A surveillance study of mycotoxins in the South African industry with specific reference to aflatoxin B_1 in feed and aflatoxin M_1 in farm gate and selected commercially available dairy milk.

A dissertation submitted to the Faculty of Health Sciences, University of Johannesburg, in fulfilment of the requirements for the degree Master of Technology: Food Technology

by

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DECLARATION

I declare that this dissertation is my own work. It has being submitted for the Degree of Master of Technology: Food Technology at the University of Johannesburg. It has not been submitted before for any degree or examination in any other Techikon or University.

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ABSTRACT

Fungi are beneficial to the food industry and are used both as a food ingredient and food additive in the production of certain foods which are consumed by Man. However, fungi are also known as food spoilage contaminants and their growth within a food substrate may sometimes be supported by the development of carcinogenic toxins which are collectively referred to as mycotoxins. Incorrect agricultural practices, insect infestation and improper post harvest storage conditions are all factors which can support the presence of these toxins which commonly occur in agricultural products, and are able to resist most food technology practices.

Although the climatic conditions in South Africa is supportive of the growth of most agricultural products, this country as well as all countries in general are not self sufficient with regards food and feed supply, thus relying on the global trade of commodities from various countries which in turn means global exposure to mycotoxin contaminated commodities such as cereals, grains and oil seeds.

A commonly occurring mycotoxin is aflatoxin B_1 which is harmful to the health of humans and animals, with the liver being the main target organ for aflatoxin toxicity and carcinogenicity. If feed contaminated with aflatoxin B_1 (AFB_1) is consumed by ruminants such as the dairy cow, this toxin may be converted to aflatoxin M_1 (AFM_1) under the influence of cytochrome P_{450} oxidase system found in the rumen micro flora and the animal's own cells. Both aflatoxin B_1 and aflatoxin M_1 are regarded to be carcinogenic to humans and tend to cause a host of diseases in animals too.

The quality of the meat and milk produced by the animal consuming the contaminated feed is thus compromised, owing to the presence of these carcinogenic toxins. The consumption of aflatoxin contaminated foods whilst breast feeding may also result in the formation of aflatoxins and their metabolites in breast milk. As milk is a primary food source for infants and continues to be an important component of a growing child's diet, it is of paramount importance that this toxin be eliminated or at the very least limited.

The most effective way of controlling levels of aflatoxin M_1 in milk seems to be through regular surveillance studies of commercial milk as well as regular analysis of dairy feed. The

South African permitted level of aflatoxin B_1 in dairy feed is 5 µg/kg (SA Fertilisers, 2009) and the permitted aflatoxin M_1 level is 0.05 µg/L in bovine milk (SA Foodstuffs, 2004).

A multi fold approach was done to address the surveillance issues of dairy feed and milk available in South Africa. Firstly, aflatoxin M_1 contamination levels in commercial milk sold at retail level was determined to check the quality and to see whether seasonal variation had an influence on the concentrations. Secondly, the milk produced by a small South African dairy was analysed, together with the feed fed to the cows on all the selected farms supplying the dairy. The feed and farm gate milk analyses were also conducted during two seasons.

During this study the immuno-affinity method was employed for the extraction of aflatoxin B_1 and aflatoxin M_1. Final confirmation was performed by high performance liquid chromatography which was coupled to a ultra-violet detector and CoBrA cell for derivitisation. Various other methods available for the detection of aflatoxins were investigated too such as solid phase extraction using C18 columns, enzyme immunoassay (ELISA), as well as certain lateral flow devices which are suitable for on- site testing of aflatoxins.

Preliminary results indicate that approximately 78% of the feed ingredients sampled were found to be contaminated with aflatoxin B_1. It was further found that over half the farm gate milk analysed was above the South African legislated levels. The contamination of commercially available milk by aflatoxin M_1 is evident in South Africa. On average aflatoxin M_1 contamination levels among fourteen selected brands were noticeably higher during the winter sampling period and this may be attributed to the increased use of compound feed in winter as opposed to pasture feeding in summer.

The application of Good Agricultural Practices both pre and post –harvest, as well as efficient quality control practices involving the use of Hazard Analysis Critical Control Program is necessary to limit the presence of aflatoxins in these commodities. The employment of fungus based bio-control products during pre-harvest, as well as certain adsorbents during post-harvest are new developments in limiting aflatoxin contamination in agricultural commodities.

A full bio-tracer investigation could follow this study, but practical difficulties in sampling of batches and compounding present challenges.

DEDICATION

To my children Tyan & Kian, any dream is possible through persistence and hard work.

"Any human being anywhere will blossom in a hundred unexpected talents and capacities simply by being given the opportunity to do so"

Doris Lessing

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1. INTRODUCTION

1.1 *Problem statement*

A fungus is a eukaryotic cell and usually a multi-cellular filamentous structure. All fungi are chemo-heterotrophic synthesizing the organic compounds they need for growth and energy, from pre-existing organic sources in their environment and using the energy supplied from chemical reactions. Fungi may be mesophillic, psychrotrophic and thermophillic and thus they are able to grow at almost any temperature. They are also aerobic and able to grow at fairly low moisture levels (Frazier & Westhoff, 1988).

Fungi are very beneficial to the food industry, as they may be grown as a food ingredient such as mushrooms and single cell protein from yeast. These organisms may also be used in food fermentations to produce oriental food ingredients such as soya sauce, miso and sake. Both the production of amylase for bread production as well as the manufacture of citric acid, employ the use of particular fungi. Certain cheeses such as Brie, Roquefort and Camembert are mould ripened to produce a certain desired flavour profile. The common human antibiotic penicillin is derived from a particular strain of fungus (Frazier & Westhoff, 1988).

However food spoilage by mould or fungus is common and is generally recognised by a fuzzy or cottony appearance on foodstuffs. Moulds can utilise a variety of foods from simple fruits to complex foods, and in doing so they reduce the shelf life of the food. Certain fungi may also produce various toxic matabolites which can occur in a variety of foodstuffs and may be harmful to both humans and animals. These toxins termed mycotoxins are naturally occurring toxins produced by filamentous fungi in many agricultural crops in the field, after harvest, during storage and later when processed into food, animal feed and feed concentrates (Smith & Henderson, 1991). The UN Food and Agricultural Organisation (FAO) estimated that approximately 25% of crops are affected annually with mycotoxins worldwide (Jelinek, 1987).

Mycotoxicosis is a poisoning associated with exposures to mycotoxins and the symptoms depend on the type of mycotoxin involved, the concentration and length of exposure as well as the age, health and gender of the exposed individual (Bennett & Klich, 2003).

The big concern regarding these mycotoxins is that their presence in foodstuff is not as easily detected as it is with common food spoilage fungi. These toxins are invisible, odourless and

very stable compounds resisting degradation by most physical and chemical food technology practices. It has been reported that at least 14 mycotoxins are carcinogenic to humans, with the aflatoxins being the most potent (Stark, 1980).

Aflatoxins are a group of hepato-carcinogenic, bi-sidi-hydro-furano secondary metabolites, so their function is not dependent on the fungi's existence. These toxins are produced by certain species of *Aspergillus* especially *A. flavus*, *A. parasiticus* and *A. nomius* and are common crop contaminants. Of these, aflatoxin B_1 (AFB_1) has been found in most feeds and foods and is highly carcinogenic (Eaton & Gallagher, 1994).

When feed contaminated with aflatoxin B_1 is consumed by ruminants such as the dairy cow, this toxin may then be converted to aflatoxin M_1 (AFM_1) under the influence of the cytochrome P_{450} oxidase system which is found in the rumen micro-flora and the animal's own cells (Yoshikawa *et al.,* 1982). The amount of aflatoxin M_1 excreted into milk can be up to 3% of the aflatoxin B_1 intake (Diaz *et al*., 2004) is affected by milk yield (Petterson *et al*., 1989; Veldman *et al*. 1992) as well as the stage of lactation (Munksgaard *et al*., 1987; Petterson *et al*., 1989; Veldman *et al*., 1992).

The results of a survey conducted in Portugal indicated that aflatoxin M_1 was found in 65.8% of commercial milk samples analysed (Martins *et al.,* 2005*)* whilst 54% of milk samples tested in Iran was found to be contaminated with aflatoxin M_1 (Tajkarimi *et al.,* 2007). It has been reported that aflatoxin M_1 contamination has also been found in milk and milk products in Nigeria, Morocco and Pakistan.

Certain data reported in literature has showed that aflatoxins may also be found in the biological fluid of humans including breast milk (El Nezami *et al*., 1995; Galvano *et al*., 1996; Gürbay *et al.,* 2010; Navas *et al*., 2005). The exposure of aflatoxin M_1 to infants and children is of great concern as they are more susceptible to the adverse effects of the toxin as their capacity for bio-transformation of carcinogens is generally slower in comparison to adults.(López *et al*., 2003).

The International Agency for Research on Cancer (IARC) (1993, 2002) have classified aflatoxin B_1 as a Group 1 (human) carcinogen and therefore monitoring the levels of aflatoxin B_1 in food and feed is important. It is documented that aflatoxin M_1 is classified as

a Group 2 carcinogen by IARC, with possible causative effects of cancer in humans. Aflatoxins are both acutely and chronically toxic to man and animals, causing acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Stoloff, 1975). Symptoms of *acute* aflatoxicosis in dairy cattle include lethargy, ataxia and enlarged fatty livers. Symptoms of *chronic* aflatoxin exposure include reduced feed efficiency and milk production, as well as jaundice and decreased appetite in dairy cows (Nibbelink, 1986). Aflatoxins have also been showed to lower an animal's resistance to diseases and interfere with vaccine induced immunity in livestock (Diekman & Green, 1992).

As milk is a primary food source for infants and continues to be an important component of a growing child's diet, it is of paramount importance that this toxin be limited or if possible, eliminated from the diet. The most effective method of controlling the levels of aflatoxin M_1 in milk are through regular surveillance studies of commercial milk as well as frequent analysis of dairy feed. Such monitoring programs still remain the primary strategy to protect the consumer (Galvano *et al.*, 1996).

The Food Standards Agency (UK) conducts surveys regularly to determine the levels of aflatoxin M_1 in commercially available milk sold in the United Kingdom (FSA, 2001). Several other countries such as Argentina, Brazil, India, Iran, Italy, Kuwait, Mexico, Morocco, Pakistan, Portugal, Nigeria, Spain, Serbia and Thailand have conducted studies on dairy feed and farm gate milk or commercially available milk, in order to determine the level of aflatoxin B_1 and aflatoxin M_1 respectively.

Aflatoxins may be present in the food chain from farm to fork so it is possible to trace this if selected feed and milk samples are analysed so as to identify possible sources of contamination. The South African permitted level of AFB_1 in feed is 5µg/kg (SA Fertilisers, 2009) and the permitted AFM_1 level is 0.05µg/kg in bovine milk (SA Foodstuffs, 2004). There does not seem to be any literature available of a surveillance or bio-tracer type study conducted in South Africa where commercially available feed and bovine milk has been sampled and analysed for the presence of aflatoxins.

1.2 *Aims and Objectives*

The overall aim of this project was two-fold, namely to determine the levels of aflatoxin M_1 present in commercially available South African milk. Secondly the aflatoxin B_1 levels of concentration in selected feed, and the aflatoxin M_1 levels of concentration in selected milk samples drawn from a chosen group of dairy farms were researched.

In order to achieve this the following objectives had to be met:

A two-phase sampling process of feed and farm gate milk from at least ten selected farms within the KwaZulu Natal region in South Africa.

To determine if there is aflatoxin B_1 present in the selected feed ingredients and if so, are the levels of contamination within the stipulated limits as prescribed by South African legislation.

To determine if there is aflatoxin M_1 present in the selected farm gate milk samples and if so, are these levels of contamination within the stipulated limits as presribed by South African legislation.

To determine if a bio-tracer analogy could apply to the results obtained for the feed and milk.

To conduct a two season surveillance study of commercially available milk, to determine the presence of aflatoxin M_1 and whether the levels of contamination are within the stipulated South African legislation.

To conduct a comparative analysis of results of selected milk samples using various test methods currently available in the market place to analyse for aflatoxin M_1.

2. LITERATURE REVIEW

2.1 MYCOTOXINS

Fungi are a natural part of the environment and are mostly aerobic. Fungi are able to grow over a temperature range of 10-40°C; a pH range of 4-8; water activity (A_w) level above 0.7 and a moisture content greater 15% (Frazier & Westhoff, 1988). The thallus or vegetative body of a fungus consists of a mass of branching intertwined filaments called hyphae and the whole mass of hyphae is referred to as the mycelium. Few kinds of fungi produce sclerotia which are tightly packed masses of modified hyphae. These sclerotia are more resistant to heat and other adverse conditions than the rest of the mycelium, allowing the fungus to thrive under almost any type of climatic conditions (Frazier & Westhoff, 1988).

Mycotoxins are toxic compounds which may be produced in the mycelia of certain filamentous fungi, and have also been known to accumulate profoundly in specialised structures such as conidia or sclerotia as well as in the environment of the surrounding fungus (Burge, 2001; Fischer & Dott, 2003). The mycotoxins are low molecular weight, non-antigenic fungal secondary metabolites, which are formed at the end of the exponential growth phase of an organism, and make no contribution to the development or metabolism of the producing organism. (Frazier & Westhoff, 1988).

Mycotoxins may be present in food as a result of the organism actually growing in the food matrix or through a more indirect route for example, in dairy milk from animals which have consumed contaminated feed. It is important that both pre-harvest and post harvest factors such as pest attack, soil condition, type of seed planted, transport and storage conditions be carefully controlled. These factors influence not just the presence of fungi, but that of mycotoxins too. Mycotoxins are thus able to develop at any stage from farm to fork as shown in Figure 1.

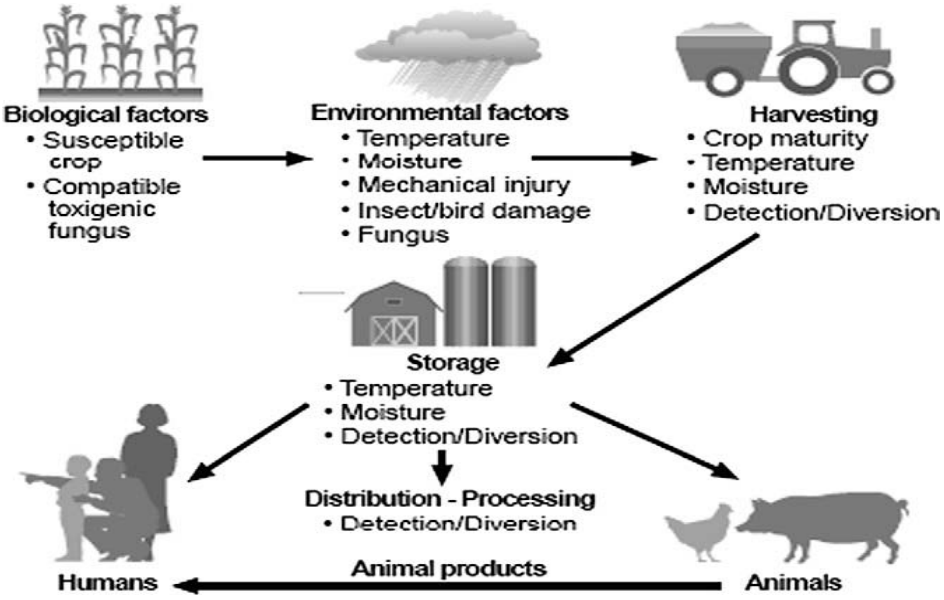


Figure 1: Factors which affect mycotoxin occurrence in the food chain (CAST, 2003).

Atmospheric composition has a great impact on mould growth with humidity being the most important variable (Gibson *et al.,* 1994). Poor storage conditions of feed ingredients such as maize and oil seeds during transport, with no aeration and at relatively high temperatures and humidity, across the seas can create the ideal conditions for mycotoxins to develop. Pre-harvest factors such as insect damage and poor harvesting techniques of cereals such as maize kernels, seem to support the growth of mycotoxins as the fungus is able to circumvent the natural protection of the integument and establish infection sites in the vulnerable interior (Lillehoj *et al*., 1980). Table 1 shows some probable routes for mycotoxin contamination of foods and feeds.

Table 1: Probable routes for mycotoxin contamination of foods and feeds (IFST, 2009).

<table>
<tr><td rowspan="5">Mould damaged foodstuffs</td><td colspan="4">Agricultural products</td></tr>
<tr><td>Major source</td><td>Minor source</td><td colspan="2">Secondary infection</td></tr>
<tr><td>Cereals</td><td>Fruits</td><td colspan="2">Consumer foods</td></tr>
<tr><td>Herbs & spices</td><td>Vegetables</td><td colspan="2">Compounded animal feed</td></tr>
<tr><td>Oilseeds</td><td colspan="3"></td></tr>
<tr><td colspan="2">Residues in animal tissues and animal products</td><td>Milk (animal & human)</td><td>Dairy produce</td><td>Meats (liver & kidney)</td></tr>
<tr><td>Mould fermented foods</td><td>Cheeses</td><td>Fermented meat products</td><td colspan="2">Oriental & other fermented products</td></tr>
<tr><td colspan="2">Fermentation-derived products</td><td>Microbial proteins</td><td colspan="2">Food additives</td></tr>
</table>

If contaminated oilseeds and grains or agricultural by-products of contaminated fruits and vegetables are used in the manufacture of animal feed, then it is highly likely that mycotoxin residues will be found in animal tissues and animal products. Ingestion of these contaminated foods by humans is of great concern, as not only does it compromise both food quality and safety, it also means that these mycotoxins are transferred to humans. The continual exposure to low levels of mycotoxins leads to immune-suppression and possibly cancer which are commonly experienced in developing countries (Bryden, 2007). Fungi can infect dairy cattle especially during periods of immune-suppression, causing a condition called mycosis (Fink-Gremmels, 2008).

2.1.1 Important Mycotoxin Producing Fungi

Whilst over 300 mycotoxins have been identified, about 20 have been shown to appear naturally in foods and feeds at significant levels and frequency to be of a food safety concern (Smith *et al.,* 1994*).* The majority of these commonly occurring toxins are produced by the genera *Aspergillus, Penicillium and Fusarium* as shown in Table 2.

Table 2: Some important toxigenic species of filamentous fungi and associated mycotoxins (IFST, 2009).

Fungal species	**Toxin**
Aspergillus flavus	Aflatoxins B_1, B_2 & cyclopiazonic acid
A. parasiticus	Aflatoxins B_1, B_2, G_1 & G_2
A. ochraceus	Ochratoxin A and Penicillic acid
A. versicolor	Sterigmatocystin & cyclopiazonic acid
Penicillium verrucosum	Ochratoxin A citrinin
P. purpurogenum	Rubratoxins
P. expansum	Patulin, citrinin
Fusarium sporotrichioides	T-2 toxin
F. verticillioides	Fumonisin B_1
F. graminearum	Deoxynivalenol, zearalenone
Alternaria alternata	Tenuazonic acid
Stachybotrys atra	Satratoxins

2.1.1.1 Aspergillus

The morphology of *Aspergillus flavus* and *A. parasiticus* are usually identified by the presence of bright yellow green conidia. *Aspergillus flavus* produces variable shaped and sized conidia with thin walls, which are smooth to moderately rough in texture. However the conidia of *A. parasiticus* are spherical with thick rough walls (Pitt & Hocking, 1997). As mentioned in Table 2, *A. flavus* produces aflatoxin B_1 and B_2 toxins, whilst *A. parasiticus* produces aflatoxin B_1, B_2, G_1 and G_2 toxins. The 'B' and 'G' in the toxin name refer to the colours blue (B) and green (G), which are the colours the respective toxins tend to fluoresce, under UV light on thin liquid chromatography (TLC) plates.

According to Cotty (1989), *A. flavus* may be divided into two morphotypes, the S and L strain. Each morphotype is divided into many clonal lineages called Vegetative Compatibility Groups or VCG's. These VCG'S are divided by a vegetative compatibility system that limits gene flow among dissimilar individuals (Papa, 1986; Bayman & Cotty, 1991).

Some of these VCG's do not produce any aflatoxins and are termed *atoxigenic* (Cotty, 1990). The timing and application of these atoxigenic strains during the growing of agricultural commodities, remains the key to the successful displacement and possible elimination of aflatoxins (Cotty & Mellon, 2006).

The S strain produces numerous small sclerotia whilst the L strain produces fewer. It has also been reported that the S strain on average produces a much higher concentration of aflatoxins than the L strain (Cotty & Mellon, 2006). Within the S strain, some isolates termed SB, produce only B aflatoxins whilst others named SBG produce B and G toxins (Cotty, 1989). Wicklow & Shotwell (1983) showed that total aflatoxin levels in sclerotia and conidia also varied amongst strains. It was found that the accumulation of aflatoxin B_1 in the conidia of *A. flavus* NRRL 6554 was 84 000 ppb, whilst the sclerotia contained 135 000 ppb aflatoxin B_1. Aflatoxin G_1 was found in both the sclerotia and conidia of *A. parasiticus.*

It has been reported that *A. flavus* can utilise a variety of substrates including proteins, elastin, mucin and complex carbohydrates (St Leger *et al*., 1997). This suggests that natural sources of proteins such as insects and mammals may provide the nutrients to support the long term survival of the fungus. Protein digestive isolates have been extracted from cottonseed, corn, peanuts, soil, insects and humans (Mellon & Cotty, 1995; St Leger *et al*., 2000). Both carbohydrate hydrolases such as pectinase and xylanase have also been observed in *A. flavus.* These enzymes are responsible for the maceration of plant tissues and *A. flavus* does vary widely in virulence to plants (Cotty, 1989). The ability of *A. flavus* to survive in harsh conditions allows it to easily out-compete other organisms for substrates found in the soil or in the plant (Bhatnagar *et al.,* 2000). The presence of sterigmatocystin, kojic acid, aspertoxin and aspergillic acid serves as a good indicator that *A. flavus* is present in a particular material (Hedayati *et al.,* 2007).

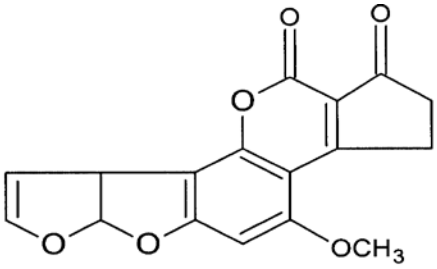
Much of this study will be based on aflatoxin B_1 (AFB_1), a low molecular weight compound produced by *Aspergillus,* which upon ingestion (by the lactating animal), is rapidly adsorbed in the gastro intestinal track through a non described passive mechanism (Yiannikouris & Jouany, 2002) and quickly appears as a metabolite termed aflatoxin M_1 (AFM_1) in milk, as soon as 12h post feeding (Diaz *et al*., 2004).

2.1.2 Aflatoxins

The discovery of aflatoxins dates back to the 1960 when more than 100 000 turkeys and other farm animals died from a disease which was traced back to Brazilian peanuts contaminated with *A. flavus* (Bash & Rae, 1969; Davis & Deiner, 1979; Sargeant *et al.,* 1961).

There are more than 25 enzymatic steps required for aflatoxin biosynthesis. Aflatoxins are biosynthesised by a type II polyketide synthase. The first stable step in the biosynthetic pathway is norsolorinic acid, an anthrax-quinone (Bennett *et al*., 1997). This is followed by a complex series of post polyketide steps. A series of increasingly toxigenic anthraquinone and di-furo-coumarin metabolites are formed (Cleveland & Bhatnagar, 1992).

2.1.2.1 Chemical composition and mode of action of aflatoxin B_1



Aflatoxin B_1

Figure 2: Chemical structure of aflatoxin B_1

Molecular formula: $C_{17}H_{12}O = 312.3$

Chemical name: (6aR,9aS) -2,3,6a,9a-Tetrahydro-4-methoxycyclopenta [*c*] furo-(3′,2′:4,5) furo [2,3-*h*] [*l*] benzopyran-1,11-dione (9CI).

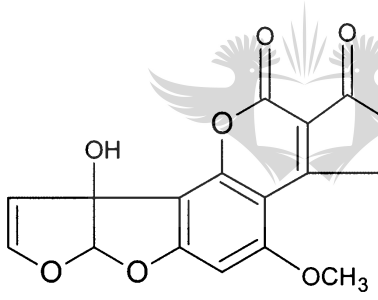
The European Food Safety Authority (2004b) has declared the presence of aflatoxin B_1 in animal feed as an undesirable substance. According to Bennett & Klich (2003), aflatoxin B_1 is the most potent and toxic hepato-carcinogenic, natural compound ever characterised. Upon ingestion of contaminated food, aflatoxins are rapidly absorbed by the gastrointestinal tract, and the circulatory system then transports the aflatoxins to the liver (Fung & Clark, 2004).

Aflatoxin entering the cell may be metabolised in the endoplasmic reticulum to hydroxylated metabolites, which may be further metabolised to glucuronide and sulphate conjugates. The aflatoxin may also alternatively be oxidised to the reactive epoxide, which undergoes hydrolysis and can bind to proteins resulting in cytotoxicity. The epoxide may then react with deoxyribonucleic acid (DNA), or protein, or be detoxified by the glutathione S-transferase enzyme (Riley & Pestka, 2005). Thus the level of metabolism determines the degree of toxicity and carcinogenicity (Patterson, 1978).

It is reported that aflatoxin B_1 is activated mainly by cytochrome P_{450} dependent mono-oxygenase which is involved in phase one metabolism. The toxicity and mutagenicity of aflatoxin B_1 may be due to the affinity of the electrophilic and highly reactive aflatoxin B_1-8-9-epoxide (AFBO) to cellular nucleophiles like DNA (Coulombe, 1993).

Between 1-3% of ingested aflatoxins binds irreversibly to proteins and DNA bases, forming adducts such as aflatoxin B_1-lysine in albumin (Skipper & Tannenbaum, 1990). Depending upon the genetic disposition of the animal, aflatoxin is metabolised by microsomal mixed function oxygenases (MFOs) to form metabolites such as aflatoxin M_1 and aflatoxin B_1-8, 9-epoxide. It has been reported that aflatoxin B1-8-9-epoxide (AFBO) is the most toxic metabolite. In the liver cells, cytoplasmic reductase acts on aflatoxins B_1 to form aflatoxicol (Campbell & Hayes, 1976). The disruption of proteins and DNA bases in hepatocytes cause liver toxicity (Tandon *et al*., 1978).

2.1.2.2 Chemical composition and properties of aflatoxin M_1



Aflatoxin M_1

Figure 3: Chemical structure of aflatoxin M_1

Molecular formula: $C_{17}H_{12}O_7$= 328.3

Chemical name: (6aR,9aR) -2,3,6a,9a-Tetrahydro-9a-hydroxy-4-methoxycyclopenta [*c*] furo [3′,2′:4,5] furo [2,3-*h*] [*l*] benzopyran-1,11-dione (9CI)

It has been reported that aflatoxin M_1 has been detected in milk of mammals following the consumption of feed which is contaminated with aflatoxin B_1 (Appelbaum *et al.,* 1982). The rate of conversion seems to be highly variable, ranging from 0.5-5 percent as reported by Neal *et al.,*(1998). However, Veldman *et al.,* (1992) have reported an increased conversion

rate of up to 6%. Also, aflatoxin M_1 is fairly resistant to most food technology practices as will be discussed later. Furthermore, it seems that aflatoxin M_1 is usually considered to be a detoxification product of aflatoxin B_1 from a mutagenic and carcinogenic process, and is the main mono-hydroxylate derivative of aflatoxin B_1 formed in the liver by means of cytochrome P450- associated enzymes (Zinedine *et al.*, 2007).

According to Neal *et al*., (1998) the human liver microsomes have a very limited capacity to catalyse the epoxidation of aflatoxin M_1. Furthermore, this epoxide also has a lower capacity to bind microsomal protein in comparison to aflatoxin B_1. It has thus been concluded that aflatoxin M_1 is a genotoxic carcinogen and is less toxic than aflatoxin B_1 (Creppy, 2002) with a classification as a Group 2B carcinogen, with possible causative effects of cancer to humans (IARC, 1993).

