g to 49 000 CFU/g and 40 CFU/g to 21 000 CFU/g in peanut samples collected from Kinshasa and Pretoria, respectively. The study shows that peanut samples from Kinshasa are more susceptible to fungi spoilage and

aflatoxin production than peanut samples collected from Pretoria. This might be due to the environmental factors, the socio-economic situation as well as lack of enforcement of food regulation. The study suggests that the DRC government should learn from South Africa in terms of policy design and implementation of food safety and security measures such as:

- To initiate an awareness programme on mycotoxin contamination among subsistence, emerging-commercial and commercial farmers.

- To design and build good warehousing which meets all safety requirements for storage of foods such as peanut.

- To also establish a good peanut-transfer system from the storage facility to the shelling plants to selling points.

- To develop adequate sanitary facilities as well as an effective pest-control programme.

- To issue and enact a regulation preventing the selling of contaminated foods and feeds.

However, there is a need for further studies to confirm the observation before one can make conclusion has would have serious implications.

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