Few and old data is available on aflatoxin M_1 metabolism and absorption particularly in humans (Caloni *et al*., 2006):

- Purchase (1963) reported that aflatoxin M_1 is less mutagenic in one day old ducklings.
- Sinnhuber *et al*. (1970) reported similar levels of aflatoxin B_1 and M_1 carcinogenicity in trout livers.
- Pong & Nogan (1971) reported that aflatoxin M_1 was as toxic as aflatoxin B_1 in rats.
- Aflatoxin M_1 is reported to have genotoxic activity (Lafont *et al.,* 1989).

Reports from two authors, Neal *et al.* (1998) and Caloni *et al.* (2006), mention that although aflatoxin M_1 is less carcinogenic than aflatoxin B_1, it is still cytotoxic.

Furthermore, Caloni *et al.* (2006) studied the absorption and cytotoxicity of aflatoxin M_1 in human intestinal *in vitro* model and the following conclusions were drawn:

- Aflatoxin M_1 is highly absorbed in differentiated CaCo-2 cells, which represent a good characterised model of human intestinal enterocytes.
- Aflatoxin M_1 is not cytotoxic in the permitted range of possible contamination, even if it causes a general cellular sufferance that does not lead to viability impairment.

Most developed countries have regulated the maximum permitted level of aflatoxins M_1, even though the World Health Organisation (WHO) considers that there is insufficient information available on the effects of the exposure levels (van Egmond, 1989).
In the European Union, the new regulation indicates that aflatoxin M_1 is regarded as a less dangerous genotoxic carcinogenic substance than aflatoxin B_1. It is however necessary to prevent the presence thereof in milk and milk products intended for human consumption and for younger children in particular (European Commission, 1998).

2.1.3 Aflatoxins – The adverse effects on human health

Kuiper-Goodman (1998) a leading figure in the risk assessment field, ranks mycotoxins as the most important non-infectious and chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives or pesticide residues.

The exposure of mycotoxins may be through the ingestion of severely contaminated foodstuff or the continuous ingestion of contaminated foodstuffs. Other routes of exposure include inhalation, direct contact and passive exposure resulting from a mycotic infection by a toxigenic fungus. As aflatoxins have been detected in the conidia of *Aspergillus,* it places the health of agricultural workers at risk, as they are continually exposed to the natural inhalation of dust in the fields (CAST, 2003).

Mycotoxins may affect many diverse cellular processes and have a wide spectrum of toxicological effects. This complexity is reflected in the very diverse range of responses by different animal species and it is likely that there will be a different response amongst different races of humans that bear on genetic basis and even amongst individuals of the same race (Kuiper-Goodman, 2004).

Mycotoxin induced adverse effects on the immune status of human adults and children have been reported (Jiang *et al.,* 2005; Turner *et al.,* 2003). A study by Azizz-Baumgartner *et al.* (2005) reported that males were more likely to die from aflatoxicosis compared to females, in spite of eating similar quantities of maize.

Ngindu *et al.* (1982) found there was a trend in that aflatoxicosis infected patients report the deaths of their dogs before they themselves were diagnosed with aflatoxicosis. Therefore

reports regarding the death of dogs, should serve as a warning of a potential aflatoxin contamination in the food supply chain.

The liver seems to be the main target organ for aflatoxin toxicity and carcinogenicity (Abdel-Wahhab *et al*., 2007) and aflatoxins can induce liver lesions identical to those of liver cirrhosis (Amla *et al.,* 1971; Shank *et al*., 1972).

It seems that aflatoxins can cross the placenta thus affecting the foetus, and resulting in an increased incidence of still births and neonatal mortality (Hendrickse, 1997; Maxwell, 1998; Wild *et al*., 1991). Also, the consumption of aflatoxin contaminated foodstuffs whilst breast feeding seems to result in the formation of aflatoxins and their metabolites in breast milk (Galvano *et al.*, 1996; Polychronaki *et al*., 2006).

Sherif *et al.* (2009) reported that there is a need for risk assessment with regards the exposure of children to mycotoxins, especially since infants and children are most susceptible to the effects of aflatoxins, due to their lower body weight and higher metabolic rate. Also infants generally possess a lowered ability to detoxify compounds, as some organs as well as the central nervous system are not completely developed to handle detoxification (NAS, 1993; WHO, 1986;). The works of Oyelami *et al.* (1997) indicated that upon autopsy, a selected group of children who died of pneumonia in Nigeria were found to have detectable levels of aflatoxins in their lungs. The presence of aflatoxins was also detected in the livers of a selected group of children, who died from Reye's syndrome in Czechoslovakia and New Zealand (Hendrickse, 1997).

Aflatoxins have also been associated with Kwashiorkor a disease in children, which occurs as a consequence of protein malnutrition (Adhikari *et al.,* 1994). Malnutrition accounts for more than 5 million deaths of under 5 year old children in the developing world. Katerere *et al.* (2008) in their review "*Infant malnutrition and chronic aflatoxicosis in Southern Africa: Is there a link?'* reported that there is mounting evidence implicating aflatoxin contamination as an important factor in the under-nutrition of infants. More recent studies seem to link aflatoxicosis to infant growth faltering (Gong *et al.,* 2002; Gong *et al.,* 2004; Polychronaki *et al*., 2007). The mechanism by which aflatoxins reduce growth rate is probably related to disturbances in protein, carbohydrate and lipid metabolism (Cheeke & Shull, 1985).

There is also some preliminary evidence to suggest that there may be an interaction between chronic mycotoxin exposure and malnutrition, immune-suppression, impaired growth as well as diseases such as malaria and HIV-AIDS (Gong *et al,* 2003; 2004). According to the United Nations, there were approximately 33.3 million persons worldwide who were affected with the HIV-AIDS disease during 2009 (UN AIDS, 2010). Is it possible that continual mycotoxin exposure leads to immune suppression which may be increasing the incidence of HIV? This may be an interesting correlation to consider, especially if one considers that Africa does have the highest number of persons infected with HIV worldwide, as well as possibly the highest exposure rate to aflatoxin contaminated commodities.

It is hypothesised that early and repeated exposures to aflatoxins *in-utero* and during childhood might result in cancer of the liver, later in life. It is estimated that 5.2 million cancer deaths occur annually with 55% of these cases being in developing countries (Dow, 1994).

2.1.3.1 *The role of aflatoxin B_1 as a carcinogen*

Aflatoxin B_1 is classified as a Group I human carcinogen with the most documented mycotoxin associated cancer being hepatic-cellular carcinoma. As indicated in Figure 4, the acute symptoms following the ingestion of aflatoxin contaminated foodstuff or any other direct exposure to aflatoxins are vomiting, diarrhea, dizziness, fever, abdominal and seizures (Etzel, 2006; Krishnamachari *et al.,* 1975;). Hepato-cellular cancer may be diagnosed years after these symptoms appear and persons who have been diagnosed with Hepatitis B infection and aflatoxin B_1 exposure, are at a higher risk of developing hepatocelluar cancer.

Fig 4: Time course of events preceding hepatocellular carcinoma (Etzel, 2006).

According to research, aflatoxin B_1 is inactive until enzymatically activated to form aflatoxin B_1-8-9-epoxide (AFBO). This carcinogen may then bond with serum albumin forming a lysine adduct (Sabbioni *et al.,* 1987). If however, aflatoxin B_1 binds with DNA then an AFB_1-DNA adduct may form. The cell will then become an initiated tumour cell. The initiated tumour cell may remain dormant or become a differentiated neoplasm. As aflatoxin B_1 is a complete carcinogen, further biochemical changes may convert neoplasm to a cancer, through the process of conversion and progression (Lu, 2003). Universal immunisation against hepatitis B has been shown to reduce incidence of hepatic-cellular carcinoma in children in Taiwan (Chang *et al.,* 1997) and Alaska (Lanier *et al.,* 2003).

Wang *et al.* 1999 have documented that hepato-cellular carcinoma is the leading cause of cancer deaths in Qidong, China, which seems to be attributed to the increased exposure of humans to aflatoxins. *Oltipraz,* a medicinal drug seems to be able to lower the biologically effective dose of aflatoxins by decreasing the metabolism of aflatoxin B_1 to its carcinogenic form, and increasing the detoxification pathways of it's metabolites.

It has been reported that the prevalence of hepatitis B and C is high in sub-Saharan Africa, and that aflatoxin consumption increases more than tenfold the risk of liver cancer (Miller & Marasas, 2002).

2.1.4 The Presence of mycotoxins, especially aflatoxins in Africa

Developed countries are undoubtedly less at risk from mycotoxin contaminated feed ingredients than developing countries. This may be attributed to both the high standards of the major food suppliers and retailers, as well as stringent regulatory enforcements detering the importation of material exceeding the legislated permitted limits (IFST, 2009).

In Africa, the chronic incidence of aflatoxins in human diets is evident by the presence of aflatoxin M_1 in human breast milk in Ghana, Kenya and Nigeria (Bhat & Vasanthi, 2003). Most of aflatoxin poisoning seems to occur in sub-Saharan Africa where groundnut and maize especially are staple food ingredients. Poverty and the limited availability of a variety of foodstuffs in the poorer countries especially in the rural areas, mean that very little variation in diet occurs. As maize is a staple food on the African continent, the average

individual is likely to consume a maize based product at every meal time. It has been reported that aflatoxin poisoning has been associated with eating home grown maize which has been stored under damp conditions (Lewis *et al.*, 2005).

Aflatoxin contaminated maize was implicated in the 2004 aflatoxin poisoning outbreak in Kenya, which has been the most severe and largest documented case worldwide. The outbreak covered more than 7 districts and resulted in 317 cases of patients who were exposed and 125 deaths. Other smaller outbreaks were reported in 2005 and 2006 with 30 and 9 deaths respectively. It was found that maize which was harvested during off-season and during early rains, contained high levels of aflatoxins which found its way into the local market distribution system (CDC, 2004; Lewis *et al.*, 2005).

There is also some truth in the fact that developing countries tend to export their best quality commodities. This situation may in turn lead to the supply of possibly contaminated foods being sold in the local market, thus resulting in the continued exposure of a community to aflatoxin contaminated commodities. Table 3 provides documented case studies of commodities found to be contaminated with aflatoxin on the African continent.

Table 3: A summary of food commodities and aflatoxin contamination levels in Africa reported in literature (adapted from: CDC, 2004; Lewis *et al.,* 2005; WHO, 2006).

Country	**Commodity**	**Frequency of aflatoxins positives**	**Concentration of Contaminant**	**References**
Botswana	Raw peanut	78% of samples	Concentration ranged from 12-329mg/kg	Barro *et al.* (2002)
Nigeria	Pre-harvest maize	65% of samples	Total aflatoxin ranged between 3-138mg/ kg in selected positive samples	Maxwell *et al.* (2000)
Nigeria	Melon seeds	32.2% of samples	Aflatoxin above 5mg/kg	Mensah *et al.* (2002)
Senegal	Peanut oil	85% of samples	Mean content of about 40 ppb	Muleta & Ashenafi (2001)
South Africa	Traditionally brewed beer	33.3% of commercially brewed beer	200 and 400mg/l	Mensah *et al.* (1999)
Kenya	Maize	Local markets	Up to 46,400µg/kg	CDC (2004)
Kenya	Maize	Government warehouse	Up to 1800µg/kg	CDC (2004)
Ghana	Maize	100%	20 - 355µg/kg	Kpodo (1996)
Ghana	Fermented maize	100%	0.7 - 313µg/kg	Kpodo (1996)

Certain documented aflatoxin contamination in food as well as cases of acute aflatoxicosis as reported in South Africa include:

- Aflatoxin contaminated dog feed resulted in the death of 16 dogs during April 2011. (Otto, 2011).

- Aflatoxin levels of about 30 times higher than legislated levels was reported in peanut butter given to school children in the Eastern Cape (MRC, 2006).
- Sorghum sampled was found to be contaminated with aflatoxin B_1 (Ruffell & Trinder, 1990).
- It was reported that seven Friesland calves died in the Eastern Cape after being fed aflatoxin contaminated maize (van Halderen *et al*., 1989).
- Bastianello *et al.* (1987) reported that ten cases of possible aflatoxicosis in dogs were investigated.
- Peanuts were found to be contaminated with *A. flavus* and *A. parasiticus* (Dutton & Westlake, 1985).

In Africa, the World Health Organisation (2006) is focusing on field projects to increase awareness and educate consumers on mycotoxins. Wagacha & Muthomi (2008) reported that there is a need for cost-benefit analysis, coupled with cost effective sampling and analytical methods which can be used for the detection of mycotoxins especially in developing countries.

2.1.4.1 Global economics of trade

It is estimated that between 25 – 50% of the world's food crops are affected by mycotoxins, so it is likely that the economic costs are considerable and 40% of productivity lost to diseases in developing countries is due to diseases exacerbated by aflatoxins (Miller, 1996). However due to the low levels of literacy and other socio-economic factors, even if certain steps were taken to make food products safe in developing countries, the consumer may be unwillingly or unable to pay for the extra costs incurred (Wagacha & Muthomi, 2008).

Mycotoxin contaminated products affect crop producers and grain handlers as market discounts need to be negotiated for contaminated grain or it may result in entire market rejection of contaminated shipments. Trade losses associated with the rejection of shipments result in re-routing of grain shipments with considerable loss in revenue, as importing agents shipping companies and brokers fees all need to be recovered (Wu, 2004).

Feed manufacturers are further affected through the loss of business, product recall, and product liability issues. Even when exporters are able to meet the new stipulated standards, compliance requirements often involves significant capital expenditures for product re-design, building of administrative systems and the maintenance of new quality control testing and certification procedures (Henson *et al.,* 2000; Maskus & Wilson, 2001b).

For international trade, it is important that some harmonisation be achieved to remove the extreme variability in standards. Major impediments to consensus are the wide variation in contamination levels worldwide and in the inability of nations to reduce aflatoxin levels in a cost effective manner (Wu, 2006). Complying with new safety regulations may be very onerous for certain developing countries, which lack the infrastructure due to limited financial resources and education.

Economic losses arising from mycotoxicosis in Africa are enormous (Wu, 2004; Fellinger, 2006; Otsuki *et al.,* 2001a). While developed countries may incur economic losses due to the trade issues of mycotoxin contaminated foodstuffs, developing countries such as those in Africa, tend to incur both the economic losses as well as health issues, as many of the individuals in these countries are malnourished and chronically exposed to high levels of mycotoxins in their diets (Miller & Marasas, 2002).

The most recent documented case of the economic effects of aflatoxicosis in South Africa, seems to be the one reported by Kellerman *et al*. (1996) which calculates that the annual mortalities of cattle from plant poisoning or mycotoxins amounts to an annual cost of R104, 506,077 for livestock in South Africa.

In a recent South African review Katerere *et al.* (2008), mention that there is a need for *new* surveillance studies, regarding the presence of aflatoxins in food commodities originating from and traded by, within Southern Africa's rural agricultural sector. Most data available are older than 20 years and the current situation is not very clear.

The Council for Agricultural Science and Technology (CAST) (2003) estimated that crop losses (to corn, wheat, and peanuts) from mycotoxin contamination in the United States amount to $932 million annually, in addition to losses averaging $466 million annually from regulatory enforcement, testing and other quality control measures.

According to Wu (2006) and Otsuki *et al*. (2001a) no international standard for aflatoxins currently exist. Until 1996, JECFA had recommended that dietary aflatoxin be kept to an 'irreducible level'. Currently more than 100 countries have their own regulations regarding levels of mycotoxins in the food and feed industry (van Egmond *et al*., 2007).

According to Otsuki *et al*. (2001a), the World Trade Organization (WTO) Agreement on Sanitary and Phyto-sanitary Standards encourages their member countries to harmonize national standards with international standards, and consider recommendations developed by other WTO member governments in international organizations, such as the joint FAO/WHO CODEX Alimentarius Commission for food safety. The Agreement does permit importing countries, to impose more stringent measures than the international standards. It also provides for 'emergency' measures to be taken to limit or ban imports. These actions should be taken on an interim basis however, until final decisions are made based on risk assessments and scientific analysis (CODEX, 2005).

Wu, a well known author in mycotoxin research and Otsuki from the World Bank, have presented some very interesting views, models and simulations regarding the effect on trade economics. (Wu, 2006; Otsuki *et al*., 2001a).

According to Wu, a nation's total export loss of a particular food crop, given a particular internationally imposed mycotoxin standard, can be calculated as the product of the price of the food crop per unit volume on the world market, the total volume of that crop exported by a particular nation, and the fraction of that nation's food export crop that is rejected as a result of a worldwide mycotoxin standard:

$$\textbf{Export Loss}_{i,\,j,\,k} = P_i * W_{i,\,j.} * R_{i\,j\,k}$$

Where:

- *i* = crop (corn, peanuts)
- j = nation
- k = international mycotoxin standard (fumonisin, or aflatoxin)
- Pi = world price for food crop i per unit volume,
- W *i*, j = total export weight (in metric tons) of crop *i* from nation j.
- R *i*, j, k = fraction of export volume of crop *i* from nation j rejected at international mycotoxin standard k

This economic model allows a sensitivity analysis on how export losses for food crops in particular nations change as a function of the strictness of the mycotoxin standard. Uniform and lognormal distributions were estimated for the parameters in order to account for the natural distribution and uncertainties surrounding nation's levels of mycotoxins from year to year, prices of food crops, and total volume exported. A Monte Carlo simulation was employed using Analytica® software to estimate uncertainty bounds and sensitivity around the results (Wu, 2004).

Otsuki *et al. (*2001b), further demonstrated their simulations and models on the trade of groundnuts between Africa and the European Union, as groundnuts are a huge export commodity for Africa. The objective of the authors Otsuki *et al.* (2001b) was to predict the trade effect of setting aflatoxin standards at differing levels. Bilateral trade flow between nine African countries (including South Africa) and 15 European countries, were examined under three regulatory scenarios: standards set at pre-EU harmonized levels (status quo), the new harmonized EU standard adopted across Europe, and a standard set by the CODEX. In addition, the trade-off between human health and trade flows were examined for each of these three regulatory scenarios. The info for these risk assessments was based on results received by joint FAO/WHO Expert Committee on Food Additives (JECFA).

According to the proceedings of the third joint FAO/WHO/UNEP International Conference on mycotoxins, stringent regulations posed by the European Union regarding the permitted concentrations of aflatoxin in groundnut has presented some serious economic and trade losses for the African Groundnut Council. The import of groundnut meal in the European Community has declined from 0.91 million metric tons in 1979/80 to 0.4 million metric tons in 1989/90. The European Commission set the standard for aflatoxin B_1 at 8ppb in groundnuts subject to further processing. As reported by Otsuki *et al.* (2001b), the Codex standard on total aflatoxin contamination in processed and unprocessed food, is set at 15 ppb. In contrast, the harmonized European standard for total aflatoxin contamination in food products is more restrictive.

The authors also developed models to determine what the effects of the value of African food export and human health risk under the new European Union harmonised standard relative to those under the alternative regulatory scenario as depicted in Table 4 below.

Table 4: The annual value of African food export and human health risk under the new EU harmonised standard relative to those under the alternative regulatory scenario (Otsuki *et al.,* 2001b).

	Relative to Codex	**Relative to the pre- EU harmonisation (1998 trade)**
Loss in value of African food Exports	$ 670 million	$340 million
Number of cancer deaths saved	2.3 persons	0.9 persons

The authors concluded that with the European Union (EU) setting a standard more stringent than the international standard suggested by Codex, it is estimated that African exporters will lose approximately 59% of export revenue from Europe for cereals, and 47% for dried fruit and nuts, compared to export revenue under the pre – EU harmonised standard. The difference is estimated to be approximately US$ 400 million for cereals, dried and preserved fruits under the harmonised standard. Trade flow of these products was found to increase by US$ 670 million, if the standards imposed were based upon those suggested by Codex guidelines instead of the EU harmonised standard.

One question thus exists, is it possible that the permitted levels of aflatoxin contamination in foods allowed by certain countries is too restrictive, so as to act as deliberate barriers of trade? This will be explored further during the discussion section of this dissertation.

2.1.4.2 Effects of climate change in Africa

Climatic conditions represent the key agro-eco-system driving force of fungal colonisation and mycotoxin production (Magan *et al*., 2003). Global warming has occurred due to the increased presence of methane, carbon dioxide, nitrous oxide and chlorofluorocarbons in the atmosphere (Chakraborty *et al.,* 1998). Globally, higher temperatures are experienced together with unusual unpredictable weather patterns.

In a report by Zvomuya (2011) commissioned by the Department of Environmental Affairs, South Africa is vulnerable to climate change, which will result in higher temperatures changes in rainfall patterns and scarcity of water resources. These climatic changes will affect crop production as well as the possibility of initiating new diseases in both humans and animals. The higher temperatures along with anticipated increase in droughts and floods will affect agricultural production. It has been found that during periods of drought, socio-economic upheavals are likely which (Giles, 2007) further affect food supply. The report also mentions that the effect of heat stress on the dairy cow will result in reduced milk production, reduced protein and milk fat content and a decreased rate of reproduction, thus affecting the future supply of milk and meat.

The issue of climate change offers a complicated, multifaceted and interrelated scenario which could have a serious impact on the availability of food and feed, particularly for developing countries (Miraglia *et al.,* 2009) as changing weather patterns can influence irrigation, crop rotations, optimal crop timing and the possible contamination of several crops (Paterson & Lima, 2010). However, Paterson & Lima (2010) have also reported that the biggest risk with respect to mycotoxins from climate change will be found in developed countries such as parts of Europe and United States of America.

In Africa certain aflatoxin producers are associated with dry hot 'agro-ecozones' with latitudinal shifts in climate, influencing the fungal community structure (Cardwell & Cotty, 2002). Anecdotal evidence from oil mills and elevators in areas prone to aflatoxin contamination indicated that high daily temperature minima lead to 'poisoned crops' (Cotty & Jaime-Garcia, 2007). Furthermore, in some regions it has been found that infection only occurs when temperature increases in association with drought (Sanders *et al*, 1984; Schmitt

& Hurburgh, 1989). According to Horn & Dorner (1999) climate influences not just the quantity, but also the type of aflatoxin producers present.

Maize is a staple food in Africa and is vulnerable to aflatoxin contamination, as recently demonstrated in Kenya in 2004. The effects of climate change will result in the restriction of certain areas in which maize may be grown profitably. If rain occurs at or near harvest 'proper crop dry down' is delayed, thus promoting aflatoxin development especially in the warmer countries. Several severe cases of maize contamination notorious for lethal aflatoxicosis has been associated with this condition of *improper drying down* of maize (Krishnamachari *et al.,* 1975; Lewis *et al*., 2005). The host susceptibility is also influenced by climate for example, the maize kernel may be compromised by a condition referred to as 'silk cut' (Cotty & Jaime –Garcia, 2007).

The identification of climate change factors is central to risk management (van der Fels-Klerx *et al.,* 2009). These factors include the molecular biology of the pathogen, the vectors involved, farming practice and land use, environmental factors as well as the establishment of new micro-habitats. The ability of certain mycotoxigenic fungi to mutate and hence respond to opportunities arising from the change is a key factor when considering the potential impact of climate change (Paterson & Lima, 2010).

Aflatoxin management technologies, detoxification and shifting of cropping patterns are all potential solutions as reported by Magan *et al.* (2003), to assist in managing the situation more effectively and generating a constant supply of safe food.

Plant, animal and human epidemics are all influenced climatically (Bosch *et al.,* 2007; Fitt *et al.,* 2006; Thomson *et al*., 2006 & Wint *et al.*, 2002) hence weather forecasts have already been developed to guide control strategies for many important diseases worldwide (Garett *et al.,* 2006). The possibility now exists to relate weather based plant disease forecasts to recent climate change models and hence predict the efforts of climate change on where, which and by how much mycotoxins will be changed (Paterson & Lima, 2010).

In summary the negative health implications of aflatoxins presents some serious concerns regarding the health of the public especially children. The lack of required quality systems and practices on the part of the African food exporter may in turn lead to reduced exports of certain commodities thus affecting revenue and employment. The intervention and support by

local Government, as well as cereal and grain seed suppliers to farmers, in the form of education and subsidies may create employment and a superior quality product which will meet international criteria. The effects of climate change will further affect food supply and in turn likely to affect the affordability of food in general. It may be necessary that the leaders of Africa involved in climate change consider the employment of task forces to better ascertain how Africa as a continent will be managing the inevitable effects of climate change.

2.2 ANIMAL FEED

A farmer's choice of what to feed their livestock will depend upon the age and species of the animal, their intended products (meat, milk or eggs) and even the time of year and geographical location (FSA, 2006). Cattle feed is usually a blend of cereals, legumes, agro industrial by-products (AIBP's) and green fodder. These products are important sources of protein supply for ruminants in particular, as they are able to tolerate much higher levels of anti- nutritional factors present, including various toxic compounds which are deleterious to the health of most other animals (Sindhu *et al*., 2002). Certain reports indicate that tree nuts, maize, cotton seed rice, figs, tobacco and spices are most frequently affected by mycotoxins (Detroy *et al.,* 1971; Diener *et al.,* 1987).

Although mycotoxins occur frequently in a variety of feedstuffs which are routinely fed to animals, it is less frequent that mycotoxins occur at levels high enough to cause immediate and dramatic loss in animal health and performance. However, mycotoxins react with other stresses to cause sub-clinical losses in performance and to the animal producer these sub-clinical losses are of greater concern and economic importance (Whitlow *et al.,* 2010).

2.2.1 The Different Components of Animal Feed

Below is a list of some of the various types of ingredients commonly used in the preparation of feed for dairy cattle:

- Cereals such as maize which may be whole or ground.

- Cotton seed & cotton seed meal.
- Distillers dried grains such as sorghum brewer's grains.
- Wheat bran or grain milling by products from barley, sorghum, rye and oats.
- Greens such as lucerne, hay, celery leaves, turnips and fodder beet.
- Animal based proteins such as feather meal and protein meal.
- Sugar derived ingredients such as molasses, condensed molasses and sugar cane.
- Rice based products such as broken rice, de-oiled rice bran, rice straw and rice husks.
- Oil cakes such as linseed meal, groundnut cake, sesame oil cake, palm kernel cake, canola meal, mustard cake and sunflower seed oil cake.
- Legumes such as cowpea, mung, urad & soya products which include soya bean meal, soya pulp and soya hulls.
- Bakery wastes & stale bread.

Other new *agro industrial by-products* (Sindhu *et al.*, 2002):

- Fruit and vegetable by-products: citrus pulp, apple pulp, pineapple bran, tomato pomace, mango piths and passion fruit seed meal
- By- products of the antibiotic industries
- By- products of the guar gum industry
- Marine waste products such frog meal, weeds, aquatic plants, green and red seaweed
- Palm oil sludge and coffee pulp

Some of the more common feed ingredients found during the sampling process will now be discussed in further detail.

FORAGE

Forage has high fibre content generally and is usually used on farms on which they are grown. Cut grass may also be turned into silage by storing in plastic sheeting, which excludes air and promotes fermentation of the sugars under anaerobic conditions (FSA, 2006). Many mycotoxins have been found to occur in forages either in the field, or in storage as hay or silage (Lacey, 1991).

SILAGE

Ensiling is a way of preserving natural forage. Silage is a popular feed for dairy cattle not just in South Africa, but in other parts of the world too. Silage is produced by harvesting a uniformly chopped forage crop at high moisture content, under anaerobic conditions and fermenting the crop with lactic acid bacteria. Lactic acid bacteria are generally used as inoculants, as they produce volatile fatty acids which inhibit yeast and mould upon aerobic exposure of silage. Properly fermented silage which has been packed properly and covered adequately has an acidic pH which is the natural preservative and may be stored for indefinite periods (Coblentz, 2006). Figure 5 shows images of a characteristic silage pit in South Africa.

Figure 5: Photographs of a characteristic silage pit found in South Africa

However the heat which may be dissipated during respiration by the forage plants can cause an increase in the surrounding temperature which interferes with the fermentation process, reducing the nutritional value of the silage by increasing the formation of forage fibre. Mould growth may also be encouraged, which may be accompanied by aflatoxin formation.

The mould species which have regularly been isolated from silage, belong to the genera *Absidia, Arthrinium, Aspergillus, Byssochlamys, Fusarium, Geotrichum, Mucor Monascus, Penicillium, Scopulariopsis* and *Trichoderma* (Jonsson *et al.,* 1990; Nout *et al*., 1993; Pelhate 1977; Woolford 1984). El-Shanawany *et al*. (2005) found that *Aspergillus* was present in 100% of silage samples collected in Egypt, and it was also found that 22.5% of silage samples analysed contained aflatoxins. It was further reported that aspergillic acid,

cyclopiazonic acid, kojic acid and sterigmatocystin were also found to be produced by certain isolates of *A. flavus.*

Aspergillus fumigatus is another frequent mould contaminant of silage (Cole *et al.,* 1977*)* and research indicates that ingestion of silage contaminated with this mould may lead to mycosis, induced abortion or mastitis (Bartels *et al.,* 1999; dos Santos *et al.*, 2003) as well as mycotic haemorrhagic bowel syndrome in new cows (Puntenney *et al.,* 2003).

HAY

Hay is a common feed ingredient for animals and is commonly packed in bales, stored in the open fields or within a sheltered area. Mould invasion of dry hay results in an increase of the dust fraction which may contain a large amount of fungal conidia (Laan *et al.,* 2006). Mould invasion can also occur if hay has been stored in wet conditions or when there are damp patches within a bale. Generally mould will grow in hay at moisture levels between 12 to 15%. It seems that *A. fumigatus* is the common mould found in hay (Shadmi *et al*., 1974) and the consumption of mouldy hay has been reported to be associated with poor semen quality in bulls (Alm *et al.,* 2002). The consumption of hay contaminated with conidia is assumed to affect the upper airways of cattle (Fink-Gremmels, 2008).

LEGUMES

Legumes are the second largest group of forage plants consumed by ruminants (Fink-Gremmels, 2008). The most prominent fungus present is *Rhizoctonia leguminicola,* causing black patch disease in certain legume leaves. This fungus produces a number of mycotoxins, one of which is slaframine the causative agent of the 'slobbers diseases' (Hibbard *et al.,* 1995; Le Bars & Le Bars, 1996) which presents symptoms such as excessive salivation and drooling in dairy cows.

Lucerne

Lucerne or green chop is a forage crop which is derived from a plant of the legume family, known for its ability to fix atmospheric nitrogen due to symbiosis between the plant and bacteria which develops in its root system. Lucerne is a rich protein and digestible fibre source used for animal feed.

According to the European Food Safety Authority (EFSA)(2009), lucerne is the most widely grown forage crop in the world and it is particularly common in hot temperature and sub-tropical regions located at high altitudes. Nearly 32 million hectares of lucerne is grown worldwide. Once the lucerne crop is ready for harvesting, the lucerne is cut as shown in Figure 6a. Fields where cutting has been completed must be left for a minimum of 24 hours or longer as shown in the Figure 6b, depending on the prevailing temperature and humidity, before being raked into windrows to dry out before being baled. The baling of lucerne as shown in figure 6c is important, as the moisture level at which lucerne is baled influences the quality. If lucerne is baled too dry, great losses of leaves occur, and this in turn implies a reduction of nutritional value. When bales are too moist however, mould growth may be encouraged (Lategan, 2007).



Figure 6a: Cutting of lucerne Figure 6b: Drying of lucerne Figure 6c: Baling of lucerne

(Lategan, 2007)

Lucerne has high crude protein content and high rumen degradability. There is often high risk of bloat, and often a low rumen ph results (Rearte & Santini, 1989; Castillo, 1999; Bargo *et al*., 2003). These effects differ though, especially if the lucerne is ensiled and fed in total mixed rations (Ruppert *et al*., 2003). Lucerne is a plant with oestrogen-mimetic activity that has been demonstrated *in vitro* and *in vivo* (Kurtzer & Xu, 1997, Zava *et al*., 1998) so it is important that consumption be controlled. A study of animal feed ingredients in Iran (Ghiasian & Maghsood, 2011) reported that *A. flavus* and *A. parasiticus* were isolated from lucerne.

Soya bean

Soya bean meal is an excellent source of rumen degradable protein. As shown in Figure 7, the highest percentage of soya beans traded in the South African market was to the seed and feed industry.

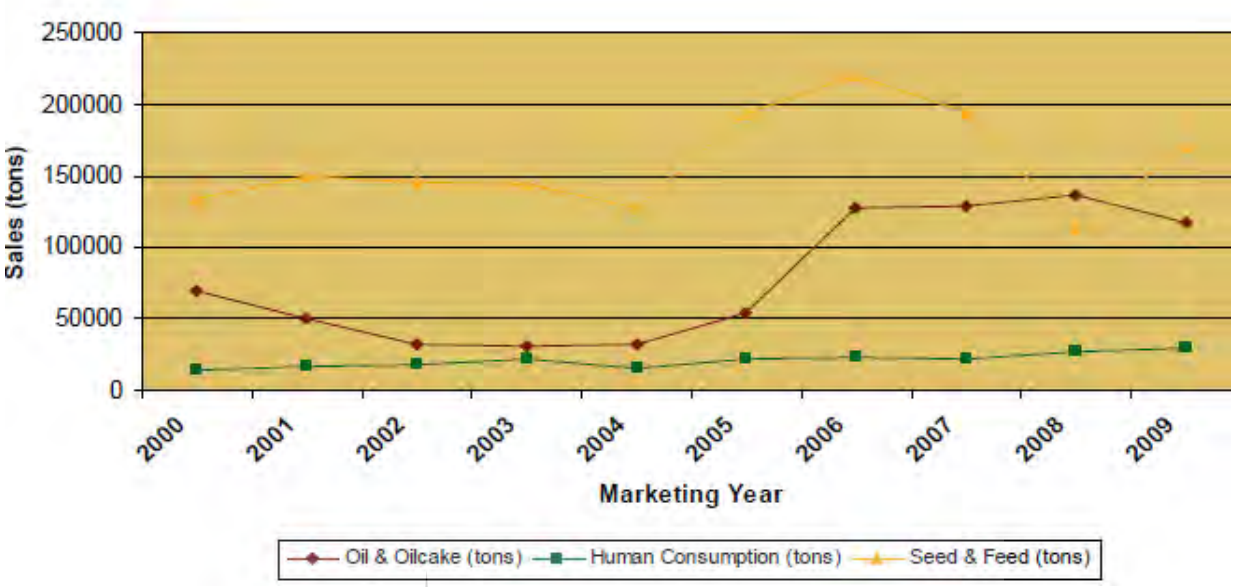


Figure 7: Graph showing the local sale of soya beans (DAFF, 2011)

In an article titled "Soya beans and Soya bean Meal in South Africa" accessed at the Agri-commodity (2010) website, it is projected by the South African Bureau for Food and Agricultural Policy (BFAP) that 605,000 hectares of soybeans could be planted by 2020, based on increased yields and growing demand for this protein. As a result, soybean production in South Africa could triple to about 1.62 million tons by 2020. It is estimated that about 1.3 million tons of oilseed meal is consumed annually in South Africa of which 70% approximately is soya bean meal. Currently more than 90% of soya bean meal is imported mainly from Argentina.

Microbiological analysis of soya bean in Argentina has reported the presence of *A. flavus* and *A. parasiticus* according to Pinto *et al*., (1991). Sutikno (2006) also found that 20% of soya bean meal sampled during 1979 - 1985 was found to be pre-dominantly contaminated with aflatoxin B_1. A report by Cotty & Mellon (1998) suggests that seed storage proteins when presented with an accessible carbon source, may predispose oilseed crops to support the production of high levels of aflatoxins by *A. flavus* during seed infection.

It has also been reported that aflatoxins tend to accumulate in soya oil cake rather than the oil. However, certain mould species such as *Aspergillus* and *Penicillium* may contaminate extracted oils with free fatty acids which can undergo oxidation leading to the formation of certain products which may contribute to the rancidity of oil, thus compromising the shelf life (Adam & Moss, 2008).

Maize

Maize is a common feed ingredient for dairy cattle in South Africa. According to the South African Feedlot Association (NDA, 2004), maize products represent 65% of the approximately 1.3 million tonnes of feed used in the feedlots annually.

Hominy chop is a white maize by-product sampled at three farms during this study and seemed to be the most cost effective animal feed source in comparison to the yellow maize, is also used regularly in animal feed.

Unpublished works by colleagues within Food Environment Health Research Group, University of Johannesburg, indicate that maize in South Africa has been found to be contaminated with a range of mycotoxins. Published reports indicate that *A. parasiticus* is the common aflatoxin producing fungus in corn (Davis & Diener, 1983). However on the African continent, maize from Nigeria was found to be contaminated with *A. parasiticus* as well as *A. flavus,* and *A. niger* (Aja- Nwachukwe & Emejuaiwe, 1994). Also in Uganda, both *A. flavus* and *A. parasiticus* were found as common contaminants in maize (Sebunya & Yourtee, 1990).

The spores of fungi are dispersed by wind settling on the soil surviving for months on end on decaying plant material. The increased consumption of maize contaminated with aflatoxin by ruminants such as the dairy cow is likely to induce a sub-clinical chronic impairment of liver function, leading to hepatic lipidosis. It seems that even a low concentration of aflatoxin B_1 contamination in maize can affect the cellular and humoral immune system resulting in immune compromised animals (Marin *et al*., 2002).

Insect damage is another factor that pre-disposes maize to mycotoxin contamination, because insect herbivory creates kernel wounds which encourage fungal colonization and the insects themselves, serve as vectors of fungal spores (Munkvold & Hellmich, 1999; Sinha, 1994; Wicklow, 1994).

It has also been reported that climatic conditions such as drought, stresses maize plants and render them susceptible to contamination by *Aspergillus* spp. Also, the warm environment inside the windowless homes and the storage of maize on the usually unprepared dirt floor tends to promote fungal growth in wet maize kernels (Azziz-Baumgartner *et al.,* 2005). Genetically modified (GM) Bt maize through the pest protection that it confers, has lowered the levels of mycotoxins in crops. In some cases, the reduction of mycotoxins afforded by Bt corn, is significant enough to have an economic impact (Wu, 2006). In 2004, Bt corn was grown on about 27% of field maize acres in the United States (USDA, 2004). In addition to the USA, seven other nations including South Africa are currently planting Bt maize (James, 2003).

According to Reuters (2010), South Africa harvested 12.815 million tonnes of maize in the 2009/2010 season, its biggest crop in decades. Surplus maize was exported to South Korea, Japan, Kuwait, Italy, Spain, Mexico and Portugal. During early 2011, concerns were that too much maize was being exported and South Africa may need to import maize to satisfy the requirements of the local market in the very near future. Currently it is predicted that South Africa will experience maize shortages so it is likely that the country will have to import maize. The imported maize may not be the Bt variety and the maize kernels can be damaged during harvesting, also the temperatures during travel as well as storage condition concerns needs to be addressed too, as these variables have a direct influence on mycotoxins contamination of maize.

Cotton seed

Cottonseed or cottonseed meal is a rich source of protein, energy and fibre for ruminants. This highly versatile seed provides one of the most desired fabrics for human apparel and is also an important food source for animal feed. Cottonseed meal may be available in varying

proportions of protein content. The hulls of cottonseed contain a very high percentage of fibre and are very palatable. The hulls may be pelleted for ease of handling and transport.

Gossypol is a toxic compound found in the cotton plant which exists either in the free or bound state. The free gossypol is toxic whilst the gossypol which is bound to protein is the non toxic compound, however whole cotton seed contains the most free form of gossypol. The amount of gossypol in cottonseed is usually used by nutritionists to make recommendations regarding the feeding of cottonseed products as over fed cattle are known to die of heart or liver failure. There is currently no treatment available for animals suffering from gossypol toxicity (Morgan, 1989).

Between 40-60% of cottonseed was imported by South Africa during 2004/2005 (DAFF, 2011). As shown in Figure 8, most of the cottonseed imported into South Africa is from countries on the African continent.

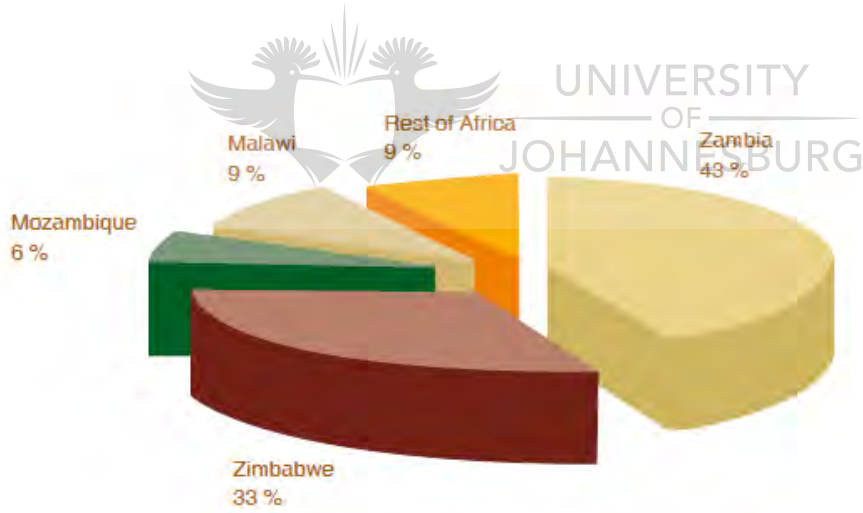


Figure 8: Pie chart showing cotton imports (DAFF, 2006).

As most of the cottonseed used in South Africa, seems to be imported from countries within the African continent, cause of concern is three fold. Firstly, has certain non-aflatoxigenic seeds been used to grow the cottonseed. Secondly, a known shortage of resources in Africa further means that certain compliance quality tests is likely not to have occurred. Thirdly, have the storage conditions during post-harvest and transport been adequately ventilated and

maintained so as not to promote the development of aflatoxins. Furthermore, Mellon & Cotty (1998) reported that aflatoxin stimulation by storage proteins in the presence of carbohydrate may occur in developing cottonseed.

Bran derived from Barley, Rice & Wheat

Bran consists of combined aleurone and pericarp. Along with germ, it is an integral part of whole grains, and is often produced as a by-product of milling and during the production of refined grains such as wheat, barley and rice. Bran is thus a rich source of dietary fiber.

Barley may also be used in the production of silage. According to a report by the Department of Agriculture, Forestry and Fisheries (DAFF), during 2009 South Africa imported a total of 6 167 tons of barley which represented an increase of 22% in quantity between 2005 and 2009 (DAFF, 2006).

Wheat is produced mainly for human consumption, although small quantities of poorer quality wheat are marketed as stock feed. Wheat imports into South Africa between 2000 and 2004 showed an increasing trend especially from countries such Argentina, USA, Russia and Australia (DAFF, 2006).

Aflatoxigenic fungi, *A. parasiticus* and *A. flavus* were isolated from certain wheaten bran and barley samples during the analysis of feed ingredients in Iran (Ghiasian & Maghsood, 2011).

South Africa does not produce rice, mainly due to the high water requirements of the crop. Nigeria and South Africa are the two largest rice importers in sub-Saharan Africa. Nigeria accounts for about 20% while South Africa is responsible for about 11% of imports into the sub Saharan Africa (Odularu, 2010). In a study by Scudamore *et al.* (1998), 72.5% of drawn samples of rice bran used in the feed industry were found to be contaminated with aflatoxins B_1. In another study by Zaboli *et al*. (2010), 30 samples of rice bran, collected in Northern Iran were all found to be contaminated with aflatoxins.

Abdullah *et al*. (2000) conducted a study determining the equilibrium moisture contents of starch based foods at different humidity levels using a static desiccator's technique. They

reported that the moisture content of rice should be maintained at 25ºC, at a level lower than 13% for long term storage.

Distillers dried grains with solubles (DDGS)

Feed by-products derived from the brewing industry are also widely used in the feed industry. Wheat and corn based distillers grains are usually used for animal feedstock. Brewer's meal, one such type of distiller's grain which is popular in South Africa was found to be a common feed ingredient during the sampling process.

In the United States of America during 2004, approximately 2.8 million metric tons of distiller's grains were fed to dairy cattle and this represented only 43% of all the distiller's grains which was used in animal feed (FDA, 2006). Distillers grains during ethanol production may contain up to three times the amount of aflatoxin originally contained in the grain, as the starch fraction has been removed (Whitlow *et al.,* 2010). These aflatoxin contaminated distillers grains can pose risks to the safety of animals consuming these products as well as the possible production of aflatoxin residues in the milk and meat produced by these animals.

Sunflower seed

Sunflower seed provides about 40 % of oilcake which is processed for animal feed respectively. Sunflower seed meal or sunflower cake offers a high roughage factor in ruminant diets. Even though sunflower oil cake is a rich source of protein, it is however regarded as a low value product, as its nutritional profile is inferior to that of soya bean.

Sunflower is the third largest grain crop produced in South Africa after maize and wheat, according to DAFF (2006).

In a study by Hadanich *et al*. (2008) it seems that the dominant micro-flora of sunflower seeds found in domestic stores is from the *Alternaria* species. However *Absidia* , *Penicillium, Stemphylium* and *Trichoderma,* spp. were detected too, but at lower concentrations. The authors found that during model tests of 20% seed moisture content and storage at 25 °C for four weeks, the *Alternaria* species were almost completely eliminated. The seeds were found

to be mainly contaminated with *Aspergillus* species and it was also reported that this mould significantly deteriorated the quality of the seed, and that of the produced oil and meal. The following properties were also affected: the reproductive ability, germinating power, oil content, lipoxygenase enzyme activity, acid value, peroxide value, fatty acid composition, UV absorbance, colour, sensorial properties, protein content, amino acid composition, colour and the smell of the meal. However, no aflatoxin production had occurred. These findings offer a comprehensive picture on the multiple destructive effects of incorrect storage.

ANIMAL BASED PROTEINS

Protein meal derived from animal based proteins is a common feed ingredient used for livestock. Feather meal was found as a feed ingredient during the sampling process. It is manufactured through the hydrolysis of non- decomposed clean poultry feathers and is thus a suitable protein source. The use of animal proteins should be carefully considered and controlled as their use has been implicated in the Bovine Spongiform Encephalopathy (BSE) disease.

PELLETED COMPOUND FEED

Animal feed is usually a combination of starch, fibre, proteins and lipids. Pelleting involves the agglomeration of these ingredients into dense pellets. Extrusion and expansion generally result in pasteurisation of the final product, destroying most undesirable micro-organisms. Heat sensitive ingredients such as vitamins, minerals, enzymes and lactose can all be applied topically (Rokey *et al*., 2006)

Pelleting also confers certain advantages to farmers as there is decreased feed wastage and reduced selective feeding of preferred feed ingredients by the dairy cow thus improved feed efficiency may be achieved (Rokey *et al*., 2006)

It is important that the particle size of the ingredients used in the manufacture of pelleted feed is uniform, so milling of ingredients may occur. This processing step may result in the re-distribution of mycotoxins, especially if aflatoxin contaminated feed is mixed with un-contaminated product.

TOTAL MIXED RATIONS

Many farmers choose to weigh and blend unique combinations of all the above mentioned ingredients as a sole feed source given to the animal over a 24 hour period, fed *ad lib.* for optimum results. Total mix rations (TMR) can be described as a mixture of both the roughage and the processed ingredients, formulated and mixed to supply the cow's requirements in a form that precludes selection. The advantages are that a wider variety of less palatable feeds can be utilized in the ration, as these are masked by the presence of other ingredients and there is also better control over the cow's diet (Neitz, 2005).

2.2.1.1 Surveys of Feed – International & Local

During the year 2010, the Biomin® survey (Rodrigues & Naehrer, 2011) analysed more than 3,300 samples of animal feed as shown in Figure 9. It was reported that 36% of samples contained at least one type of mycotoxin, whilst 42% of samples contained two or more mycotoxins, and only 22% of samples were free of mycotoxins.

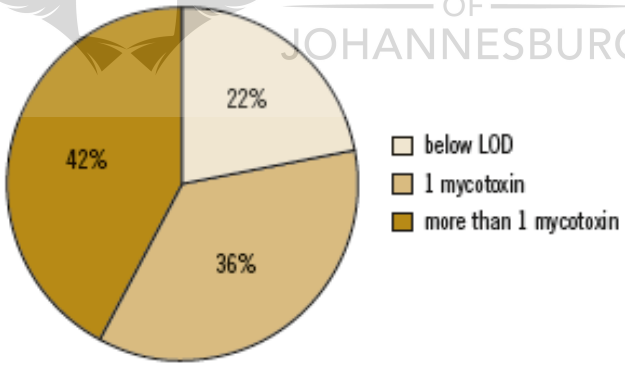


Figure 9: Pie chart showing the occurrence of mycotoxins in the feed samples analysed (Rodrigues & Naehrer, 2011).

In the Kwa-Zulu Natal, Mngadi *et al*. (2008) conducted a study on 23 samples of ready-to-consume animal feed as well as selected feed ingredients. The authors reported that 21 samples were contaminated with the mycotoxigenic fungus, *A. flavus.* The analysis of canned pet food also revealed the presence of *A. flavus,* which is odd considering the high acidity of

the product, the extreme levels of processing and the presence of preservatives in the product. It was also found that *A. parasiticus* co-occurred with *A. flavus* in two of the samples.

It was found that seven of the 23 samples analysed reported levels of aflatoxins equal to or above legislated levels of 20µg/ kg for animal feed as per USA and Canadian law. Analysis of cotton oil seed cake, sunflower oil cake and molasses meal had four times this legislated level. It was documented that whilst seventeen of the samples had tested positive for mycotoxins, eight of these samples were found to contain more than one toxin. In addition, ten samples were found to contain aflatoxins which were above the stipulated legislated limit mentioned earlier (Mngadi *et al.,* 2008).

This survey indicates the severity of the mycotoxin contamination of animal feed products and ingredients in South Africa. As discussed earlier in this review, these toxins impact not only on the health of the animal which consumes the contaminated feed, but there is also the carry-over of these toxins to humans, which manifests itself in a host of diseases. There is thus a definite need for more stringent controls, on the quality of both imported and locally produced agricultural commodities especially with specific focus on mycotoxin contamination.

All the ingredients discussed in this review, have been found in literature to be contaminated with moulds and or aflatoxins. This is confirmed by van-Egmond (2002) and Bennett & Klich (2003) who reported that cereals, oilseeds, and a long list of other commodities are commonly attacked by aflatoxigenic moulds. Binder *et al*. (2007) further highlighted that the mould –flora found on forage crops may lead to a significantly different spectrum of toxins which needs to be considered too.

Williams *et al.* (2004) further concluded that at latitudes between 40°N and 40°S of the equator, fungal invasion is responsible for the contamination of stored produce which has been inadequately dried and that the contamination often begins before harvest and can be promoted by production and harvest conditions.

2.2.2 Feed Storage Areas

The storage management of feed is important as it determines whether any pre-harvest damage of grains by moulds will be further exacerbated through incorrect storage temperatures, high humidity and possible insect infestation. The estimated losses of staple food grains in store may amount to 10% worldwide (Anon, 1979) but can reach up to 50% in tropical regions (Hall, 1970). It is estimated that between 15 and 35% of high moisture rice in southern India was lost in only nine days due to incorrect storage conditions as documented by Vasan (1980).

There seems to be a correlation between the socio-economic status of the majority of sub – Saharan countries and the exposure to mycotoxins. For example, instead of maize being stored in granaries as is traditional practice, it is usually found to be stored inside (usually poorly aerated) homes during periods of food shortage which may facilitate contamination with mycotoxins (Azziz-Baumgartner *et al.,* 2005).

The cleanliness of the storage facility and stock rotation of feed ingredients is important in preventing contamination by fungi. The First-In-First-Out (FIFO) principle should apply to ensure effective stock rotation. It should be a documented policy on the farm, that prior to further loading the storage areas with newly delivered raw material, the area should be emptied completely and cleaned out. The cleaned area should then be dried prior to storing any feed ingredients.

Figure 10 shows some photographs of the various types of storage areas which are commonly used on dairy farms in parts of Kwa-Zulu Natal, South Africa. Grain silos were seen on many farms but their access was usually restricted.

Figure 10: Photographs of various types of feed storage facilities used on dairy farms.

Wallace & Sinha (1981) considered the storage of grain to be a man made ecosystem which should be examined in a more holistic ecological manner, to enable a proper understanding of the processes occurring and to improve the post harvest management of stored food commodities (Magan & Aldred, 2007). The spoilage of stored grain by fungi is determined by a range of factors which can be classified into 4 groups namely: intrinsic nutritional factors, extrinsic factors, processing factors and implicit microbial factors (Sinha, 1995). These factors have been used to demonstrate the 'storage ecosystem' in Figure 11.

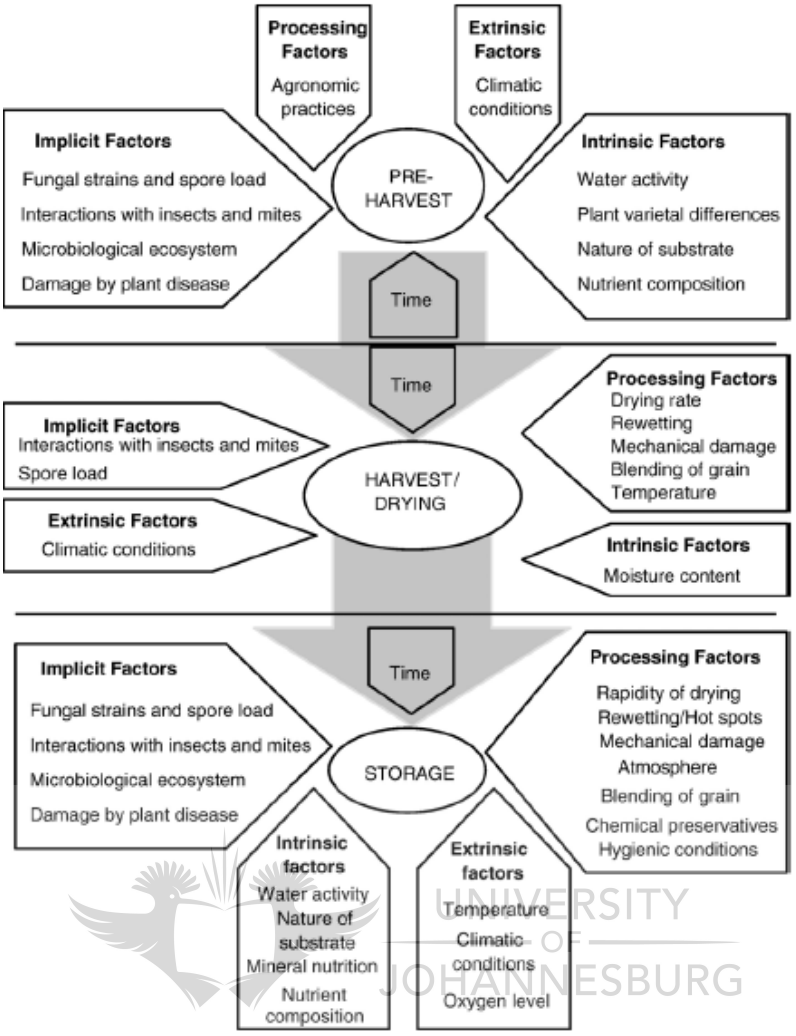


Figure 11: The interaction between extrinsic and intrinsic factors in the food chain which influence mould spoilage and mycotoxin production in stored commodities (Magan *et al.*, 2004).

The investment in small expensive per tonne silos for on-farm storage is a cost effective option for stock feed grains especially when offset against the high cost of transporting grain to the farm on a regular basis. The key element of a successful commercial on –farm grain storage system is to ensure the protection of grain against infestation and contamination by insects and fungi. Grains should be dried to moisture levels below that at which moulds can develop, before it is actually transferred to storage (Viljoen, 2001).

Bolted steel silos as seen in Figure 12, are manufactured from corrugated steel sheets and are relatively cheap and effective for the on-farm storage of grain. Conical concrete or metal

floors within the silos, are considered to be more expensive but is generally more effective in preventing insect infestation and ensures all grains are removed from the silo, prior to re-filling thus preventing the presence of pockets of old grain which may encourage insect infestation (Viljoen, 2001).

Figure 12: A photograph of bolted steel silos which are commonly used for grain storage (Viljoen, 2001).

Bunkers and storage sheds are also used as on-farm storage facilities. Steel mesh structures lined with plastic sheeting is another type of storage structure, but it is not as cost effective to maintain. Bunkers consisting of plastic sheeting can function as beneficial fumigation enclosures. Small sheds which can be sealed gas tight for fumigation by phosphine, are also commonly used. The use of shipping containers is also common, with a polyethylene liner which improves gas tightness for fumigation.

Brooker *et al.* (1992) described the moisture migration in steel bins as follows: When the temperature outside the bin decreases, a temperature differential is created across the walls. The air in the silo thus develops a continuous convection movement. The air near the walls is cooled thus raising its relative humidity, and resulting in an increase in the moisture content at the bottom of the silo. This increase in moisture can create a deterioration spot. The dry air then rises through the central part of the bulk mass, and picks up moisture from the grain. When this warm moist air makes contact with the cool upper grain surface, the moisture may

be deposited and another deterioration zone can occur. This moisture migration is shown in Figure 13.

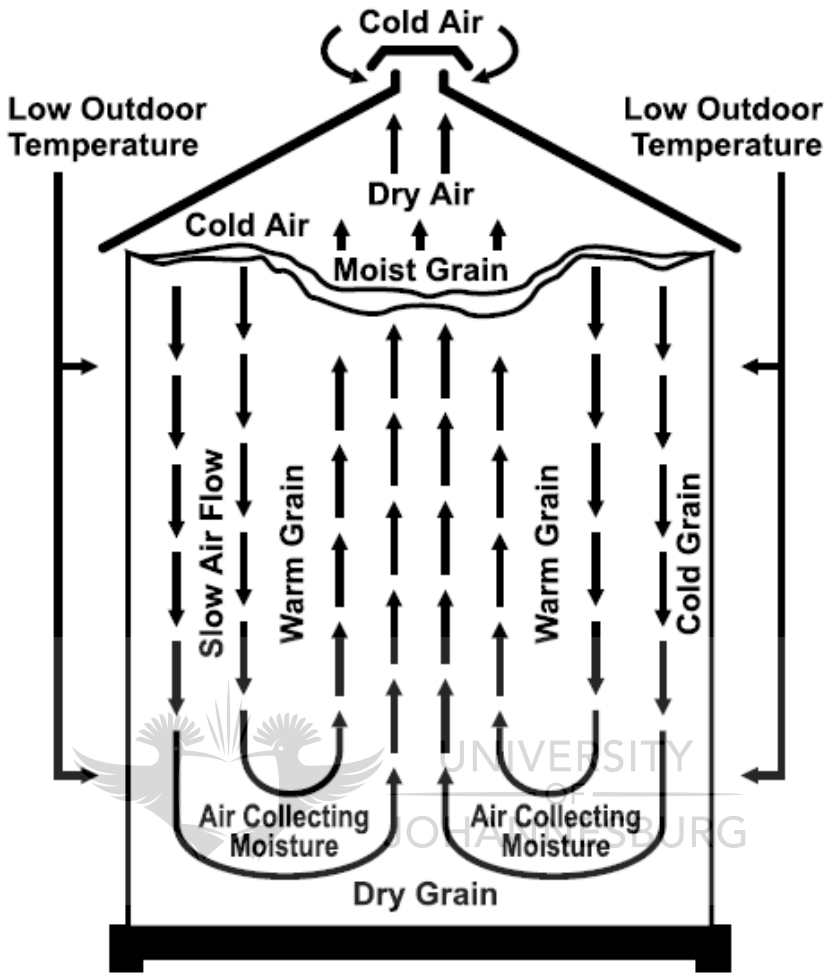


Figure 13: A diagram showing the moisture migration in an unventilated bin during winter and autumn (Canadian Agriculture, 2001).

Aeration units such as the one shown in Figure 14 are efficient in ensuring adequate ventilation. Air may be blown in or drawn out by means of a fan which is attached to a bin equipped with either perforated ducts or a perforated floor. When air is blown in the last part of the bulk to cool, will be the top layer. It is important to monitor the top of the bulk to determine whether the whole bulk is cooled or whether spoilage has begun. If the air is drawn out by reversing the airflow, the last section to cool is the bottom layer. In this case, spoilage may occur at the bottom of the bin where it is much more difficult to control or monitor.

Figure 14: A photograph of an aeration unit which is attached to a grain silo (Canadian Agriculture, 2001).

Spoilage moulds such as *Aspergillus* and *Penicillium* have been known to be found in stored or ensiled feeds (Fink-Gremmels, 2008). These so called storage flora tend to produce Microbial Volatile Organic Compounds (MVOC's). These compounds, are formed as a part of the moulds metabolism, and may be responsible for that typical mould scent encountered during visits to some of the farms storage sheds. MVOC's are implicated in Sick Building Syndrome (SBS) in humans (Fischer & Dott, 2003) and have been known to cause eye irritations, respiratory distress and asthma (Kim *et al*., 2007).

Mould infection may spread quickly during the transport of cereals and grains especially when transport extends over a few months as during the export of grains across the sea. This may be attributed to the warm temperatures within the shipping container coupled with high humidity at sea which creates conditions conducive to mould contamination. To minimise cost and for purpose of efficiency, shipping containers are usually stacked to maximum capacity so there is very little room for any ventilation, thus creating the ideal conditions for possible insect and mould infestation.

2.2.3 Feed Trade in South Africa

Dairy farming in South Africa does not yield huge profits. It is estimated that 70% of expenditures on a dairy farm is attributed to cattle feed. In the year 2010, due to increased global grain prices, the cost of feed ingredients rose sharply due, along with other farm requisites as seen in Figure 15. According to DAFF, farm debt was further increased in 2010 by 9.7% from R51.94 billion to R57.07 billion.

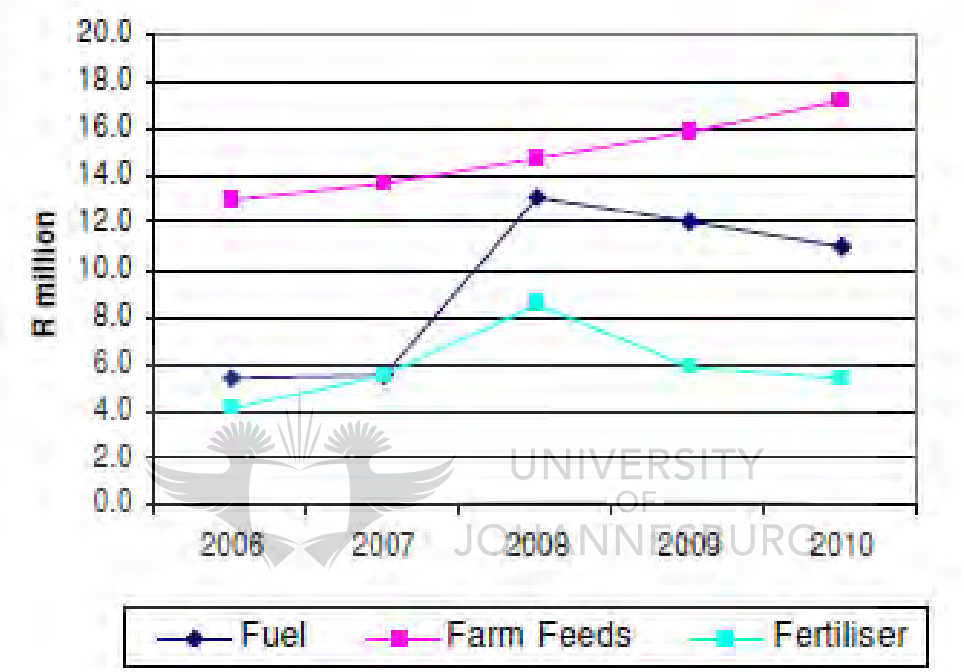


Figure 15: A graph showing the expenditure in selected farm items between 2006- 2010 (STATS SA, 2011).

In 2010, South Africa had produced a bumper maize crop, which has lead to some price relief on the current maize pricing (Reuters, 2010). In a report commissioned by the Department of Environmental Affairs (Zvomuya, 2011), it is estimated that even without the effects of climatic change, the price of maize is expected to increase by 63% whilst the price of soya bean and wheat can expect to incur increases of 72% and 39% respectively. The effects of climate change will result in a possible further price hikes for these commodities.

In an article titled ‘Facing dairy challenges’ it seems that sagging milk prices and a four-year drought, as well as higher fuel and feed prices have taken their toll on dairy farmers in the southern Cape. As a result, fewer farmers are investing in updating machinery and rather choose to diversify their businesses, to contend with the drop in milk producer prices. One farmer was quoted ‘the farm gate price farmers get for their milk is as low as it was a decade ago, but the cost of running the farm has increased’. The dairy farmers in the southern Cape contribute largely to about a quarter of the country’s milk production, but many have had to reduce their herds at great losses to afford fodder **(**Zvomuya, 2011).

The adverse weather conditions experienced in the past year and the steep electricity price tariffs passed in South Africa recently coupled with continued fuel price hikes, is placing a lot of pressure on all sectors of industry in South Africa. Furthermore, the effects of global warming will result in prices increases of most agricultural commodities and possible cereal and grain shortages. The use of agro industrial by-products (AIBP’s) are generally less fibrous, more concentrated, highly nutritious and cheaper than crop residues (Aguilera, 1989) and may be the only way forward for cattle feeding, provided the safety and quality of the products are confirmed prior to application.

2.2.4 Legislation

Various organisations such as the World Health Organisation (WHO), Codex Alimentarius Joint Expert Committee for Food Additives and Contaminants (JECFA) and the European Food Safety Authority (EFSA) are involved with the regular assessment of mycotoxins. Each country also has their own legislative authorities involved in prescribing the permitted maximum levels of mycotoxins in foodstuffs.

A limit of 5µg AFB_1 in animal feed destined for consumption by dairy cow and a limit of 20µg AFB_1 in feed for cattle are applied in the European Union countries (European Community, 2003).

A limit of 20µg AFB_1/kg is the prescribed level by the FDA for dairy feed and 100µg AFB_1/kg is applicable for cattle feed (FDA, 2009). These levels apply in United States of America and in certain countries in Africa and Latin America (FAO, 2004).

In South Africa, the Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Amendment R227 of 2009) stipulates that dairy feed compound with a moisture content of 120g/kg should be permitted to have only 5μgAFB_1/kg or 5 ppb, whilst feed for cattle should contain a maximum tolerable level of 50μg AFB_1 /kg.

A summary of the permitted levels of aflatoxin B_1 in dairy feed is shown in Table 5.

Table 5: A summary of the permitted levels of aflatoxin B_1 allowed in dairy cattle feed.

Regulatory Body	**Dairy cattle feed permitted level**
European Union	5μg AFB_1/kg
Food Drug Administration	20 μg AFB_1/kg
South Africa	5μg AFB_1/kg

2.2.5 The Stability and degradation of Aflatoxin B_1

The fact that aflatoxins has been implicated in certain negative effects on both human and animal health has prompted extensive research, to provide ways to eliminate the toxin or at the very least reduce the levels present in food and feed. The applications of various physical, chemical and microbiological methods have been explored to remove, destroy or at the very least limit the toxin to levels prescribed by the relevant regulatory bodies.

It is important that the method of choice to eliminate or reduce the toxin meet the following requirements (Jemmali, 1989; Ellis *et al*., 1991; Park & Liang, 1993):

- The method must not result in the formation of other toxins or leave any harmful residues that will compromise the safety of product
- The nutritional quality of the food is to remain unaffected
- The sensory properties of the food or feed should remain acceptable
- The process or ingredients employed should be economically feasible

- It must be capable of destroying the spores and mycelia of aflatoxigenic fungi

2.2.5.1 Physical Methods

- ***Sorting Trimming and Cleaning*:** It was reported that between 40-80% reduction in aflatoxins was achieved when mould damaged seeds, kernels or nuts were removed from the intact commodity (Park, 2002).

- ***Milling*:** It has been documented that milling does not contribute to any reduction in aflatoxins as the contamination may just be re-distributed. However during the wet milling of corn, mycotoxins have been found in the steep water, gluten fibre and germ whilst the starch seems to be relatively free of any mycotoxins (Bennett *et al.,* 1996a; Lauren & Ringose, 1997; Park 2002; Ryu *et al*., 2002).

- ***Brewing*:** It seems that aflatoxin B_1 is stable at boiling temperatures of the mash cooking step but seems to be sensitive during mash malting, wort boiling and final fermentation (Chu *et al.,* 1975).

- ***Heating*:** Aflatoxins possess high decomposition temperatures ranging between 237°C and 306°C. The degradation of aflatoxin by heat is influenced by the moisture content, pH and ionic strength of the food. Cotton seed meal with 30% moisture heated at 100°C for 1 hour, resulted in 74.8% reduction of aflatoxin B_1 and aflatoxin B_2, whereas heating a similar meal with 6.6% moisture resulted in only 32.7% of the toxins being destroyed (Mann *et al.,*1967). Raters & Matissek (2008) demonstrated that when soya protein, was exposed to heat treatment of 170°C, the effect of the process resulted in complete degradation of aflatoxin B_1.

- ***Extrusion*:** The reduction in the concentration of aflatoxins in the final product depends on several factors, including extruder temperature, screw speed, moisture content of the extrusion mixture and residence time in the extruder, with the extrusion temperature and residence times being the greatest contributors to aflatoxin reduction (Bullerman & Bianchini, 2007). The greatest reduction in mycotoxin concentrations in extruded products seems to occur at temperatures greater than 160°C.

Hameed (1994) demonstrated that extrusion alone reduced the aflatoxin concentration by between 50-80%. The addition of ammonia or bicarbonate resulted in the reduction of the overall aflatoxin concentration by approximately 95%. Saalia & Phillips (2011) demonstrated that extrusion conditions which reduced throughput in the single screw extruder, promoted greater aflatoxin reduction.

- ***High pressure / autoclave:*** It was found that rice which was contaminated with aflatoxin B_1 and cooked in a pressure cooker, resulted in between 78% and 88% reduction of the toxin (Park *et al*., 2005).

- ***Irradiation*****:** Research by Nkama & Muller (1988) indicated that when the moisture content of rice was increased from 14% to 18.7%, the rate of aflatoxin B_1 destruction increased between 40% and 63%.

- ***Ultraviolet light*****:** Aflatoxins are sensitive to UV radiation and it has been found that aflatoxin B_1 absorbs UV at 362nm which may lead to the formation of up to 12 photo-degradation products (Samarajeewa *et al.,* 1990).

- ***Gamma rays*****:** Following gamma irradiation, the toxicity of peanut meal contaminated by aflatoxin B_1 was reduced by between 75% and 100% at irradiation of 1kGy and 10kGy respectively (Temcharoen & Thilly, 1982).

- ***Solar irradiation*****:** Ultra-violet rays from sunlight play an important role in the photo-destruction of aflatoxin. However, peanut protein can bind aflatoxins and this complex is less susceptible to photo-degradation than the free toxin (Shantha & Murthy, 1980; 1981).

- ***Extraction*****:** Materials such as cottonseed, peanut and oilseed which are extracted with solvents are more suited for animal feed ingredients, as there is no formation of toxic by-products, or reduction in the protein quality. The use of this kind of processing is limiting though, due to its high cost and sophisticated disposal issues of the toxic extracts (Rustom, 1997).

- ***Adsorption*****:** Some adsorbents can bind to the aflatoxin and remove them from aqueous solutions. Certain inorganic binders such as Bentonite, which is a clay derived from volcanic origin, is a porous material onto which mycotoxins is adsorbed

and trapped by electric elementary charges. Doyle *et al*. (1982) found that Bentonite removed between 65% and 79% of aflatoxin M_1 from milk. It has also been reported that hydrated sodium calcium alumina-silicate (HSCAS : NovaSil clay) has a high affinity for aflatoxin B_1. It was found that more than 80% of the toxin was removed from feed and cereals, and *in vivo* studies further demonstrated that HSCAS prevents the mutagenicity and toxicity of aflatoxin B_1 (Phillips *et al*., 1988).

- ***Natural organic binders*:** Alfalfa and oat fibres have also been researched by Smith *et al*. (1980). The extracted cell wall of *Saccharomyces cerevisiae* was able to bind *in vitro* a large range of mycotoxins according to Devegowda *et al.* (1998). Yiannikouris *et al.* (2004a) demonstrated that ß-D-glucan fraction of the yeast cell wall is directly involved in the binding process of mycotoxins. Mendoza *et al.* (2009) reported the use of *Lactobacillus casei* strains to bind aflatoxins. It seems that organic binders are more effective against a large range of mycotoxins and are biodegradable. The clays however, tend to accumulate in the manure and then in field and may harm soils and pastures, and also these binders have not yet been approved for use in the European Union.

In summary, high temperature causes varying degrees of mycotoxin reduction but most mycotoxins are moderately stable in most feed processing systems. Aqueous cooking and steeping reduces mycotoxins concentration, whilst it has been reported that roasting and extrusion cooking at high temperatures appear to reduce mycotoxin contamination even further (Bullerman & Bianchini, 2007).

2.2.5.2 Chemical Methods

Chemicals such as acids, bases, oxidising agents, bisulphites and gases may react with aflatoxins and convert them to less toxic and mutagenic compounds. There has been some research on certain natural chemical compounds which have been found to be effective against the detoxification of aflatoxin B_1 in both animals and humans as reported by Tedesco *et al.* (2008).

The possible use of the following chemical compounds has been documented:

- *Coumarin* which is a natural antioxidant contained in plants, (Tulayakul *et al.,* 2007)
- *Lupeol*, which is isolated from a medicinal plant *Crataevanurvala* (Preetha *et al*., 2006)*;*
- *Aquilegia vulgaris* is a perennial herb indigenous to central and southern Europe and Asia (Jodynis-Liebert *et al.,* 2006)
- *Laurencia obtusa* and *Caulerpa prolifera* are marine extracts from the Red Sea in Egypt (Abdel-Wahhab *et al.,* 2006)

It has been reported that currently ammoniation and treatment with sodium bisulphite is used to treat maize, cottonseed and peanut meal destined for animal feeding (Rustom, 1997).

2.2.5.3 Microbiological Methods

Research by Park & Liang (1993) found that *Flavobacterium aurantiacum* as well as other acid producing moulds and bacteria are able to inactivate aflatoxins. They postulated that this was a result of acid production and subsequent conversion of aflatoxin B_1 to aflatoxin B_{2a} which is a 100 times less mutagenic than the parent toxin.

Although certain treatments may reduce the levels of specific mycotoxins, no single method has been developed which is actually equally effective against a wide variety of mycotoxins which may occur together in various commodities (Shapira & Paster, 2004). According to the Biomin® survey, "Mycotoxins inseparable from animal commodities and feed", the best way to deactivate mycotoxins is with a triple assault using adsorption, bio-transformation and bio-protection. This is due to the fact that grains and cereals seem to be affected by more than one mycotoxin and not all mycotoxins can be destroyed by one deactivating agent.

In summary, the application of any of the above discussed methods will result in a contributing cost factor which needs to be accounted for during the final pricing of the product. With the current electricity and fuel increases as well as expected future anticipated price increases for feed ingredients, any method of mycotoxin elimination or reduction will need to be cost effective and easily implemented, not requiring sophisticated technology or highly specialised training.

Government intervention is required to provide resources for the training and education of mycotoxins which should be exercised on a global scale, especially if one considers the negative health implications caused by these resistant toxins. This in turn will ensure that safe-to-consume commodities are available to consumers, thus securing future food supply chains which will be capable of feeding growing populations. The implementation of a universal, cost effective, rapid, user friendly and on–site aflatoxin detection system is necessary in the quality assurance chains of both food and feed.

2.3 COW'S MILK

Cow's milk has been considered to be an important element of the human diet for centuries. The neutral flavour of milk, its versatility in culinary preparations, as well as the perceived nutritional benefits makes this beverage an important component of the human diet.

There is currently an effort to promote an increase in the drinking of cow's milk as it has been found that there is a global decline of milk consumption. In the United Kingdom, the Dairy Council (2010) in conjuction with various health professionals have launched many campaigns to promote the drinking of milk.

The global dairy industry seems to be allocating more resources to the development of new innovative milk based beverages, as according to Innova Market Insights, there were more than 8,500 new product introductions (NPI) in the worldwide dairy market between January and November 2009 (McMurray, 2010). Some of these NPI's may actually be a part of a new segment of innovative dairy snacks, which seems to have established a firm place in the dairy industry and snack market in both Europe and the USA (Zenith International, 2011). These snacks are dairy based, smaller portion sized and packaged for mobile consumption. It is estimated that this new dairy snack market will reach over 260,000 tonnes by the year 2014 (Zenith International, 2011).

Cow's milk is however considered a human allergen and certain individuals may also be lactose intolerant. Therefore it is imperative that foods in South Africa which contain milk or

milk derived ingredients be labelled accordingly detailing the presence of a food allergen as prescribed by the South African Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972) Regulations: Government Gazette No.9236 R146 of 1 March 2010.

2.3.1 Composition and health benefits of bovine milk

Cow's milk contains approximately 87.7% water, 3.3% total protein, 4.9% carbohydrate (which is predominately lactose with trace amounts of monosaccharide and oligosaccharides) 3.4% milk fat (which contains approximately 65% saturated, 30% monounsaturated, and 5% polyunsaturated fatty acids) and 0.7% minerals (Neitz, 1995).

The composition is dependent upon the animal's nutrition, the season, the management of the dairy cow, genetics and the stage of lactation. The modification of the milk fatty acid (FA) composition, by altering the feed of ruminants through diet formulation and nutrition management of the dairy cow, has attracted the attention of researchers regarding the possibility of improving the human diet (McGuire & McGuire, 2000; Bauman *et al*., 2006).

Regular milk consumption also seems to stimulate protein synthesis in the human body after exercise. A recent study at McMasters University in Canada showed that females who consumed milk after resistance training exercise, gained lean muscle and strength whilst losing body fat (Dairy Council, 2010). It has also been reported that bioactive peptides from milk casein and whey proteins, seem to reduce blood pressure and the effects of osteoporosis.

While the consumption of saturated fat, mainly of C12:0, C14:0 and C16:0 FAs, is associated with cardiovascular diseases, the unsaturated FAs are regarded as beneficial for human health as it contains certain anti-carcinogenic compounds including conjugated linoleic acid (CLA), sphingomyelin, butyric acid and ether lipids (Parodi, 1999; 2004). So the regular consumption of dairy milk is important and beneficial in the diet of both young and old persons.

2.3.1.1 The effect of lucerne & forage related feeds on the quality and sensory attributes of milk

Lucerne

Research by Castillo *et al*. (2006) showed that the proportion of lucerne pasture in the cow's diet during the different seasons of the year, influenced the fatty acid profile of milk. It was reported that the conjugated linoleic acid (CLA) and trans 18:1 fatty acids were positively correlated with the proportion of lucerne pasture in spring diets, and higher when compared to diets based on conserved forages and concentrates during winter, when lower proportions of lucerne were generally present in diets of dairy cows.

Forage, Hay & Silage

Cow's milk is recognised to be an important source of fat soluble vitamins, especially vitamin A (retinol) and vitamin E (tocopherol) and the presence of these vitamins in milk is largely influenced by the type of feed consumed by the dairy cow.

Silage is considered to be a richer source of pro-vitamins A and tocopherols compared to hay, due to the higher losses of these compounds during grass drying and hay storage. Ensiled grasses and legume forages seem to contain higher levels of available tocopherols than maize silage. Red clover silage however, is reported to be a richer source of available tocopherols than maize silage (Kalač, 2011).

The carotenoids which are present in milk are important for human health as they are precursors of vitamin A and also contribute to the oxidative stability of milk. Higher lipid anti-oxidative power was noted in milk from cows fed grass silage, in comparison to those fed maize silage. Fresh forage seems to be the richest source of carotenoids, followed by silage and lastly hay. Maize silage is however a poorer source of carotenoids than the silage of other crops, especially if the maize was damaged by frost (Kalač, 2011).

Some volatile and non volatile minor components of milk can be used as tracers of the type of forage eaten by dairy cows. The volatiles are namely hydrocarbons, terpenes, alcohols, aldehydes and ketones, acids, esters and sulphur compounds. Some of these originate from plants and may be produced during wilting and drying and also during the fermentation of

silage. Milk can develop a bad aroma if poor quality silage is fed to the dairy cow as some sensory active compounds can be produced by the rumen (Kalač, 2011).

If cows are fed on a substantial diet of red clover silage or soya, it may result in considerable levels of estrogenic equol being found in milk. Equol is a phytoestrogen and is important in human health as it prevents cardiovascular diseases and certain hormone related diseases (Kalač, 2011). It is thus possible to develop milk with desired functional properties to promote human health, by managing the quality of feed. This further highlights the importance of ensuring that the dairy cow diet is free of mycotoxins and other toxic compounds, so that safe, good quality milk may be produced.

2.3.2 The dairy industry in South Africa

The coastal areas in South Africa are better suited for milk production because of mild temperatures, as well as good rainfall thus ensuring that good quality natural and planted pastures are grown. Milk production by province is shown in Figure 16 and it is clear that the western and eastern Cape Provinces, followed by KwaZulu Natal are the major producers.

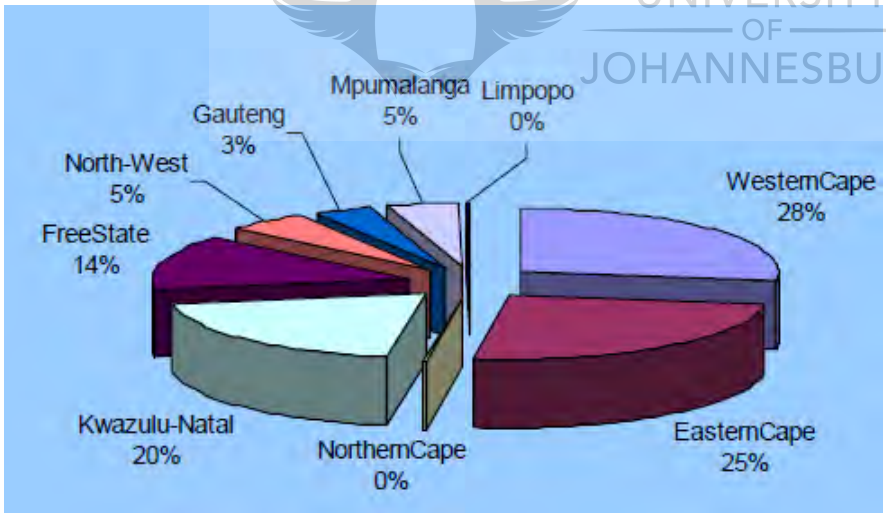


Figure 16: Pie Chart showing milk production per province in 2009 (DAFF, 2010)

The South African dairy industry is dominated by five major milk buyers and almost 50% of the dairy market is controlled by only two buyers (De Waal, 2008). According to a dairy

report by Scholtz & Grobler (2009) milk buyers are only involved in the secondary industry and not in the primary industry of milk production.

Most dairy products sold in the retail trade are standard products and niche markets are limited (Scholtz & Grobler, 2009). The South African dairy market is divided into the so called liquid market which has approximately 60% market share, comprising of pasteurized and ultra high temperature (UHT) liquid milk. The remaining 40% is the concentrated products market, which comprises hard and semi-soft cheeses.

The consumption of fresh milk may be considered a luxury in rural parts of South Africa probably due to lack of availability or high perceived cost. Or could it be that many homes in rural South Africa do not have electricity and thus no refrigerators? Nevertheless milk replacers which are milk based, such as Nestle® Condensed milk as well spray- dried milk powders, have a firm place on the shelves of formal and informal sectors of the South African retail markets.

2.3.2.1 Milk Production

Between January 2007 and August 2009, the number of milk producers in South Africa decreased by 11.3%. The annual production did however not decrease, thus possibly implying expansion of production for those remaining.

In 2008, 5% more milk was produced, in comparison to 2007. In 2009, production decreased by 4.1%. According to the Quarterly Review by Department of Agriculture, Fisheries and Forestry (DAFF 2010), the 2008 season ended with a production deficit of 37 million litres whilst a 46 million deficit was forecasted for 2010, as shown in Figure 17.

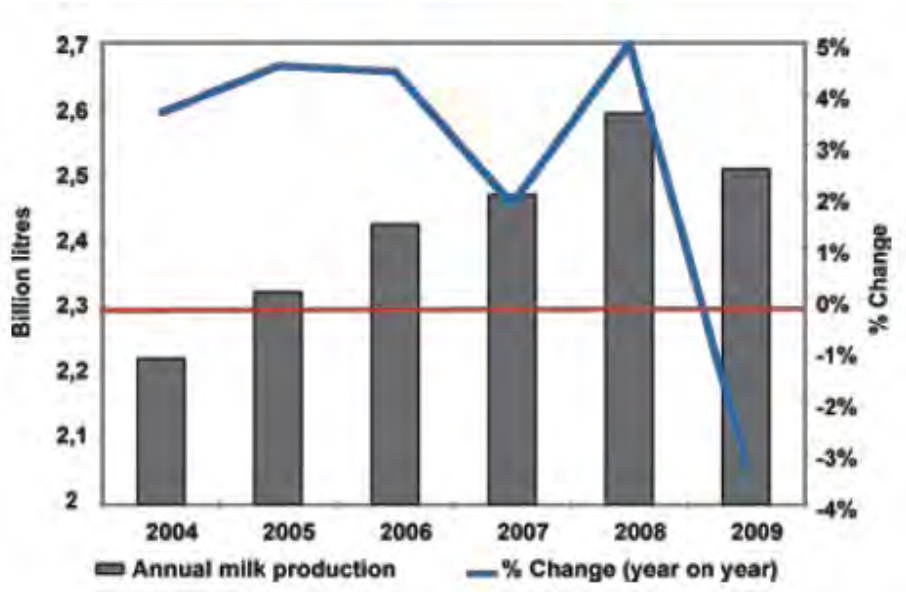


Figure 17: Graph showing annual milk production and percentage change during 2004-2009 (Lactodata, 2010).

This decline in production may possibly indicate that dairy farmers may be resorting to either cattle farming as beef sales yield better profits in comparison to milk, or diversifying their business completely to generate more income due to the poor profits of dairy farming.

The Dairy Mail (2011) reported that 2 058 million litres of milk was produced during the year 2010. Milk production was marginally higher during the first three months of 2011 compared to the corresponding period in 2010. However, during April 2011, the production was lower than April 2010 and this was due to adverse conditions caused by heavy summer rainfall (MPO, 2011).

Looking toward the future, a continual growth in the population will require a continual supply of milk. If South Africa cannot provide milk from the local industry, it will need to look at importing milk and milk derived products so as to satisfy the needs of the local consumer as well as possibly those of consumers in neighbouring countries for international trade purpose. This may result in South African consumers paying higher prices for dairy products especially if the exchange rate is not in the favour of the Rand.

2.3.2.2 Export and import of dairy products

Even though production deficits were recorded by DAFF, the dairy industry managed to generate foreign revenue, through the export of products to the value of R521 million in 2009, as shown in Figure 18. Export destinations were Zimbabwe, Zambia, Angola, Malawi, Mozambique, Tanzania, Korea, Japan and Tanzania.

Figure 18: Graph showing the export of milk and dairy products (DAFF, 2010).

Since 2005, South Africa has been a net importer of dairy products on a milk equivalent basis (NAMC, 2010). As shown in Figure 19, milk powders and cream make up almost half of dairy product imports. Internationally the main milk producing regions of the world are as follows: European Union 31%, New Zealand 30%, Australia 12%, USA 5% and the balance 22% are the cumulative total of the other regions in the world.

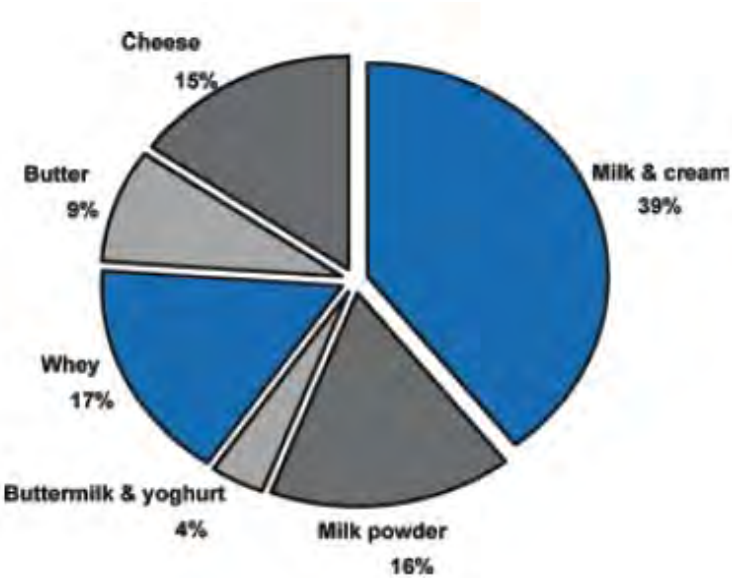


Figure 19: Pie chart showing the percentage composition of imports, mass base 2009 (Lactodata, 2010).

Some imported milk and dairy products available at major retailers in South Africa are priced more competitively in comparison to locally produced products. It may be that South Africa imports dairy products at a competitive price and is able to trade internationally, especially to neighbouring countries with these products, as it is clear that local production of milk cannot satisfy the trade requirements of both local and international markets.

2.3.2.3 Milk producer prices

There is a basic price per litre for milk as well as certain premiums or levies which apply, depending upon the quality of milk supplied (with specific reference to butterfat content, protein content, bacterial count and somatic cell count). This trade and technical information is generally dictated by The Milk Supply Agreement which is a legal binding contract between the milk producer and milk buyer.

The current opinion of many figures involved in the dairy industry is that the producer price of milk needs to be adjusted. Dairy farmers seem to be resorting to other businesses to provide income and this may result in a shortage of milk produced locally, thus affecting local food security as South Africa may become a net importer of dairy products.

Figure 20 indicates that milk price increased from 1999/00 to 2003/04 and declined from 2004/05 to 2006/07 before a sharp increase from 2007/8 to 2008/09. The period 2004/05 – 2005/06 experienced a decline in prices as processors were positioning themselves for an expected increase in production but this led to no growth in production which forced processors to review their pricing. Consumption of milk started at a low rate from 1999/00 to 2002/03 and increased continually from 2004/05 to 2007/08.

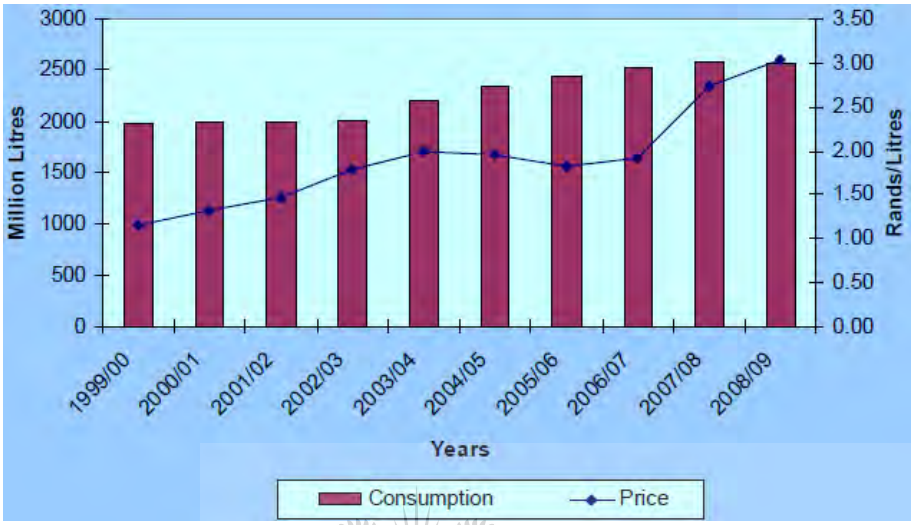


Figure 20: Graph showing milk consumption and milk producer price (DAFF, 2010)

The chairperson of the Milk Producers Organisation (MPO) Dèan Kleynhans was quoted in an article titled "Price dips could damage Dairy Industry" (I-Net Bridge, 2011) that milk producer prices are currently at least 7% lower than a year ago, while input prices have increased sharply since 2010. Internationally, producer prices have already been adjusted to align with global dairy prices. Import parity is currently in the region of R4, 00/ litre while the local average producer price is about R2, 80/litre. Producers with a substantial volume of good quality milk usually negotiate higher prices than the average producer. Although they have the advantage of countering transport and transaction costs they are still at the mercy of milk buyers, especially during periods of surplus raw milk supply (Scholtz & Grobler, 2009).

Information provided by Dr Koos Coetzee at the recent directors meeting of the MPO (MPO, 2010), indicated that farmers in the USA and European Union, receive various government subsidies which increases their total income per litre of milk. Coetzee further highlighted the

fact that South African farmers receive no such subsidies. In the EU, dairy farmers are paid subsidies for the use of certain inputs too (DAFF, 2010).

Quick, committed intervention is required by the key role players in the South African Dairy industry especially Government, to reduce the crippling effects experienced by dairy farmers due to adverse climatic conditions and spiralling electricity and feed expenditures. It is the responsibility of milk processors who are the price leaders in the dairy industry to strategically adjust milk prices in order to balance future requirements. If adjustments are not made timeously, 2012 may prove to be an even more difficult year for dairy farmers (Dairy Mail, 2011). Whilst it is expected that farmers produce good quality milk, it is necessary to ensure that fair pricing be negotiated for the milk producer prices paid to farmers.

2.3.3 The Role of aflatoxins in the health of the dairy cow

It is important to bear in mind that the dairy cow is exposed to a complex mixture of mycotoxins as they are exposed to a wide range of feed ingredients and natural forage, which have all been documented to contain various toxins. Continual exposure of low level mycotoxins in feed or a very concentrated exposure of mycotoxin contaminated feed can cause acute toxicity. However mycotoxins seem to be more likely to cause chronic problems of increased disease and decreased milk production (Fink-Gremmels, 2008).

Kiessling *et al.* (1984) proposed that the rumen micro-flora and micro-fauna are the first line of defence in ruminating animals, and are effective in degrading aflatoxins into less toxic metabolites. However, it may not always be the case as some aflatoxins may escape the degradation process. Furthermore the detoxifying capacity of the rumen micro flora is saturable and varies with changes in the diet or as a consequence of metabolic diseases such as rumen acidosis (Fink-Gremmels, 2008). Once the detoxifying capacity of the rumen becomes exhausted, the unchanged mycotoxins become absorbed via the duodenum.

Once ingested, mycotoxins exert their effect through four mechanisms according to Hagler & Whitlow (2007) and Fink- Gremmels (2008) which cause metabolic, hormonal, inflammatory and immunological reactions as documented below:

- Intake reduction or feed refusal

- Reduced nutrient absorption and impaired metabolism
- Alterations in the endocrine and exocrine systems
- Suppression of the immune system
- Reproductive system affected

In the rumen, aflatoxin B_1 is converted into aflatoxicol, but the percentage of aflatoxicol which remains is unknown, as an inter-conversion resulting again in the formation of the parent aflatoxin B_1 has only been described according to Nakazato *et al*. (1990). The aflatoxin B_1 that escapes rumen degradation is partially converted by hepatic metabolism into aflatoxin M_1 (Kuilman *et al*., 2000) which is excreted with dairy milk at a transfer rate that varies between 1% and 6% (EFSA, 2004b).

Aflatoxin intoxication in dairy cattle results in the formation of epoxides as in other animals species and is characterised by liver cell injury, a fatty liver syndrome, poor feed conversion and a significant reduction in milk yield (Colvin *et al.,* 1984; Cook *et al.,* 1986; Cockcroft, 1995). It has been reported that when cows were fed on an aflatoxin free diet, milk production had increased by over 25% (Guthrie, 1979). It was also reported by Guthrie (1979) that a decline in the reproductive efficiency of dairy cattle occurred when they consumed 120 ppb aflatoxins in a field situation.

As high yielding dairy cows are considered more sensitive to aflatoxins than fatting cattle (Appelbaum *et al.,* 1982) milk yield is affected. The low producer prices paid to farmers for milk and the high feed prices coupled with the loss of income from decreased animal production of milk are factors which can lead to greater poverty among farmers, reinforcing conditions conducive to poor human health (Miller & Marasas, 2002). It is thus important that certain legislative restraints be implemented to control the situation of aflatoxins contamination and this should be driven aggressively by government and the regulatory bodies concerned.

2.3.4 Legislation

There were approximately 60 countries which regulated the permitted levels of aflatoxin M_1 in milk during 2003, this is almost a threefold increase compared to 1995 (FAO, 2004).

The European Free Trade Association (EFTA) and some countries in Asia, Africa and Latin America apply a limit of 0.05µg AFM_1/kg in cow's milk and a maximum level of 0.025µg AFM_1/kg in infant formulae (European Community, 2006).

A limit of 0.5µg AFM_1/kg in milk is applied in the USA (FDA Compliance Policy Guide 7106.10) as well as in certain Asian and Latin America countries (FAO, 2004). The Joint FAO/WHO expert Committee on Food Additives and Contaminants concluded that this higher limit is sufficient to protect human health (FSA, 2001).

In South Africa, according to the Foodstuffs, Disinfectants and Cosmetics Act,1972 (Act No 54 of 1972. Regulations 1145 of October 2004) a prescribed level of 0.05µg AFM_1/ kg in cow's milk is permitted.

A summary of the permitted levels of aflatoxin M_1 is shown in Table 6.

Table 6: A summary of the permitted levels of aflatoxin M_1 allowed in dairy milk

Regulatory Body	**Dairy Milk**
European Union	0.05µg AFM_1/ kg
Food Drug Administration	0.5µg AFM_1 / kg
South Africa	0.05µg AFM_1 / kg

Based on a thumb rule of 1.7% carry over rate, cows consuming diets containing 30µg aflatoxin / kilogram dairy feed can produce milk containing aflatoxin residues above the FDA action level of 0.5µg AFM_1/ kilogram in milk. Thus in Europe, an illegal milk residue can occur, if there is more than 3µg of AFB_1 / kilogram feed (Hagler & Whitlow, 2007). As South Africa legislation mirrors European legislation, the same figures apply.

2.3.5 Stability of aflatoxin M_1 in milk

Dairy milk is a natural secretion and which needs to undergo processes such as homogenisation and pasteurisation in order to be stable and safe to consume. Although the

presence of aflatoxin M_1 extends to other dairy products such as cheese and yogurt, only the effects of aflatoxin M_1 stability in whole milk will be discussed.

2.3.5.1 Physical Methods

- ***Effects of freeze drying***

There was no loss in the concentration of aflatoxin M_1 in naturally contaminated freeze dried skim milk stored for four months at 22°C, according to research by Wiseman & Marth (1983c). However, Kiermeier & Mashaley (1977) found that storing the product at 18°C for 15 days caused a loss of approximately 9.6% of aflatoxin M_1 which was originally present in milk.

- ***Ultraviolet energy***

Studies of Yousef & Marth (1985a; 1986) reported that UV irradiation at low temperatures degraded aflatoxin M_1 in milk. The sensory properties of milk are also negatively affected when exposed to irradiation, according to findings by Li & Bradley (1969).

- ***Effects of refrigeration & freezing***

The outcomes of the research performed by various authors seem to present variable results of the stability and degradation of aflatoxin M_1 in milk when frozen or refrigerated.

Kiermeier & Mashaley (1977) indicated that storage of naturally contaminated milk at 4°C for a period of one to three days caused a reduction of between 11 % and 25% of aflatoxin M_1. Stoloff *et al. (*1975) however, found that all of aflatoxin M_1 was recovered from artificially contaminated raw milk, which was stored at 4°C for up to 17 days.

Researchers McKinney *et al.* (1973) reported that aflatoxin M_1 in naturally contaminated raw milk, decreased steadily whilst being frozen at -18°C, and after a period of 120 days at frozen storage, 87% of aflatoxin M_1 originally present in the milk was lost. Authors Kiermeier & Mashaley (1977) reported that artificially contaminated milk which was frozen and stored for 6 days resulted in the concentration of aflatoxin M_1 being lowered by almost 32%. When the same researchers used naturally contaminated milk, the comparable loss of aflatoxin M_1 was recorded to be between 2% and 8%. Stoloff *et al.* (1975) recorded that the

concentration of aflatoxin M_1 present in the milk analysed, did not decrease during first 68 days of frozen storage.

Owing to the variability of the information included above it seems that no final conclusion may be drawn for now. However, it seems that frozen storage for a few months does not seem to affect the presence of aflatoxin M_1 in whole milk.

- ***Effects of heat***

In order for milk to be safe for consumption it is generally heat processed using pasteurisation or sterilisation which is used to kill certain bacteria and thus increase shelf life. The findings of various authors are listed in Table 7 with specific reference to the effects of such application processes.

Table 7: The effects of various heat processes on the aflatoxin M_1 concentrations in natural and artificially contaminated milk

Nature of contamination in milk	**Type of heat treatment applied**	**Changes in AFM_1 Concentration observed**	**Reference**
Natural	Pasteurised	No change	Allcroft & Carnaghan (1962)
Natural	• Pasteurisation • Sterilisation • Evaporation • Roller Drying • Spray drying	32-64% loss 81% loss 64% loss 61-76% loss 86% loss	Purchase *et al.*(1972) Diaz *et al.* (1995)
Artificial	Pasteurisation at 63°C /30min	No change	Stoloff *et al.* (1975)
Natural	Pasteurisation at 77°C /16sec	No change	Stoloff *et al.* (1975)
Natural	Pasteurisation	No change	Van Egmond *et al.* (1977)
Natural	Sterilisation	No change	Van Egmond *et al.* (1977)

- ***Adsorption***

Bentonite, Vermiculite and other clays have been evaluated for its efficiency in the removal of the toxin from buffer solutions (Masimango *et al*., 1978). The use of a 5% Bentonite solution at 25°C for 60 minutes in milk, was found to adsorb 89% of aflatoxin M_1 originally present in the milk analysed (Appelbaum & Marth, 1982c).

It is however important that the type of particulate used does not remove any nutritional compounds from milk and comply with the other requirements as documented by Jemmali, (1989), Ellis *et al.* (1991) & Park & Liang (1993).

2.3.5.2 Chemical Methods

Certain findings have indicated that when hydrogen peroxide was combined with riboflavin or lactoperoxidase, it was found that between 47- and 52% of aflatoxin M_1 originally present in the milk was degraded (Appelbaum & Marth, 1982b).

Appelbaum & Marth, (1982c) also found that if milk was naturally contaminated with aflatoxin M_1 and treated with 0.4% of sodium bisulphite at 25°C for 5 hours, a reduction in 45% of the total aflatoxin M_1 originally present in the milk, was observed.

In summary, Figure 21 simulates the feed to food transfer of mycotoxins in bovine milk. As noted in the Figure, bovines which are exposed to feed contaminated with aflatoxin B_1 results in the formation of aflatoxin M_1 residues in milk, at a carry-over rate dependent on a range of factors including the level of contamination of aflatoxin B_1 in the feed consumed. The dilution or concentration of milk occurs in tankers when milk is usually combined from a variety of farms. The milk is then processed during homogenisation and pasteurisation and the presence of the aflatoxin M_1 compound remains unaffected. The frequency of milk consumption coupled with the concentration of aflatoxin M_1 residues which are present in the milk, together with each individual's different physiological conditions, will determine the effects on human health. Risk management with Government's involvement in all areas concerned, from the employment of good agricultural practices through to the implementation of documented legislation, remains the only solution to effectively control and manage the situation of mycotoxin contamination.

Concentration of cereals at harvest

Bovine exposure, feed inclusion rate

Carry-over rate to milk

Concentration in milk

Human exposure

Exposure assessment

Risk management

Figure 21: Simulation of feed to food transfer of mycotoxins in bovine milk (Coffey *et al*., 2009)

2.4 METHODS AVAILABLE TO ANALYSE FOR AFLATOXIN B_1 AND AFLATOXIN M_1

A review of the occurrence of aflatoxins in food products has shown that aflatoxins occur frequently in food products at levels that are of a substantial concern (Pittet, 1998). Thus systematic and comprehensive monitoring of aflatoxins for consumer protection is a big challenge especially if looking toward the future, since an increased production of food products is estimated. For example, the world peanut production doubled within the last 20 years to 29 million tonnes (FAO, 2000).

The method of analysis chosen, should be characterised by appropriate criteria namely accuracy, applicability (matrix and concentration range), limit of detection, limit of determination, precision, repeatability, reproducibility, recovery, selectivity, linearity, sensitivity and measurement uncertainty (IFST, 2009). In addition to these required performance criteria, economical, fast and efficient procedures coupled with the ability for automation are highly desired features (van Rhijn *et al.,* 1992).

There are a wide range of methods available to analytical scientists ranging from newly described *multi toxin liquid chromatography tandem mass spectrophotometer(LCMS)* methods to rapid methods based on immunological principles (Shephard, 2009).

Methods routinely used for mycotoxin analysis are based on thin layer chromatography (TLC), high performance liquid chromatography (HPLC) or enzyme linked immunoassay (ELISA). Due to the demand for reliable and comparable methods, performance requirements have been established at national and international level for implementation as official methods, by bodies such as the European Committee for Standardisation (CEN) or the Association of Official Analytical Chemists International (AOAC) (Anklam *et al.,* 2002).

According to an AOAC report (2008) topic advisor Hans van Egmond documented that during 2006 and 2007, there were many scientific reports related to studies of aflatoxin M_1 levels in milk from various countries.

Analysis of the methodologies used for surveys of aflatoxin in milk showed the following:

- Enzyme linked immunoassay 39%

- Liquid Chromatography 31%
- Thin Layer Chromatography 18%
- Ridascreen® Immunoassay 4%
- Fluorimetry 4%
- Lateral flow immunoassay 4%

2.4.1 Extraction Methods Available

The official methods as validated by AOAC International rely on the use of Immuno-affinity columns (IACs). The AOAC method 2000.08 which involves the analysis of aflatoxin M_1 in milk using IAC by liquid chromatography (with Final Action 2004) is the newest official method. (AOAC, 2008).

Solid phase extraction (SPE) to provide extracts for analysis by high performance liquid chromatography (HPLC) is also used as documented by Manetta *et al*., (2005). Analysis by high performance liquid chromatography generally follows the clean-up procedures by IACs and SPE, and involves the separation of analogues with detections based on the natural fluorescence of the aflatoxin compounds. Recently, the use of various derivatisation methods have been employed to assist with the detection of the fluorescing compounds.

2.4.1.1 Immuno-affinity columns

Immuno-affinity columns (IACs) for clean-up steps prior to HPLC or TLC (Scott & Trucksess, 1997) have become increasingly popular in recent years as they offer high selectivity. Immuno affinity columns are easy to use and their application for purification of samples which are contaminated with several mycotoxins is already well tested. As mycotoxins are low weight molecules, they are only immunogenic if they are bound to a protein carrier. If this problem is overcome, antibodies can be produced and bound to an agarose, sepharose or dextran carrier. The analyte molecules which are the mycotoxins are bound selectively to the antibodies after a preconditioning step, and subsequent to a washing step the toxin can be eluted with a solvent causing antibody denaturation. Interfering substances do not interact and the column is therefore washed to remove the matrix (European Mycotoxin Awareness Network, 2009).

A popular brand of IAC's available in South Africa is Vicam® as shown in Figure 22 which is based on monoclonal affinity chromatography. These columns are composed of monoclonal antibodies specific for aflatoxin M_1, which are immobilised on sepharose and packed into small cartridges. A milk sample is loaded on the affinity gel column and after washing to remove impurities, the aflatoxin M_1 compound is eluted from the column with an organic solvent such as pure acetonitrile. As the aflatoxin M_1 antibody specifically recognises the aflatoxin M_1 analyte, the column should not adsorb any other contaminants (Vicam®, 1999).

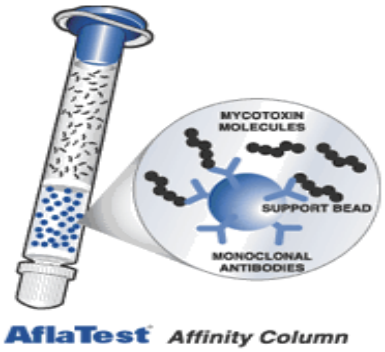


Figure 22: Vicam® Immuno-affinity column (Vicam®, 1999)

The IACs have been commercialised into systems in which the IAC eluate is used directly for aflatoxins quantification based on derivatisation and the subsequent reading of florescence using a commercial fluorometer (Shephard, 2009). These columns provide a combination of extraction and clean up stages for final confirmation by HPLC. Excellent performance and results as low as 0.1ng/g have been demonstrated in a collaborative trial carried out at an international level (Stroka *et al*., 2001).

This product and procedure is easy to use, rapid, accurate, toxin specific and ideally suited for high throughput testing (Gilbert 1984; Cahill *et al.,* 1999*)*. As reported by Shephard (2009), the use of IACs also results in the economic use of organic solvents. Larger aliquots (up to 1ml) may be injected into the HPLC, provided the elution strength of the injection solvent matches or is less than that of the HPLC mobile phase (Stroka *et al.,* 2000). Large injections of this nature clearly benefit the detection and quantification limits of the analysis.

The success of IACs as a purification medium for a single mycotoxin class has resulted in the development and commercialisation of multi-mycotoxin IACs, which contain antibodies specific to more than one mycotoxin.

2.4.1.2 Solid Phase extraction

Solid phase extraction (SPE) is an increasingly useful sample preparation technique which is quick and easy to perform as the process may be automated, resulting in a reduction of solvent use and analysis time. Various SPE cartridges are available in a wide variety of chemistries, adsorbents, and sizes suited to most products ranging from food to veterinary chemistry.

Solid phase extraction products are excellent for sample extraction, concentration and clean-up processes which are often used to prepare liquid samples and extract semi-volatile or non-volatile analytes. These cartridges may also be used with solids which have been pre-extracted into solvents. Bovine milk generally is processed using *reversed phase* or *ion exchange* solid phase extraction conditions. These processes will now be described in more detail.

Reversed Phase Solid Phase Extraction

Reversed phase solid phase extraction involves a polar (usually aqueous) or moderately polar sample matrix (mobile phase) and a non polar stationary phase. The analyte of interest is typically mid- to non polar. Materials such as the alkyl- or aryl-bonded silicas of the LC-18 cartridge are in the reversed phase. In the columns the hydrophilic silanol groups at the surface of the raw silica packing has been chemically modified with hydrophobic alkyl or aryl functional groups, through a reaction with the corresponding silanes. Retention of organic analytes from polar solutions such as water on to these SPE materials is primarily due to the attractive forces between the carbon-hydrogen bonds in the analyte, and the functional groups on the silica surface. These attractive forces are commonly called van der Waals or dispersion forces. To elute an adsorbed compound from a reversed phase SPE tube, a non-polar solvent must be used to disrupt the forces that bind the compound to the packing (Supelco, 1998).

Ion Exchange Solid Phase Extraction

Ion exchange solid phase extraction can be used for compounds which are charged when in a solution (usually aqueous, but sometimes organic). Anionic compounds can be isolated on LC-SAX bonded silica cartridges. The primary retention mechanism of the compound is due to the electrostatic attraction of the charged functional group on the compound, to the charged group that is bonded to the silica surface. In order for a compound to be retained by ion exchange from an aqueous solution the pH of the sample matrix must be the same, at which both the compound of interest and the functional group on the bonded silica are charged. Also there should be no other product of the same charge, as the compound in the matrix, as it may interfere with the adsorption of the compound of interest. A solution having a pH that neutralizes either the compound's functional group, or the functional group on the sorbent surface, is used to elute the compound of interest. When one of these functional groups is neutralized, the electrostatic force that binds the two together is disrupted and the compound is eluted. Alternatively a solution which has a high ionic strength or which contains an ionic fraction that could displace the adsorbed compound, must be used to elute the compound (Supelco, 1998).

Figure 23: A photograph showing the various types of solid phase extraction cartridges available (Wikipedia, 2011).

2.4.1.3 *Enzyme Linked Immunoassay (ELISA)*

Enzyme linked immunoassay's are well defined in their applicability, analytical range and validation criteria and merely require use of a laboratory to ensure the ability to perform the assay satisfactorily and within the constraints of the matrix and analytical range as specified by the manufacturer (Shephard, 2009).

In a competitive enzyme immunoassay for the quantitative analysis of aflatoxin M_1, the basis of the test is the antigen–antibody reaction. A polystyrene micro titre plate as shown in Figure 24, is a 96 well plate which allows for high sample throughput and low volume measurement, resulting in time and cost saving.

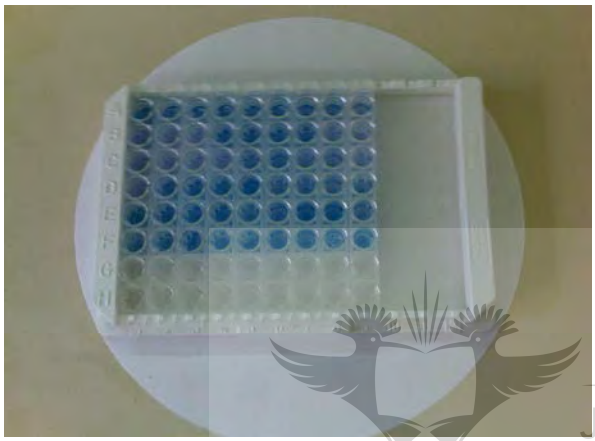


Figure 24: A photograph showing a micro-titre plate

The wells in the micro titre strips are coated with specific antibodies against aflatoxin M_1. After a washing step the conjugate is added. Free aflatoxin M_1 and aflatoxin-M_1-conjugate compete for the aflatoxin M_1 binding sites as shown in Figure 25. Any unbound enzyme conjugate is removed during the second washing step. Traditionally ELISA's typically involve chromogenic reporters and substrates that produce some kind of observable color change to indicate the presence of antigen or analyte. A stop solution is then added and the colour is measured with a spectrophotometer (Salimetrics, 2008).

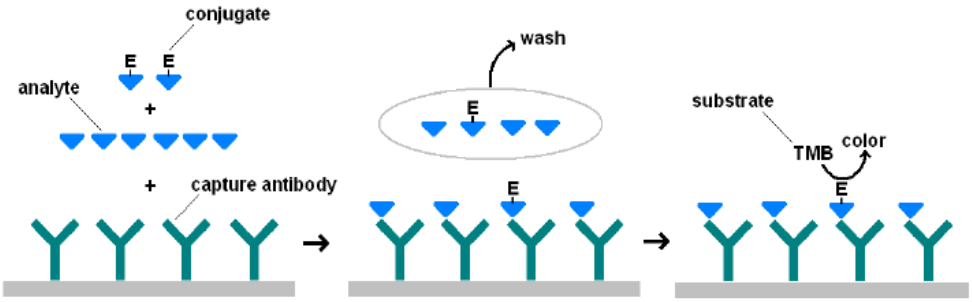


Figure 25: An illustration showing a competitive assay where there is a high concentration of analyte (Salimetrics, 2008)

2.4.2 Chromatography

Chromatography is the separation of two or more compounds or ions due to the distribution between two phases, one which is moving and the other which is stationary. These two phases can be solid-liquid, liquid-liquid or gas-liquid.

Although there are many different variations of chromatography, the principles are essentially the same. High performance liquid chromatography (HPLC) and thin layer liquid chromatography (TLC) will now be discussed.

2.4.2.1 High performance liquid chromatography (HPLC)

The separation of aflatoxins has been performed for many years using HPLC (Sydenham & Shephard, 1996) and used by many authors such as, Elgerbi *et al.* (2004) Nachtmann *et al.* (2007) and van Egmond & Dragacci, (2001) during their studies of aflatoxin determinations. Although both normal and reversed columns have been used during HPLC analysis, the vast majority of separations are performed on reversed – phase systems with mobile phases composed of water, methanol and acetonitrile mixtures.

Chromatographic performance has improved with column technology, particularly with the reduced size of the column packing material (Shephard, 2009). The introduction of packing materials with particle size 1.7µm in Ultra Performance Liquid Chromatography has brought total run times down to about 3 minutes, using a mobile phase of methanol-water with 0.1% formic acid and mass spectrometric detection (Ventura *et al.*, 2006).

The HPLC detection of aflatoxin analogues is achieved through the application of fluorescence detection. However various analogues exhibit solvent dependent quenching in HPLC solvent systems. In the aqueous mixtures used for reverse phase chromatography, the fluorescence of aflatoxin B_1 is significantly quenched (Kok, 1994). This is generally overcome by derivatisation of this analogue at the reactive 8,9–double bond of the dihydrofuran moiety.

Derivatisation – The Coring® Cell

To enhance their natural fluorescence, aflatoxins require derivatisation with a suitable fluorophore so that they may be detectable at low ppb levels. The Coring Cell formerly the CoBrA (or Kobra) cell is an electrochemical cell which generates a reactive form of bromine, a derivatising agent from potassium bromide present in the mobile phase. The derivatisation of aflatoxin M_1 occurs within four seconds at ambient temperatures. This technology provides rapid, reliable and easily maintained post column derivatisation (Dr. Weber consulting, 2005)

Manetta *et al.* (2005) found that HPLC coupled with post column derivatisation improves the analysis of aflatoxin M_1, and also the method was simple and quick to automate and the reproducibility was improved.

An added advantage of automated post column derivatisation is that the derivatisation can be switched off and thus the decrease in peak heights of the peaks representing aflatoxin B_1 may be observed as a confirmation of their presence (Shephard, 2009).

2.4.2.2 Thin Layer Chromatography

Thin layer chromatography or TLC is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or a combination of solvents. TLC is a quick, inexpensive micro-scale technique that can be used for the following applications:

- To determine the number of components in a mixture
- To verify a substance's identity
- To monitor the progress of a reaction
- To determine appropriate conditions for column chromatography
- To analyze the fractions obtained from column chromatography

In thin layer chromatography a solid phase referred to as the **adsorbent**, is coated onto a solid support as a thin layer (about 0.25 mm thick). The stationary phase is typically alumina (AlO_3 **.**H_2O)n or silica gel (SiO_2**.**H_2O)n. The surface of the silica gel and the aluminium atoms on the surface of the alumina is very polar and due to the presence of the -OH groups, they can form hydrogen bonds with suitable compounds as well as van der Waals dispersion forces and dipole-dipole attractions.

The mixture (say A plus B) to be separated is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom. A solvent or mixture of solvents called the **eluant**, is allowed to flow up the plate through capillary action. At all times the solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. Any one molecule will spend part of the time, sitting still on the adsorbent with the remainder moving up the plate with the solvent. A substance which is strongly adsorbed (say, A) will have a greater fraction of its molecules adsorbed at any one time, and thus any one molecule of A will spend more time sitting still and less time moving. In contrast, a weaker adsorbed substance (B) will have a smaller fraction of its molecules adsorbed at any one time, and hence any one molecule of B will spend less time sitting and more time moving. Thus, the more weakly a substance is adsorbed, the further up the plate it will move. The more strongly a substance is adsorbed, the closer it will stay near the origin (University of Wisconsin, 2004; Penn State, *date accessed 25 July 2011*).

As the distance travelled by a substance relative to the distance travelled by the solvent front depends upon the molecular structure of the substance, TLC can be used to identify substances as well as to separate them. The relationship between the distance travelled by the solvent front and the substance is usually expressed as the Rf value:

$$\textbf{Rf value} = \frac{\text{distance travelled by substance}}{\text{distance travelled by solvent front}}$$

The Rf values are strongly dependent upon the nature of the adsorbent and solvent.

2.4.3 Mass Spectrophotometer

The coupling of HPLC to mass spectrophotometry via atmospheric pressure ionisation techniques such as electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) has resulted in the formation of new methods for the analysis for aflatoxins. The advantages of LC-MS OR LC-MS/MS lie in improved detection limits, the confirmation provided by on line mass spectral fragmentation patterns and the ability to filter out by mass any impurities that interfere in spectro-photometric detectors (Shephard, 2009).

2.4.4 Portable Methods – Lateral Flow Devices

Whilst chromatographic methods are specific and sensitive, they are also time consuming, laborious and multi-complex. In addition these technologies are unaffordable to farmers and some laboratories in developing countries. Over the past few years, the focus has been on the development of a point-of-care (POC) testing units which are highly accurate, rapid and analytical (Ngom *et al.,* 2010). Rapid disposable membrane based assay tests have been developed in multiple formats such as test strips, dip sticks and flow through tests. Immuno - graphic assay (ICA), test strips or lateral flow assays (LFAs) or lateral flow devices (LFDs) are the common names used when referring to such testing units.

Lateral flow assays are pre-fabricated strips of a carrier material containing dry reagents which are activated when a fluid sample is applied. They are used mostly for diagnostic purpose such as to determine pregnancy, to test for failure of internal organs, to test for the presence of infection or contamination with specific pathogens including bio-warfare agents as well as to test for the presence of toxic compounds in food, feed or the environment or for the testing of illicit drugs (Posthuma-Trumpie *et al.,* 2009).

Dip sticks work like an ELISA, with carrier membranes instead of micro titer plates but requires a time of between 30 min to 3 hours to obtain the test results. These test strips however, may be completed quickly within 5 to 10 minutes. Flow-through membrane based immunoassays are comparable with lateral-flow test strips in rapidity and ease of use. But these are qualitative or semi-quantitative tests and the interpretation of results may be difficult especially when the test result is close to the cut-off level (Zheng *et al.,* 2006). Although dip sticks and flow-through immunoassays have been developed for mycotoxins,

they are not as commercially successful as test strips (Krska & Molinelli, 2009). This review will therefore focus on membrane-based test strips, also called lateral-flow devices (LFDs)

Principle of Lateral Flow Devices (LFDs)

The work reported by Krska & Molinelli (2009) will now be used to demonstrate the workings of the lateral flow test (LFT) devices.

Lateral flow devices are based on a test format which involves the flow of a sample along an analytical nitrocellulose membrane, which occurs due to capillary forces. This methodology results in fast and easy to handle immunoassays which can be either qualitative (with a defined cut-off level) or quantitative (when used with a photometric reader). Due to their ready availability, ease of production and ease of conjugate formation with antibodies, colloidal gold is used in most test strips developed for mycotoxins.

The test strip components such as sample pad, conjugate pad, analytical nitrocellulose membrane, and absorbent pad are immobilized on a plastic backing card for better handling. The pads which are usually comprised of cellulose or glass fibre material will overlap with the analytical membrane by a few millimetres, in order to ensure that the sample will flow along the strip. The absorbent pad at the end of the strip allows the absorption of excess liquid, thus ensuring that no backflow occurs on to the membrane, as demonstrated in Figure 26

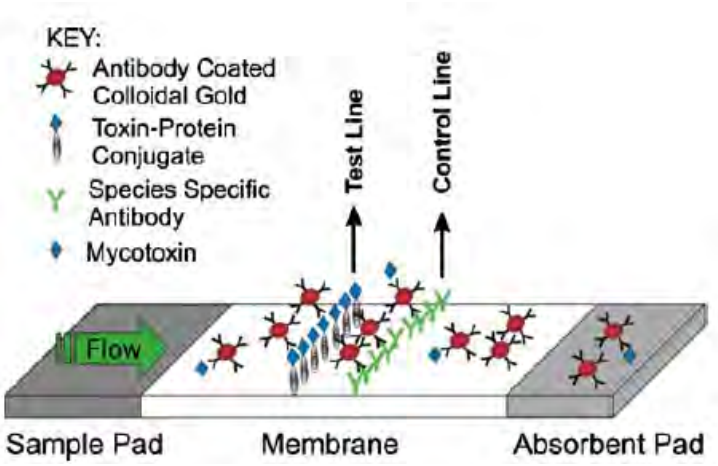


Figure 26: A schematic representation of a lateral flow test with competitive format (Krska & Molinelli, 2009).

The signal reagent may either be mixed with the sample extract in a micro-well or previously immobilized on the strip in the conjugate pad. The test strip is then inserted into the well or the sample extract is applied directly to the strip (signal reagent previously immobilized) and the mixed content then migrates on to the nitrocellulose membrane, which contains a test zone and a control zone. In a competitive assay as shown in Figure 25, the mycotoxin–protein conjugate coated on the test zone captures the free antibody–colloidal gold particle complex, allowing the colour particles to concentrate and form a visible line. The intensity of the test line is dependent on the analyte concentration and may be measured with a photometric reflectance strip reader. A species-specific antibody coated on the control zone will capture loaded and unloaded antibody–colloidal gold particle complex. One line will therefore always be visible in the control zone regardless of the presence of a target analyte, confirming correct test development (Krska & Molinelli, 2009).

2.4.4.1 *Charm® Rosa® System*

The Charm® Rosa® Safe Level Aflatoxin M_1 Quantitative (SLAFMQ) test is an example of a colloidal gold lateral flow immunoassay lateral flow test strip. Colloidal gold particles with a diameter of approximately 40 nm are prepared by controlled reduction of tetra-chloroauric (III) acid tri-hydrate with citric acid tri-sodium salt. Because of surface plasmon resonance effects, the 40 nm colloidal gold particles have a deep red colour which is exploited for test strip signalling.

In the Charm® system, the aflatoxin M_1 in a milk sample competes with the antibody gold beads for binding to 2 test lines. The remaining unbound binder forms on the control line. The test and control lines are compared with a reflectance reader. A reading in parts per trillion (ppt) concentration is then determined with an algorithm (AOAC, 2008). A negative interpretation with a reading of 400 ppt and a positive interpretation with a reading >400 ppt was designed to detect 500 ppt, the USA and CODEX violative level at 90% positive with 95% confidence (Salter *et al.,* 2006).

The Charm® system is shown in Figure 27. The basic cost (excluding the cost of balances, pipettes etc) to implement the Charm® System as at June 2011* was as follows**:**

- Rosa® Reader M for mycotoxins: *ZAR 33,564.43 exc. VAT*
- Rosa® Incubator for mycotoxins 4 place, 10 min, 45 degrees with display: *ZAR 5,274.24 exc. VAT*
- Thermal printing system / 7.5V: *ZAR 4,649.05 exc. VAT*
- Mycosoft software program: *ZAR 2,240.91 exc. VAT*
- Latflow / SL /Aflatoxin 3 LINE/ Milk / 100 Kit *ZAR 9,534.96 exc. VAT*

*****Prices supplied by local distributor, Anatech Analytical Technology

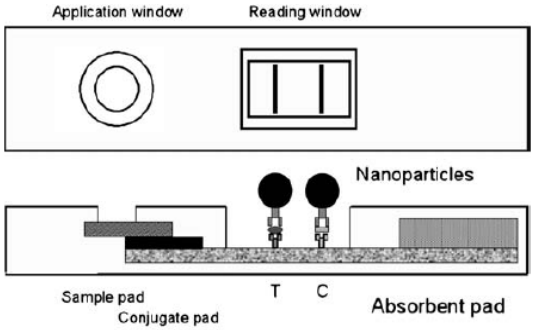
Charm® Test Strips & Standards | Charm® Incubator | Charm® Reader

Figure 27: Components of the Charm® System (Charm Sciences Inc, 2009).

2.4.4.2 Idexx Snap® System

This is another type of quantitative lateral flow test strip designed to test for aflatoxin M_1 in milk, based on the sandwich format as seen in Figure 28a. The methodology which is described in figure 28a is that the nano particle labelled anti-analyte antibody 1, is dried at the conjugate release pad. The anti-analyte antibody 2 is sprayed at the test line (T). Anti-species immunoglobulin G is sprayed at the control line (C). The sample then flows from sample pad to the conjugate pad and into membrane. The strips are mounted within a plastic unit for ease of handling as seen in Figure 28b (Posthuma-Trumpie *et al.,* 2009).

Top view (28a)



Side View

(28b)

Figure 28a: Lateral flow immunoassay in sandwich format. (Posthuma-Trumpie *et al*., 2009); Figure 28b: Lateral flow immunoassay housed in plastic casing as demonstrated in SNAP® device (Idexx, 2008).

The Snap® system components as shown in Figure 29 which is required for the screening of aflatoxin M_1 in milk is outlined below together with the pricing:

- Snapshot Reader: *ZAR 32,264.00 exc. VAT*
- Snap® Portable Heater (24V or 220V): *ZAR 2,869.00 exc. VAT*
- Snap® Aflatoxin M_1 Test includes 20 Snap Devices: *ZAR 1,991.00 exc. VAT*

*Prices supplied by local distributor, DEHTEQ (Pty) Ltd. June 2011

SNAP ® Portable Heater

SNAPSHOT® Reader

Fig 29: Components of the Snap® system (Idexx, 2008)

2.4.4.3 Advantages & Disadvantages of Lateral Flow Devices

Gold colloid-based immune-chromatographic test strips for the detection of mycotoxins are easy to handle, rapid to test and allow for on-site pre-screening. One of the many advantages of rapid immunoassay-based tests is that the sample clean-up stage may be omitted, and as it is a one-step assay no washing step is necessary. Sample pre-treatment is often not necessary if the sample is a liquid. These devices are considered to be cost effective with no real analytical skill required, however training regarding the test procedure is recommended. Also as lateral flow devices are designed for single use application, no contamination with previously tested sample can occur (Posthuma-Trumpie *et al.,* 2009).

A drawback of these devices is however even though the test procedure is relatively easy to perform, any imprecise sample volume may reduce the precision of the test and sample pre-treatment is obligatory when the sample is not a fluid. Depending upon the matrix being tested, some obstruction of pores on the pad may occur, due to certain matrix components. It has also been reported that the large differences observed between spiked samples and naturally contaminated samples, may contribute to calibration and validation problems (Krska & Molinelli, 2009).

It is reported that with any methodology, there are concerns about the sensitivity, precision, and reproducibility of the method and the subsequent rate of false-positive, false-violative (positive test result with non-actionable levels in the sample), and false-negative results (Henry *et al.,* 2001). Rapid screening methods need to provide detection at the action level, but not be overly sensitive as to cause the loss of milk due to false violatives (Charm, 1994). Since these methods are considered for screening technology, false positives are less serious as such samples would need to be tested by a fully quantitative method (Shephard, 2009).

The use of these lateral flow devices in the determination of aflatoxin M_1 in milk has not yet been officially approved by CEN or AOAC yet as their introduction to the market place is fairly new.

2.4.5 Documented analytical methods used by other authors

Surveillance studies have been conducted in many countries to determine the incidence levels of aflatoxin M_1 in bovine milk and derivatives thereof, and proposed maximum levels for this mycotoxins (Galvano *et al*., 2001; Kamkar, 2006).

A summary of the analytical methods used by various authors in their respective countries, to conduct surveillance studies of aflatoxin B_1 in feed and aflatoxin M_1 in milk, is shown in Table 8.

Table 8: A summary of the various documented methods used to analyse for aflatoxin B_1 and aflatoxin M_1

Country	**Type of milk**	**Method used for analysis**	**Reference**
Argentina	Farm gate + retail cow's milk	Ridascreen® ELISA	López *et al*. (2003)
Brazil	Cow's milk + dairy feed	Ridascreen ® ELISA for milk + TLC for feed	Sassahara *et al.* (2005)
FSA	Farm gate + retail cow's milk	IAC + HPLC	Food Standards Agency (2001)
India	Infant milk powder, infant liquid milk and weaning food(all based on cow's milk)	Ridascreen® ELISA	Rastogi *et al.* (2004).
Iran	Raw cow's milk	HPLC	Tajkarimi *et al.* (2007).
Iran	Buffalo, cow, camel, sheep and goat milk	Ridascreen ® ELISA	Rahimi *et al*. (2010).
Italy	Raw cow's milk + dairy feed	ELISA Immunoscreen M1 and ELISA Immunoscreen Afla	Decastelli *et al.* (2007)

Country	Type of milk	Method used for analysis	Reference
Italy	Cow's milk Pasteurised and UHT	IAC + HPLC	Nachtmann *et al.* (2007)
Kuwait	Cow's milk -Retail, UHT and powdered milk + human milk + cattle feed	Ridascreen® ELISA	Dashti *et al.* (2009)
Morocco	Pasteurised cow's milk	IAC +HPLC	Zinedine *et al.* (2007)
Nigeria	Human milk, cow's milk, yogurt, ice cream, unripened cheese, cultured whole milk	Ph, acidity, Total plate count and fungi count	Atanda *et al.* (2007).
Pakistan	Buffalo, cow, camel, sheep and goat milk	IAC + HPLC	Hussain *et al*. (2010)
Portugal	Raw cow's milk, powdered cow's milk + traditional cheeses	IAC + HPLC	Martins *et al.* (2005)
Russia	Cow's milk Homogenised	SPE	Komarova (2000)
Serbia	Cow, sheep and goat raw milk + retail milk	IAC +TLC	Polovinski-Horvatović *et al.* (2009)
Spain	UHT Processed cow's milk	TLC	Blanco *et al*. (1988)
Thailand	Raw cow's milk	IAC + HPLC	Ruangwises & Ruangwises (2010)
Turkey	Pasteurised cow's milk	Ridascreen ® ELISA	Çelik *et al.* (2005)

IAC: Immuno-affinity column SPE: Solid Phase Extraction

TLC: Thin layer Chromatography ELISA: Enzyme Linked Immunoassay

In summary, there are various methods available to analyse for mycotoxins and aflatoxins in particular each with its own advantages and disadvantages. However, any method which is proposed to become official must be validated in a collaborative trial study, resulting in defined method performance characteristics (European Commitee for Standardisation, 1999). It is important that the framework for the design and methodology of such collaborative trial studies, as well as the statistical evaluation are also defined in appropriate protocols (Horwitz, 1995). Any method which has been successfully validated according to these protocols, may only then be recognised as an official method for use in legal cases or for international trade purpose (provided an accredited laboratory is used to conduct the test).

3. MATERIALS AND METHODS

3.1 Sampling of feed and milk

Feed ingredients and farm gate milk were sampled from ten selected dairy farms and a dairy processing facility within the KwaZulu- Natal area of South Africa. A farm control document system was devised to facilitate the sampling process whereby each farm and feed sample were assigned a number and code for traceability and confidentiality purpose. This document may be found in Appendix A.

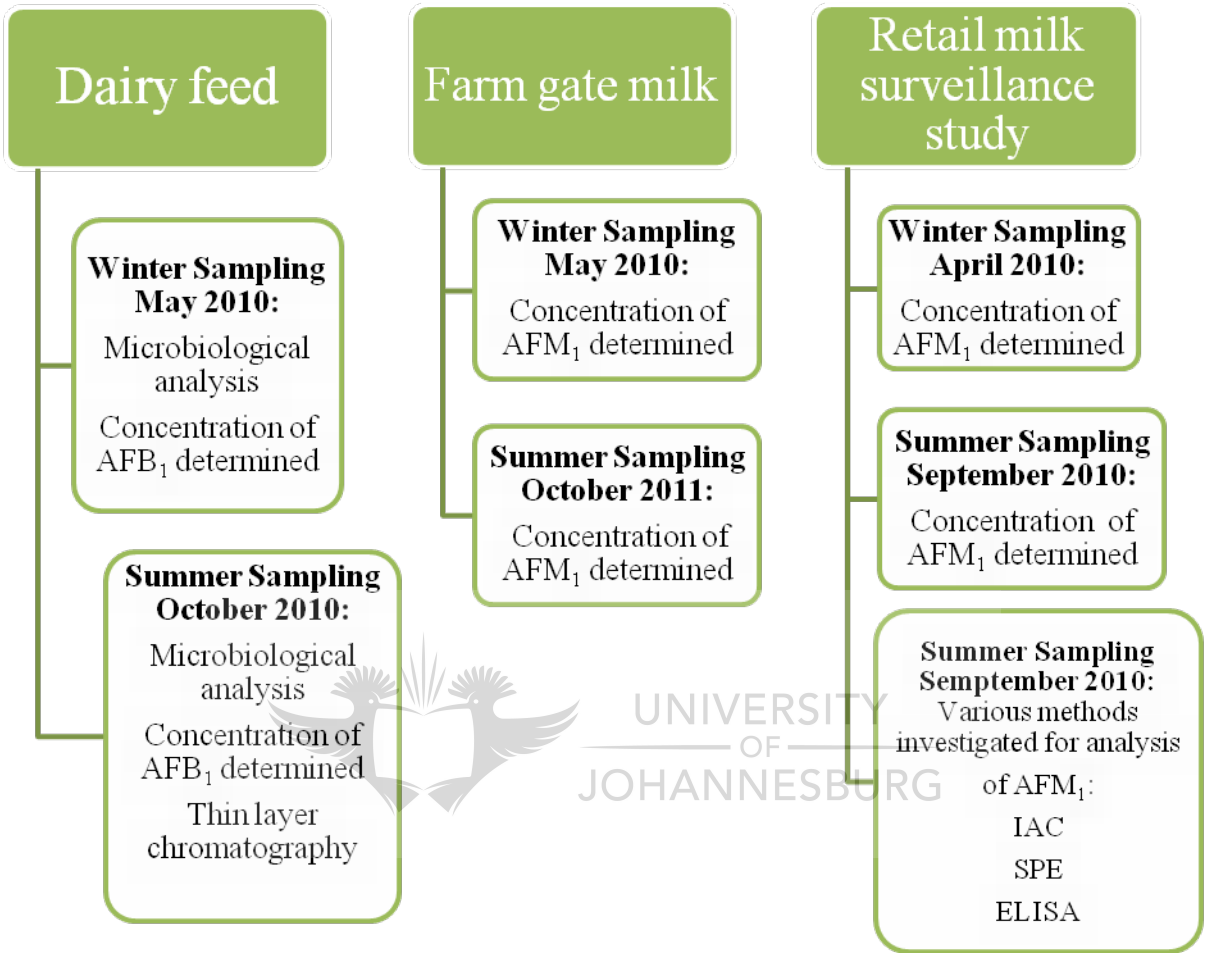
The winter sampling of feed was performed during late May 2010. Ten farms were visited and all the feed ingredients present during this time were sampled in triplicate, sealed in air tight bags and transported in cold cooler boxes. These samples were then analysed at the first available opportunity. The second round of feed sampling occurred during summer, in September 2010.

The winter sampling of farm gate milk occurred during late May 2010. Milk from each of the ten farms were then sampled over a ten day period by the respective tank drivers and coded accordingly. These samples were frozen immediately and shipped to Gauteng using the dairy's logistics transport chain. Upon receipt of the samples it was noticed that there were some inconsistencies of sampling. Some milk samples had leaked whilst other samples were not coded correctly and thus insufficient sampling lead to limited sample analysis for farm 3 and farm 10.

The summer sampling of farm gate milk from nine farms were done during early October 2010. The farmers were presented with empty labelled and coded PET milk bottles and asked to sample milk over a five day period and freeze immediately. The samples were delivered to the dairy processing facility where they were collected and securely delivered to Gauteng. It should be noted that no milk samples were obtained from farm number ten during the summer sampling, as the farmer chose to diversify his business and was no longer involved in milk production.

During the surveillance sampling commercially available milk was purchased during winter in April 2010 and during summer in September 2010 from selected retail outlets in Gauteng. The same brands of full cream, low fat, fat free, UHT processed milk, organic fresh milk and powdered infant formulae were selected during both sampling periods

A summary of the procedure used for the seasonal sampling of feed, farm gate milk and the surveillance sampling is shown in Figure 30.



IAC : Immuno-affinity column

SPE : Solid phase extraction

Elisa : Enzyme linked immunoassay

Figure 30: A summary of sampling procedure and analysis conducted on feed and milk

3.2 Materials

3.2.1 Dairy Feed

Microbiological Analysis

The following equipment was used, in order to accomplish the objectives of the study: a standard microscope equipped with an Axiocam MRC camera (208060245) and Axiom release (4.5 SPI 03/2006 Zeiss, Germany) and incubators (Sigma, RSA). All reagents and other consumables (potato dextrose agar, glucose, yeast extract, sodium propionate, di-potassium hydrogen sulphate, ox-bile, sodium propionate, agar powder, lactic acid, streptomycin, chloramphenicol and disposable petri dishes) were obtained from Sigma Aldrich RSA.

Vicam® Afla-Test® Immuno-affinity Analysis

The method used was as prescribed by Vicam®, the manufacturers of the columns. The columns and reagents required were included in the Aflatest® immuno-affinity columns kits. These kits were obtained from Vicam® USA who are represented in South Africa by Microsep and included universal calibration standards, microfibre filters 1.5 µm, 11 cm, fluted filter paper, Tween 20, culture tubes, disposable cuvettes, 15 x 85 mm test tubes, 12 x 75 mm cuvettes with cups, plastic beakers and funnels. Non iodized sodium chloride, methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from Sigma and Merck, (RSA). In addition a Series- 4 -Fluorometer detector, a four position pump stand, a blender with stainless steel container, a calibrator bottle, a digital scale with AC adapter and graduated cylinders were also obtained from Vicam® USA. Mycotoxins Standards were obtained from Sigma Bioscience (RSA), Merck (RSA) or Medical Research Council (RSA).

Thin Layer Chromatography

Thin layer chromatography required aluminium backed thin layer chromatography plates (20 x 20cm) pre-coated with silica gel which was obtained from Merck, RSA. Capillary pipettes, variable hot air drier, chromatographic tanks and ultra-violet (UV) light was also obtained from the supplier. Dichloromethane, ethyl-acetate, propan-2-ol toluene, ethyl-acetate and formic acid were obtained from Sigma Aldrich, RSA.

High Performance Liquid Chromatography

The high performance liquid chromatography unit used was from the Shimadzu Corporation (Kyoto, Japan). The model was a LC-20AB liquid chromatography unit equipped with CBM-20A communication bus module, a degasser coded LC-20AB, a column oven coded CTO-20, an auto sampler with code SIL-20A, a fluorescence detector with code RF-10AxL, a

refractive index detector coded RID-10A and a photodiode array detector SPD-M20A which was linked to LC solutions version 1.22 Software Release.

3.2.2. Dairy milk

Vicam® Afla-M_1 Immuno-affinity analysis

The method used was as prescribed by Vicam®, the manufacturers of the columns. The columns and reagents required were included in the Afla M_1® immuno-affinity columns kits. These kits included universal calibration standards, microfibre filters 1.5 µm, 11 cm, fluted filter paper, Tween 20, culture tubes, disposable cuvettes, 15 x 85 mm test tubes, 12 x 75 mm cuvettes with cups, plastic beakers and funnels were obtained from Vicam® USA who are represented in South Africa by Microsep. Non-iodized sodium chloride, methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from Sigma and Merck, (RSA). In addition a Series- 4-Fluorometer detector, a four position pump stand, a blender with stainless steel container, a calibrator bottle, a digital scale with AC adapter and graduated cylinders were also obtained from Vicam® USA. Mycotoxins Standards were obtained from Sigma Bioscience (RSA), Merck (RSA) or Medical Research Council (RSA).

Solid Phase Extraction

The method employed is the same as that used by Manetta *et al.* (2009). The cartridges used for the analysis were the Biotage® Isolute SPE. Non iodized sodium chloride, methanol (HPLC grade) acetonitrile (HPLC grade) *n*- hexane, dichloromethane and acetone were obtained from Sigma Bioscience (RSA) and Merck (RSA) A four position pump stand and centrifuge were also required to perform the analysis.

High Performance Liquid Chromatography

The high performance liquid chromatography model used was from the Shimadzu Corporation (Kyoto, Japan). The model was a LC-20AB liquid chromatography unit equipped with CBM-20A communication bus module, a degasser coded LC-20AB, a column oven coded CTO-20, an auto sampler with code SIL-20A, a fluorescence detector with code RF-10AxL, a refractive index detector coded RID-10A and a photodiode array detector SPD-M20A which was linked to LC solutions version 1.22 Software Release.

Ridascreen® ELISA

For analysis using the Ridascreen® ELISA, all the reagents required for analysis was included in the kit. The kit included 6 x aflatoxin M_1 standard solutions-0 ppt (zero standard); 5 ppt, 10 ppt, 20 ppt, 40 ppt, 80 ppt; aflatoxin M_1 in milk buffer; 1 x Conjugate (1.3 ml) peroxidase conjugated aflatoxin M_1 concentrate; 1 x substrate/chromogen (10ml)stained red, contains tetramethylbenzidine; 1 x stop solution (14ml) contains 1 N sulphuric acid; 1 x buffer 1 (20 ml) sample dilution buffer; 1 x buffer 2 (12 ml) conjugate dilution buffer; 1 x washing Buffer (salt) for preparation of a 10 mM phosphate buffer (pH 7.4) contains 0.05 % Tween 20. The kit was obtained from r-Biopharm, AG, Darmstadt, Germany, who are represented in South Africa by Amersham. Pasteur pipettes, graduated pipettes in variable sizes ranging between 20 µl and 200 µl were also obtained from the supplier. A micro -titer plate spectrophotometer (450 nm) and centrifuge were also required to perform the analysis.

Lateral Flow Devices

The materials, reagents and equipment required were for the Charm® and Snap® test units were all supplied with use of the respective portable systems.

3.3 Methods for Feed Analysis

3.3.1. *Fungal isolation screening and identification*

A serial dilution technique was employed where 1 g of the food or feed sample was diluted in 9 ml sterile Ringer solution and vortexed. A sample of 1 ml of this suspension was then transferred to 9 ml of Ringer solution and vortexed. This was repeated with further samples of Ringers to obtain a dilution factor of 10^{-6}. Then 1 ml of each suspension was aseptically added to sterile petri dishes and mixed with 20ml of molten potato dextrose agar (PDA) or Ohio agriculture experimental agar (OAEA) medium (Kaufman *et al.,* 1963) at 50^{o}C. This was allowed to set and incubated at 25°C for between 7 to 14 days (Klich, 2002). From the 4th to the 7th day, plates were screened for different types of fungal colonies, and counted manually. The number of fungal colonies per gram of sample was then calculated and expressed in colony forming units per gram (cfu/g). The hyphae and conidia from each colony representing each fungal spore were then transferred aseptically, forming three spots

on three different media (PDA, CYA & MEA) for identification purposes. The plates were then incubated at 30°C for between 4 to 7 days. Determination of each spp. of fungi was done using the keys of Klich & Pitt (1988) and Klich (2002) for *Aspergillus spp*., Pitt & Hocking (1997) for *Penicillium* and Nelson *et al*. (1983) for *Fusarium spp*. This was done by observing both macroscopic characteristics of the colonies on various media used as well as the microscopic morphology and measurements of the conidiophores (after staining mycelia with 0.1% fuschin dissolved in lactic acid or with lactophenol blue solution).

3.3.2 *Immuno-affinity Column*

Representative feed samples of 50g weight was mixed with 10g of sodium chloride and placed in a blender jar with 200ml of methanol-water (80/20 v/v) and blended for 5 minutes. The mixture was then filtered through fluted filter paper into a clean vessel. From the filtrate 10ml was collected and diluted to 50ml with purified water and then further filtered through a microfiber filter paper. From the filtrate, 10ml were collected and passed through the immuno-affinity column after which the column was washed successively with 10ml of purified water. The aflatoxins was then eluted with 1.0ml of methanol and collected in a glass cuvette. AflaTest® developer was added to the eluate in one of the cuvettes, mixed well and placed in a calibrated Vicam® series 4 fluorometer and the aflatoxin concentration was read after 60 seconds. Finally, the extract was then dried under nitrogen (N_2) and stored in a deep freezer for analysis by thin layer chromatography or for final detection by high performance liquid chromatography.

3.3.3 *Thin Layer Chromatography (TLC)*

The plates were lightly pencilled across in each corner 15 mm from each edge (These are the two lines at 90^{o}C parallel to two edges, so that they cross at the origin to assist in the measuring of retardation factor (R_F) values. Four equal 10 x 10cm plates were guillotined and the crosses became the origin of a two dimensional chromatograph. The identification of each sample to be run was pencilled at each corner diagonally opposite to the origin. The extracts which were obtained from extraction with immune affinity columns were dissolved with 200µl of acetonitrile: water (1:1, v/v) of which 20µl of the extract were spotted onto the origin of the plates in 2µl portions. The origin was dried at each stage with a steam of warm

air using a hot air drier. The mobile phase, dichloromethane-ethyl-acetate-propan-2-ol (DEI) was prepared and transferred to chromatographic tanks as shown in Figure 31. The tanks were left for about 30min in order to be saturated by the solvent systems. The plates were inserted into the chromatographic tank with the origin in the bottom left hand corner and allowed the solvent reach the top of the plate.

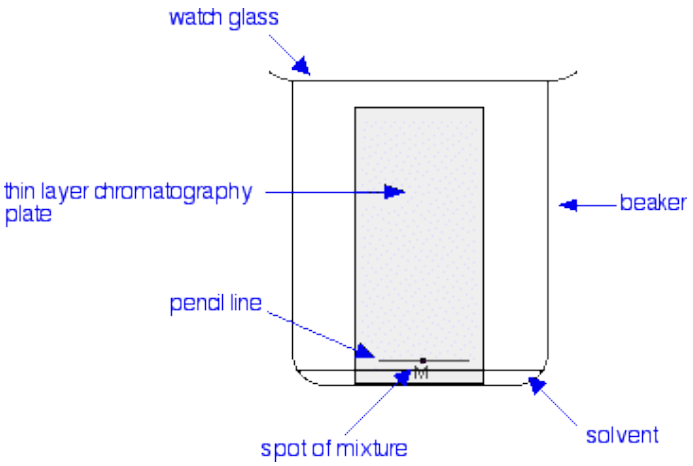


Figure 31: A thin layer chromatography chamber (University of Wisconsin, 2004)

The plates were immediately removed before the solvent over-runs and dried using warm air. The plates were then cooled and transferred again into the chromatographic tank where toluene-ethyl-acetate-formic acid (TEF) was placed at the right to the first run, thus the origin was now at the bottom right hand corner. The solvent was also allowed to run until the top of the plate, the TLC plates were then removed and allowed to dry and observed under ultraviolet light (UV) at 254 and 365nm. The spots were then marked at the centre in order to calculate the R_F values.

3.4 Methods for Milk Analysis

3.4.1 *Immuno-affinity Columns*

Representative milk samples of 50ml volume was mixed with 1g of sodium chloride and centrifuged at 2000*g* for 10 minutes. The skim portion of the milk was then filtered through a microfiber filter. About 10 ml of the filtered skim milk was passed completely through Afla® M_1 Test affinity column at a rate of about 1-2 drops/second until air came through the

column. The column was then washed successively with a 10% methanol solution. The affinity column was then eluted by passing 1.0 ml methanol: water (80:20v/v) solution through the column at a rate of 1-2 drops/second and all of the sample eluate (1ml) was collected in a glass cuvette. This eluate was then divided amongst 2 cuvettes, 1.0 ml of AflaTest® developer was added to the eluate in one of the cuvettes, mixed well and placed in a calibrated Vicam® - series- 4- fluorometer and the aflatoxin concentration was read after 60 seconds. The other cuvette of eluate was then air dried under a stream of nitrogen. The residue was then dissolved in 200µl of acetonitrile prior to injection of 10µl into the high performance liquid chromatography unit.

For recovery analysis, samples were run in triplicate by mixing control samples of milk with 5, 10 and 20 ng/ml of AFM_1. These samples were then extracted prior using the above mentioned immuno-affinity columns then injected into the HPLC.

3.4.2 *Solid Phase Extraction*

Representative milk samples of 50ml volume were mixed with 1g of sodium chloride and centrifuged at 3000*g* for 10 minutes. The skim portion of the milk was then filtered through a microfiber filter. About 10g of the filtered sample was then diluted with 10ml of deionised water. The solid phase extraction cartridges were mounted on a four position pump stand as shown in Figure 32. The Biotage® Isolute Sax SPE-C_{18} cartridge was then conditioned with 5ml acetonitrile and 10ml water. The diluted milk samples were then cleaned up on SPE-C_{18} cartridge. Typical flow rate was 7ml/min for 6ml columns. The cartridges were then washed with 10ml water and 20ml of acetonitrile/water (20:80 v/v) solution. This was followed by 10ml of *n*-hexane. The aflatoxin M_1 was then eluted with 6ml of dichloromethane / acetone (95:5,v/v) which was then evaporated under a gentle stream of nitrogen. The residue was then dissolved in 200µl of acetonitrile prior to injection of 10µl into the high performance liquid chromatography unit.

Figure 32: A photograph of a typical solid phase extraction manifold. The cartridges drip into the chamber below, where tubes collect the effluent. A vacuum port with gauge is then used to control the vacuum applied to the chamber (Wikipedia, 2011).

3.4.3 *Enzyme Immunoassay*

As per test procedure suggested by manufacturer r-Biopharm (2007) and reports of Dashti *et al.* (2009) and Karimi *et al*. (2007).

Milk samples were centrifuged at 10 min/ 3500 *g*/10 °C. After centrifugation, the upper cream layer was removed completely by aspirating through a pasteur pipette. The 100 µl skimmed milk portion or defatted supernatant was placed directly in the well.
The position of the standards and sample places was recorded. Then 100 µl of the standard solutions (as supplied) and prepared milk samples were added to the separate wells in duplicate. The plate was shaken manually and incubated for 30 min at room temperature in the dark.

The liquid was poured out of the wells and micro-well holder was tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. All the wells were then filled with 250 µl washing buffer (as supplied) and the liquid poured out again. This washing step was repeated twice. Then 100 µl of the diluted enzyme conjugate (as supplied) was added and mixed gently by shaking the plate manually. This plate was then incubated for 15 min at room temperature in the dark. The liquid was poured out of the wells and the micro-well holder was tapped upside down

vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells.

All the wells were then filled with 250 µl washing buffer (as supplied) and poured out the again. This washing step was repeated twice. Then 100 µl of chromogen (as supplied) was then added to each well. This was mixed gently by shaking the plate manually and incubated for 15 min at room temperature in the dark. Finally, 100 µl of the stop solution (as supplied) was then added to each well. This was mixed gently by shaking the plate manually. The absorbance was measured at 450 nm against an air blank and read on micro titer plate Spectrophotometer within 15 min after the addition of stop solution.

Calculation:

The calculation was performed without the use of Ridasoft® software. The course of the standard curve was shown in the Quality Assurance Certificate enclosed in the test kit.

Remark for calculation without software:

Absorbance standard (or sample)

Absorbance zero standard x 100 = % absorbance

3.4.4 Lateral Flow Devices

Charm® System

The method employed was as prescribed by the manufacturers, Charm Sciences Inc. (2009). All the milk samples were well mixed prior to testing. The test strips were labelled with the sample identification code and then placed in the ROSA® incubator. The test strip was then held down in the incubator whilst the peeling tape was pulled back gently to the **'peel to here'** line to expose the sample compartment as per the schematic representation in Figure 33

Figure 33: A graphical representation showing a test strip being placed in a Charm® incubator (Charm Sciences Inc, 2009).

A milk sample was then slowly pipette to a volume of 300µl. This sample was placed in the sample compartment at the ROSA® incubator indicator line as shown in Figure 34 below.

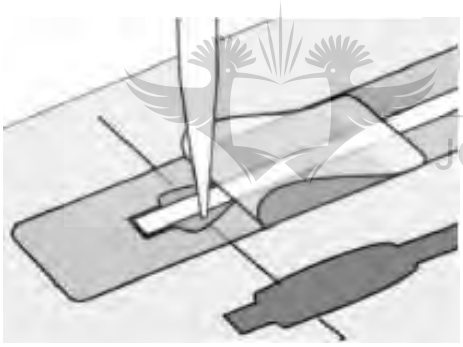


Figure 34: A graphical representation showing the position on the test strip where the milk sample should be placed (Charm Sciences Inc, 2009).

The tape was then re-sealed over the sample compartment. It is important to note that each test strip was completed *within 1 minute*. The lid of the Rosa® incubator was then closed and latched as evident by the presence of a red light. The samples were then incubated for 15 minutes. At this time interval, a beeping and alternating red and yellow blinking light began to flash on the Rosa® incubator.

The test strips were then carefully removed from the incubator. The test strips were held such that the sample compartment was in the down position until interpreted. If the test was invalid as per the schematic shown below in Figure 35, then the test strip was discarded. If the test strip was valid, it was inserted into a calibrated (MRL Aflatoxin M_1 Quantitative Test mode) Rosa® reader.

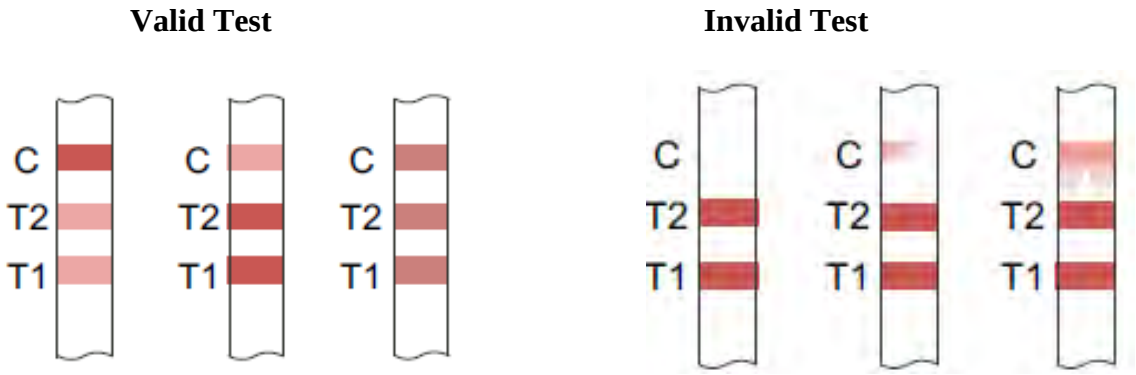


Figure 35: Charm® test strips indicating the validity of the test (Charm Sciences Inc, 2009).

It is important to note that the qualitative interpretation of the concentration is relative to the EU MRL of 50 ppt. So a result less than 40 ppt is a **negative result.** A result greater than or equal to 40ppt is a **positive result.**

SNAP® System

The SNAP® device was placed on the pre-heated heating block, which was set at approximately 45°C. The milk sample was well mixed and 50µl was slowly pipette (avoiding air bubbles) using the pipette supplied into the sample tube which contained the reagent pellet. This tube was well mixed to ensure that the reagent pellet was properly dissolved as demonstrated in Figure 36 below.

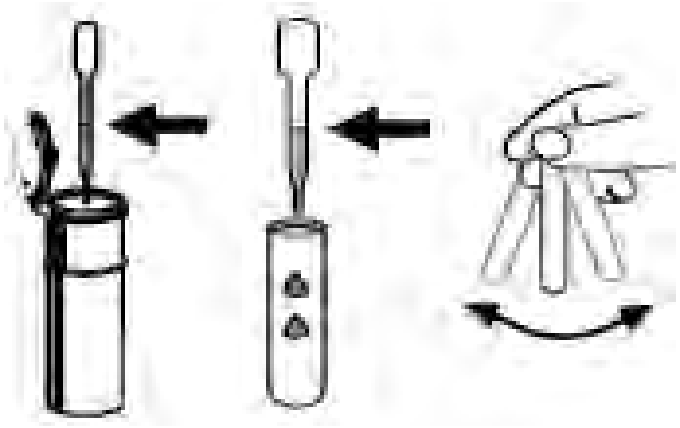


Figure 36: A graphical representation of how a milk sample should be prepared for application using the Snap® device (Idexx, 2008).

The entire contents of the sample tube was then poured into the sample well of the Snap® device. The lateral flow Snap® device, is shown in Figure 37.

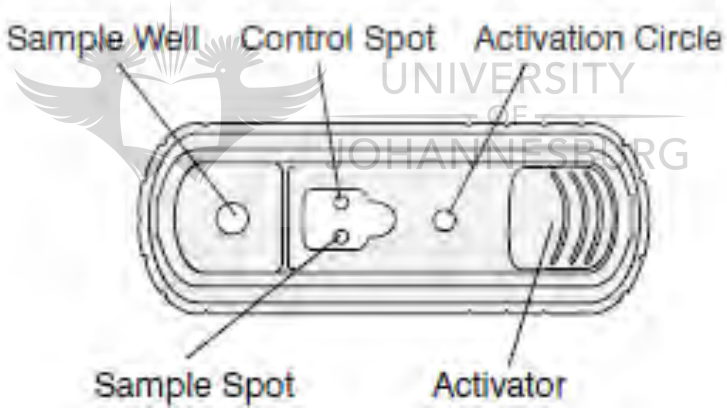


Figure 37: A diagram of the layout of a Snap® device (Idexx, 2008).

Once the sample was poured, it began moving toward the result window and toward the green activation circle. When the green activation circle begun to disappear, the activator was pushed firmly until it snapped flush with the body of the Snap® device as shown in Figure 38.

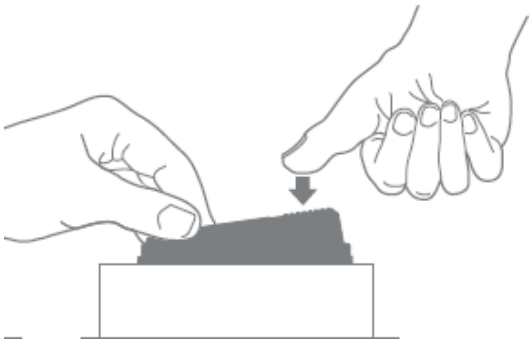


Figure 38: Graphical representation of the action involved in pushing the activator so it snaps flush with the body of the Snap® device (Idexx, 2008).

A timer was then set to 4 minutes. Thereafter the Snap® device was removed from the heater block and the control and sample spots were visually inspected. If the test was valid, the Snap® device was placed in the Snapshot® Reader.

A negative reading as seen in the Figure 39 indicates that the ration was below or equal to 1.05.

Figure 39: The schematic representation of a negative result using Snap® device (Idexx, 2008).

A presumptive positive result as shown in Figure 40 indicates that the ratio is 1.06 or higher.

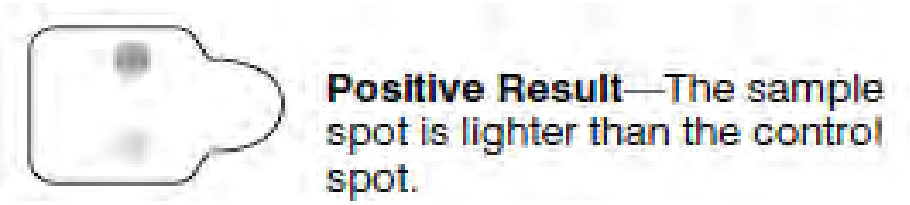


Figure 40: The schematic representation of a positive result using the Snap® device (Idexx, 2008).

3.5 High performance liquid chromatography (HPLC)

The determination and quantification of mycotoxins aflatoxins from feed and milk samples were performed using high performance liquid chromatography. Mycotoxin extracts were re-dissolved in 400 µl of methanol and filtered through a 0.2 µm Millipore filter and filtrate used as the analyte solution. The chromatographic separation of analytes and standards was performed by passing the solution through a reverse phase symmetry column C18 column (Waters®, USA). The oven temperature was maintained at 30°C. Peak areas and retention times of mycotoxins were used to determine the amount of specific mycotoxins per sample based on those of standard mycotoxins using the calibration curve. The HPLC analysis of aflatoxin contamination in feed and milk was done according to Trucksess *et al.* (2008) and Reiters *et al*. (2009) in which the HPLC was coupled with a fluorescence detector (RF 10AXL) coupled with a Coring cell (CoBrA cell) from Dr. Weber Consulting, which was the electrochemical cell for the derivatisation aflatoxins. The mobile phase was composed of methanol-acetonitrile- water (20:20:60) containing 119 mg of potassium bromide and 100 µl of nitric acid. The HPLC conditions were 362nm for excitation and 440 nm for emission and at a flow rate of 1ml / min.

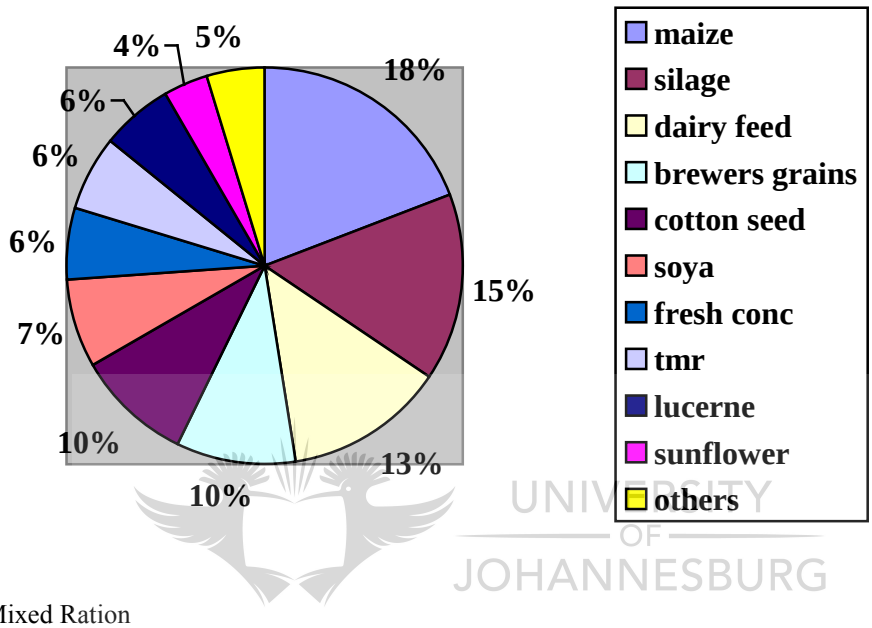
3.6 Statistical Analysis

The Statistical analysis was performed using Sigma Plot for Windows version 11, Systat Software Inc. The statistical methods used in this study were based on normal confidence intervals and analysis of variance (ANOVA) and is reported as mean, SD and SEM. The levels were considered significantly different at $P < 0.001$.

4. RESULTS

4.1 Fungal Isolation and Screening of Dairy Feed

The total feed ingredients which were sampled and analysed from the ten farms are shown in Figure 41.



*tmr: Total Mixed Ration

Figure 41: A pie chart showing the ratios of the various feed ingredients sampled from the ten farms and analysed

Maize (18%), silage (15%), dairy feed(13%), brewer's grains(10%) and cotton seed(10%) make up the largest number of samples analysed as seen in the chart above, followed by soya and the remainder of the feed ingredients listed. Table 9 shows a list of all the various fungi commonly found during the microbiological examination of the sampled feed ingredients

Table 9: A summary of the various moulds and fungi found during the microbiological analysis of the feed ingredients

During this study 86 samples were analysed for the presence of fungi contamination. It was found that 100% of samples tested positive for the presence of fungal growth. *Aspergillus flavus* was a common contaminant found in *all* the feed ingredients sampled. Most samples were contaminated with a combination of fungi from the three genera namely, *Aspergillus, Fusarium* and *Penicillium.*

Below are some photographs (Figure 42- 45) showing the mould contamination found on some of the feed samples.

Figure 42: Photograph of *Fusarium sambucinum*

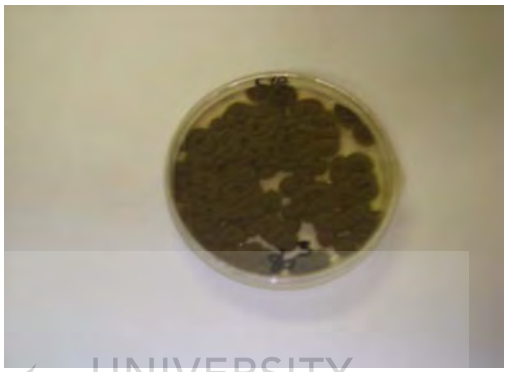


Figure 43: Photograph of Aspergillus *clavatus*

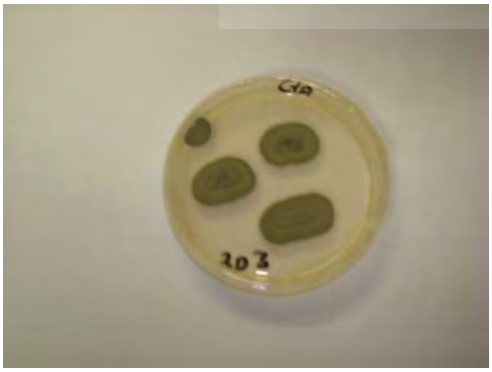


Figure 44: Photograph of *Penicillium crustosum*

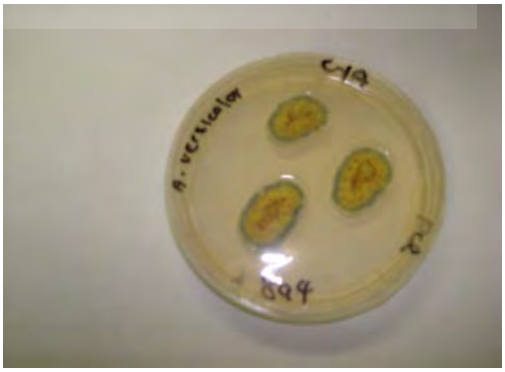


Figure 45: Photograph of *Aspergillus versicolor*

4.2 Mycotoxin Analysis

4.2.1 *Mycotoxin Analysis in Dairy Feed*

The immuno-affinity (IAC) method was used for extraction and clean-up of feed samples. Following the extraction, samples were analysed by high performance liquid chromatography (HPLC) which was used as the final confirmatory procedure. The HPLC was coupled with a UV detector and CoBrA cell as shown in chromatogram in Figure 46

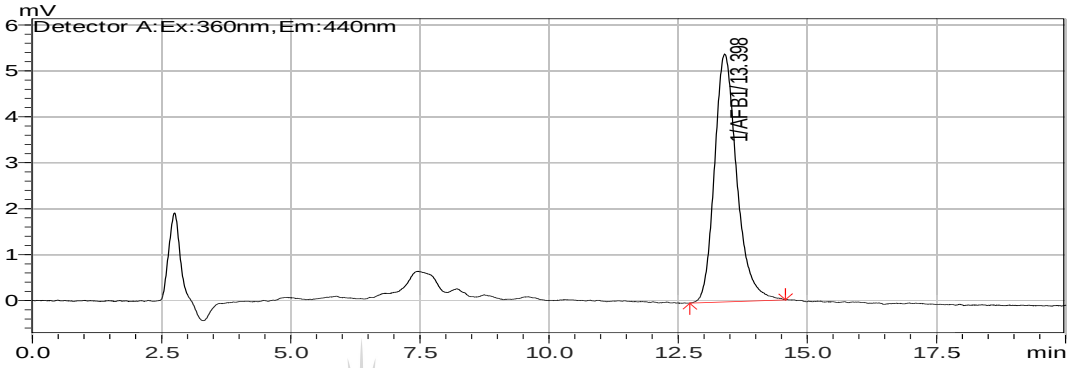


Figure 46: Chromatogram of Aflatoxin B_1 standard 20 µg/ml at 20µl injection using high performance liquid chromatography, coupled with a UV detector and a CoBrA cell

The chromatogram illustrates a clear peak with no interferences in the region where aflatoxins B_1 is eluted. The clarity and perfection of the shape of the peak may be attributed to the presence of the derivitising agent which clearly enhances the natural fluorescence of the aflatoxins, making them more detectable at low concentrations.

Selected feed samples which were not contaminated with mycotoxins, were used for the mycotoxin recovery using mycotoxins standards. The samples were spiked with known levels of mycotoxin standards and analysed with the other samples as per the documented methodology. The recoveries of standard toxins are shown in Table 10.

Table10: Mycotoxin recovery from samples which were spiked with mycotoxin standards using Vicam® fluorometry assay

Aflatoxin standard	**Spiked concentration/ g in animal feed**	**Means recovery (µg)**	**Percentage (%)**
Aflatoxin B_1	100 µg / 25g	102 (±1.3)	102%
Aflatoxin B_2	100 µg / 25g	98 (±2.5)	98
Aflatoxin G_1	100 µg / 25g	95 (±0.9)	95
Aflatoxin G_2	100 µg / 25g	97 (±1.34)	97

Table 10 shows a summary of the mean recoveries obtained from animal feed samples spiked with aflatoxin standards using high performance liquid chromatography. The recovery rate was between 97-102% which was adequately sufficient to continue with the analysis of the feed samples using the HPLC coupled with UV detector and CoBrA cell.

A total of 102 feed sample ingredients were analysed for aflatoxin B_1. A summary of the feed ingredients analysed with both the winter and summer aflatoxin B_1 contamination values are reported in Table 11. The total mix composites were a blend of all the ingredients which was sampled from each farm combined in equal quantities.

Table 11: A summary of the aflatoxin B_1 quantification values and statistical analysis results of the seasonal feed ingredients, obtained from clean by Vicam® immuno-affinity and high performance liquid chromatography coupled with UV detector and CoBrA cell.

Farm/ Sample ID Winter	**Ingredient**	**Vicam® IAC (ppb)**	**HPLC AFB_1 (ppb)**	**Farm/ Sample ID Summer**	**Ingredient**	**Vicam® IAC (ppb)**	**HPLC AFB_1 (ppb)**
1A 1	Brewer's meal	0	0	1A 54	Lucerne	2	**22.9**
1B 2	Maize Silage	6	**15.2**	1C 55	Cotton seed	0	0
1C 3	Fresh concentrate	70	**7.4**	1D 56	Maize chop	0	0
1D 4	Lucerne +hay	56	**5.0**	1E 57	Maize Silage	0	0
1E 5	Maize chop	24	**6.0**	1F 58	Brewer's meal	0	**111.3**
1F 6	Soya oil cake	18	**3.4**	1G 59	Soya Crushed	0	0
1G 7	Sunflower oil cake	48	**12.0**	1H 60	Maize meal	0	**25.7**
1H 8	Bran	0	**12.7**	2A 61	Fresh concentrate	0	**24.0**
1I 9	Cotton seed	4	**6.3**	2B 62	Feather meal	0	0
1J 10	Maize meal	8	**8.7**	2C 63	Maize chop	0	**21.3**
2A 11	Maize Silage	2	**16.5**	2D 64	Soya crushed	0	**29.5**
2B 12	Lucerne	248	**4.0**	2E 65	Sunflower oil cake	0	**46.3**
2C 13	Cotton seed	96	**17.2**	2F 66	Maize meal	2	**47.5**
2D 14	Cotton oil cake	92	**7.5**	2G 67	Brewer's meal	0	**8.70**

Farm/ Sample ID Winter	**Ingredient**	**Vicam® IAC (ppb)**	**HPLC AFB_1 (ppb)**	**Farm/ Sample ID Summer**	**Ingredient**	**Vicam® IAC (ppb)**	**HPLC AFB_1 (ppb)**
2E 15	Fresh concentrate	56	**13.0**	2H 68	Maize Silage	0	**100**
2F 16	Maize meal	72	**14.3**	3A 69	Dairy feed	0	**19.3**
2G 17	Brewer's meal	2	**19.2**	4A 70	Soya crushed	2	**264**
3A 18	Dairy meal	200	**12.6**	4B 71	Maize meal	0	**11.6**
3B 19	Dairy feed	256	**10.4**	4C 72	Maize chop	0	0
4A 20	Cotton oil cake	38	**13.2**	4D 73	Brewer's meal	0	**15.3**
4B 21	Feather meal	12	**9.5**	4E 74	Maize Silage	2	**56.0**
4C 22	Maize chop	60	**159.4**	4F 75	Fresh concentrate	2	**15.1**
4D 23	Total Mix Ration	4	**513.9**	4G 76	Lucerne	0	**909.1**
4E 24	High protein concentrate	18	0	4H 77	Total Mix Ration	16	**87.3**
4F 25	Brewer's meal	40	**452.8**	4I 78	Cotton seed +mix ration	0	**741.3**
5A 26	High protein concentrate	100	**441.8**	5A 79	Fresh concentrate	0	**32.1**
5B 27	Mix maize +cotton oil	20	**7.8**	5B 80	Maize Silage	2	**26.4**
5C 28	Total mix meal	64	**4093.2**	5C 81	Maize chop	0	**179.2**
5D 29	Brewer's meal	2	**8.1**	5D 82	Soya Crushed	12	0
5E 30	Lucerne +hay	12	**10.0**	5E 83	Brewer's meal	0	**10.2**
6A 31	Silage	8	0	6A 84	Dairy meal	4	**258.6**

Farm/ Sample ID Winter	**Ingredient**	**Vicam® IAC (ppb)**	**HPLC AFB_1 (ppb)**	**Farm/ Sample ID Summer**	**Ingredient**	**Vicam® IAC (ppb)**	**HPLC AFB_1 (ppb)**
6B 32	Dairy meal (own mix)	2	**15.4**	6B 85	Maize Silage	2	**200.0**
7A 33	Total Mix Ration	50	**10.0**	7A 86	Maize chop	4	**19.6**
7B 34	Total Mix Ration	8	0	7B 87	Soya Crushed	0	0
7C 35	Maize Silage	4	**422.0**	7C 88	Cottonseed	0	**16**
8A 36	Maize Silage	12	**145.7**	7D 89	Cotton oil seed	18	**328.3**
8B 37	Maize chop	6	0	7E 90	Sunflower seed	0	**1348.0**
9A 38	Dairy meal high energy	12	**18.4**	8A 91	Maize chop	0	0
9B 39	Cow pea silage	20	**14.6**	8B 92	Maize chop	0	0
10A 40	Dairy meal	0	**7.7**	8C 93	Maize Silage	2	**20.4**
10B 41	Dairy meal	42	**27.6**	8E 94	Maize Silage	2	0
1T 42	Total mixed composite	8	**39.7**	9A 95	High energy feed	1	**346.4**
2T 43	Total mixed composite	2	**15.6**	10A 96	Dairy feed	0	0
3T 44	Total mixed composite	12	0	1T 97	Total mix composite	2	**110.0**
4T 45	Total mixed composite	6	0	2T 98	Total mix composite	10	**60.3**
5T 46	Total mixed composite	10	**9.3**	4T 99	Total mix composite	18	**102.2**
6T 47	Total mixed composite	72	**16.8**	5T 100	Total mix composite	2	**2259.0**
7T 48	Total mixed composite	4	**663.1**	7T 101	Total mix composite	6	**2260.3**

Farm/ Sample ID Winter	Ingredient	Vicam® IAC (ppb)	HPLC AFB_1 (ppb)	Farm/ Sample ID Summer	Ingredient	Vicam® IAC (ppb)	HPLC AFB_1 (ppb)
8T 49	Total mixed composite	12	**16.7**	8T 102	Total mix composite	12	**1182.3**
9T 50	Total mixed composite	0	**14.1**				
10T 51	Total mixed composite	10	0				
 52	Sunflower seed crushed	0	0				
53	Sunflower seed crushed	0	0				

Method/sample	**n**	**Mean (ppb)**	**Range (ppb)**	**Std. Dev**	**SEM**
Vicam® IAC (winter)	53	36.37	0.00 - 256.00	56.665	7.784
HPLC (winter)	53	138.45	0.00 - 4093.21	573.022	78.711
Vicam® IAC (summer)	49	2.53	0.00 - 18.00	4.788	0.684
HPLC (summer)	49	230.92	0.00 - 2260.34	514.31	73.474

Vicam® IAC: Immuno-affinity

HPLC: High Performance Liquid Chromatography

Std Dev: Standard Deviation

SEM: Standard Error of Mean

The values which are highlighted in bold print in Table 11 exceed the legislated level of permitted aflatoxin contamination. There seems to be no real trend in feed ingredients or particular farms which show preferred contamination by aflatoxin B_1. Cereals such as brewer's meal and maize chop show high levels of contamination and so did the oil seeds such as soya and sunflower oil cake. Lucerne from farm 4 showed a particularly high incidence of contamination. Compounded dairy feed and silage (both maize and cowpea) were also reported to be heavily

contaminated. The highest recorded level of contamination was sample 28 from farm 5, where a total mix meal which is actually a compound feed had reported a aflatoxin B_1 concentration of 4093 ppb which is over 800 times that permitted by South African legislation.

The statistical analysis summary based on the Vicam® IAC results reported statistically, the difference in the mean values of the two groups is greater than would be expected by chance; there *is* a statistically significant difference between the input groups (P = <0.001).

Based on the HPLC results reported statistically, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is *not* a statistically significant difference between the input groups (P = 0.395).

The mean aflatoxin B_1 contamination values obtained for the feed were found to be higher in summer (230.92) when compared to winter (138.45) as shown in Figure 47.

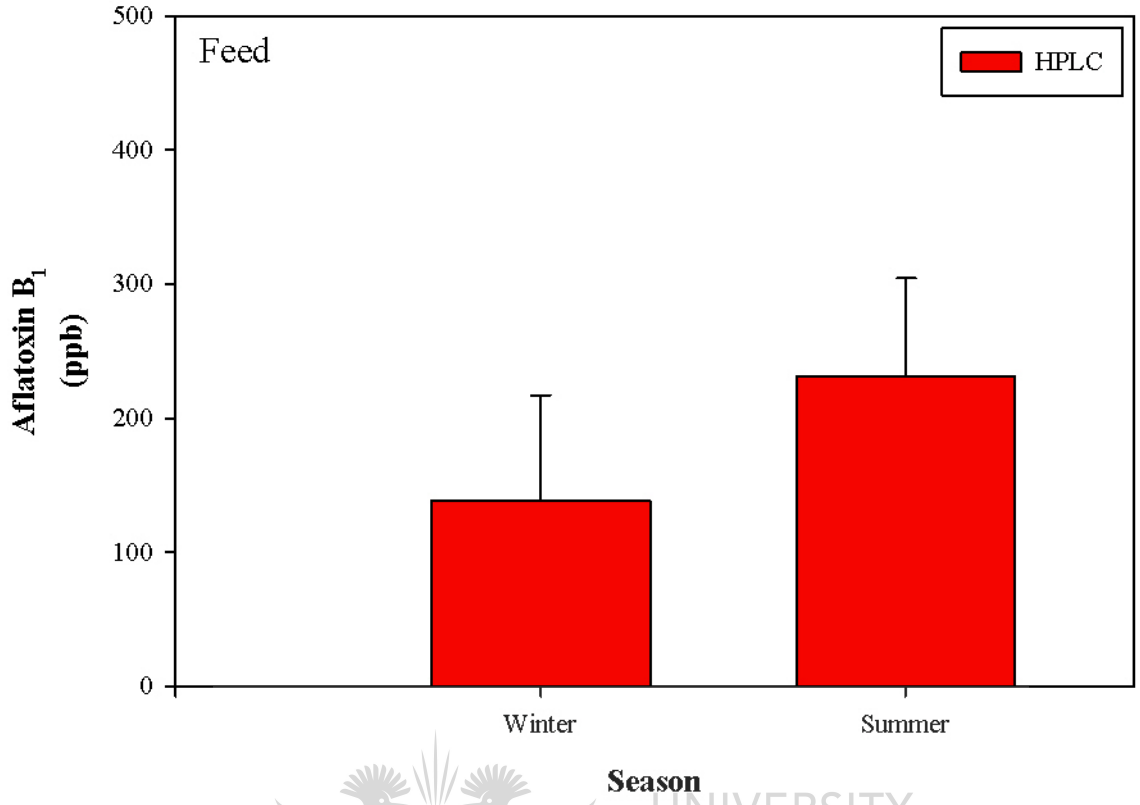


Figure 47: A graphical representation of the average contamination levels of aflatoxin B_1 in feed samples during the *winter* and *summer* sampling periods

The mean aflatoxin B_1 contamination values obtained for the feed exceeds not just South African and European Union permitted levels of 5 ppb aflatoxin B_1 but also that of the more lenient limit as prescribed by the FDA of 20 ppb.

Figure 48 furthermore illustrates that the aflatoxin B_1 concentrations found during HPLC analysis of most of the feed ingredients were found to exceed the legal limit.

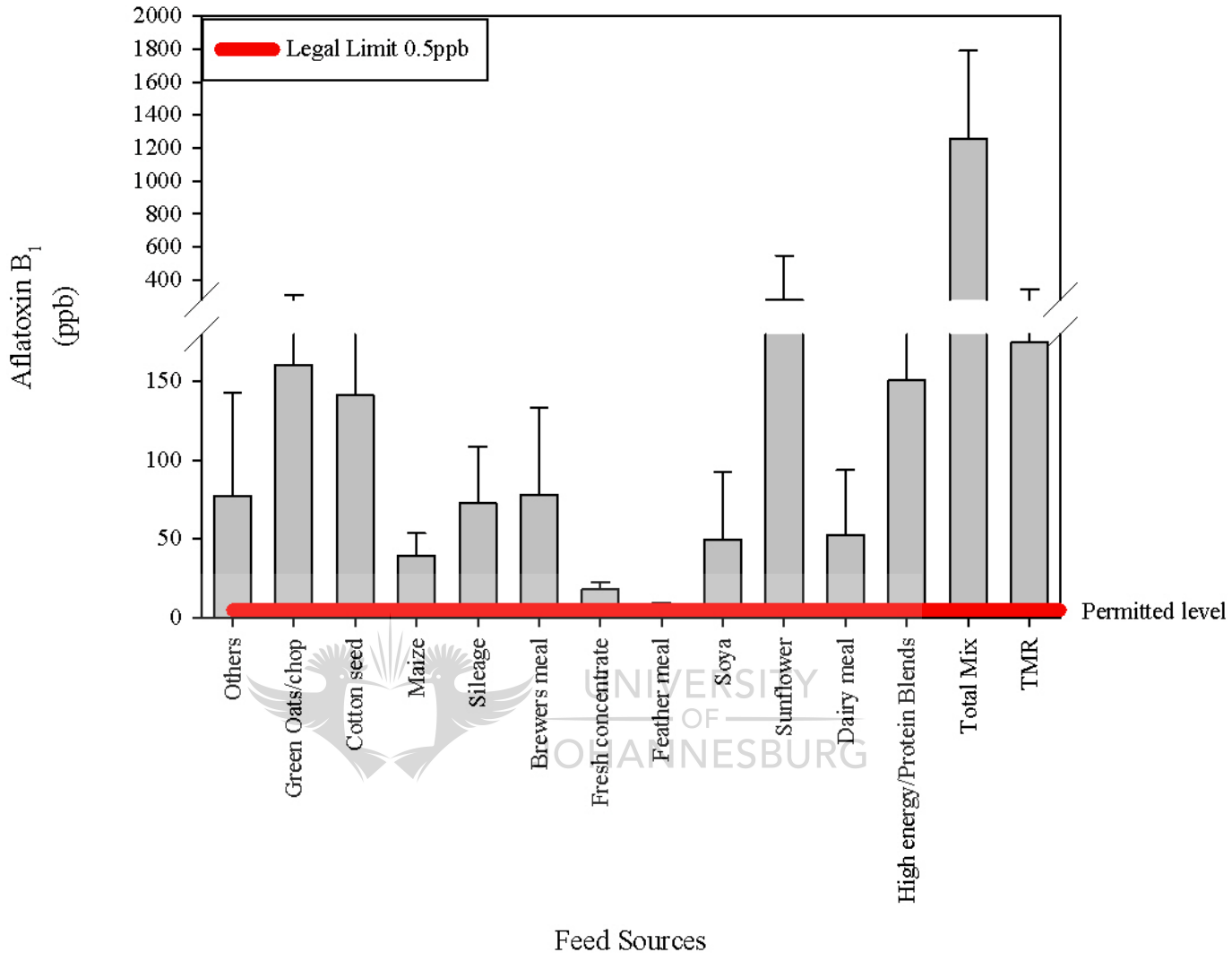


Figure 48: Quantification of aflatoxin B_1 by HPLC in the various feed ingredients together with the permitted legislated level.

Approximately 80 (78%) of the samples were found to be contaminated with aflatoxin B_1, whilst 76 (74%) of these samples were found to exceed the legislated level of 5ppb aflatoxin B_1.

4.2.2. Thin Layer Chromatography of Feed

All the feed ingredients sampled during the summer session were used to determine the presence of aflatoxins in the feed samples. The extracts used were obtained using the Vicam® immuno-

affinity procedure. The procedure for thin layer chromatography (TLC) used was the procedure outlined in the materials and methods section of this dissertation.

Upon viewing the TLC plates under the UV light it was found that no other visible fluorescent aflatoxins compounds could be seen with the exception of the aflatoxins standard used.

4.2.3 Mycotoxin Analysis in Milk

4.2.3.1 Seasonal Farm Gate analysis of milk

Samples of raw milk were obtained from the same ten farms as the feed samples. Immuno-affinity columns were used for extraction of feed samples, followed by confirmatory analysis using HPLC with fluorescent detection coupled with a CoBrA cell as shown in Figure 49.

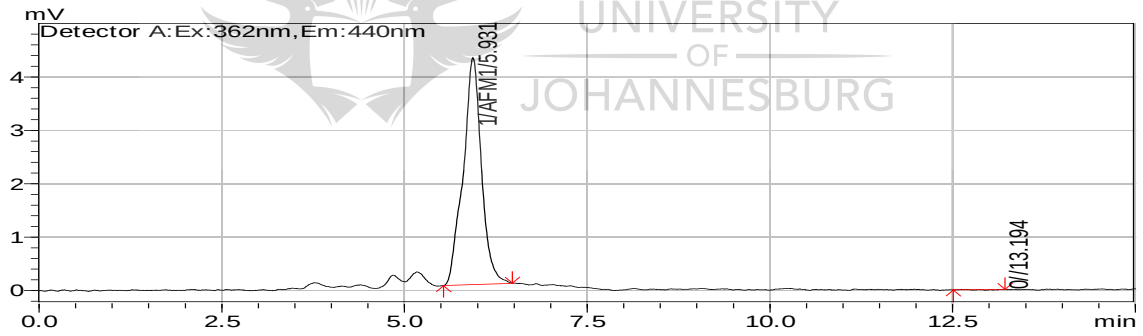


Figure 49: Chromatogram of aflatoxin M_1 standard at 8 µl/ml and 40µl injection using high performance liquid chromatography (HPLC) coupled with a UV detector and a CoBrA cell.

The chromatogram illustrates the efficiency of the proposed method as there are no interferences in the region where AFM_1 is eluted. The retention time of 6 minutes is a bit short but still ensures there will be no interference from un-retained compounds thus permitting a high sample throughput. These findings are comparable to the chromatogram reported by Manetta *et al.* (2005).

The clarity and perfection of the shape of the peak may be attributed to the presence of the derivitising agent which clearly enhances the natural fluorescence of the aflatoxins, making them more detectable at the low concentrations.

The chromatograms shown in Figures 50 and 51 respectively show the differences between a sample analysed for aflatoxin M_1 *with* and *without* a CoBrA cell.

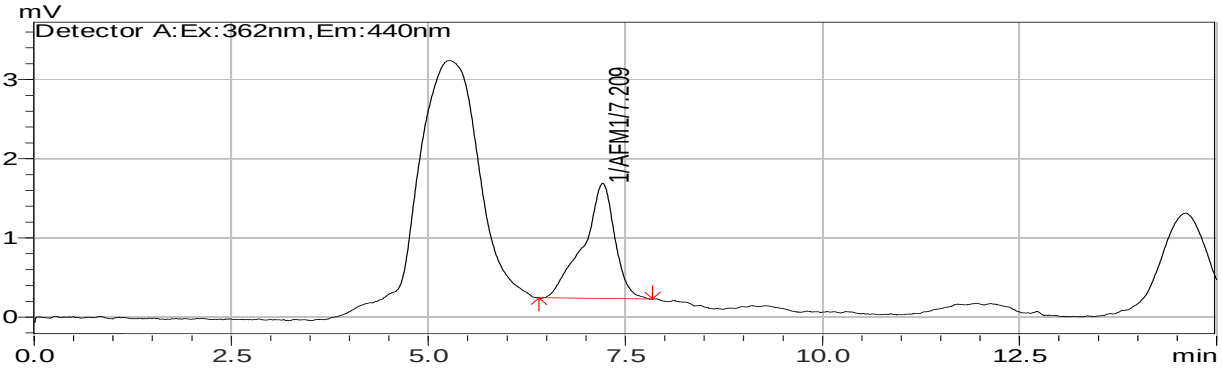


Figure 50: Chromatogram of aflatoxin M_1 of a milk sample as per high performance liquid chromatography coupled with a UV detector **with** a CoBrA cell

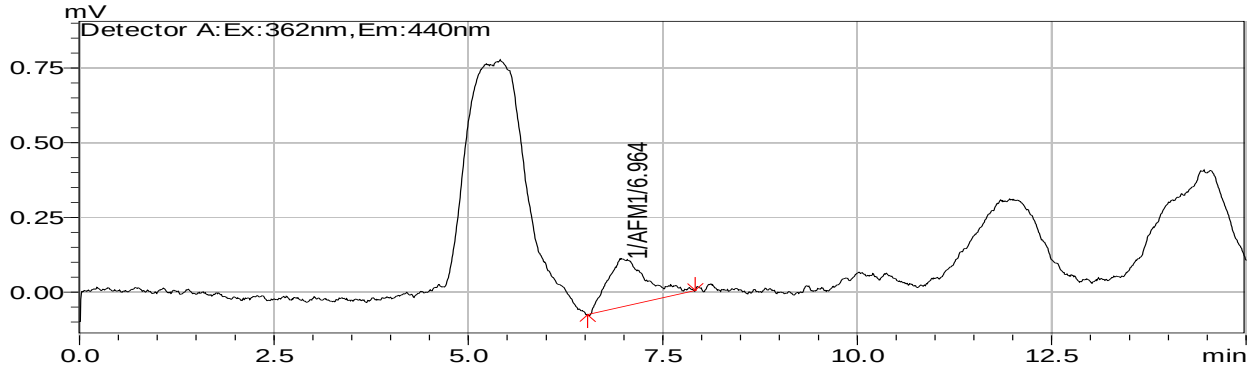


Figure 51: Chromatogram of aflatoxin M_1 in the same milk sample using high performance liquid chromatography coupled with a UV detector but **without** a CoBrA cell.

The peaks shown in both Figures occur at the same time however, the aflatoxin M_1 peak formed in the chromatogram without the CoBrA cell (Figure 51) is not as high and good as the aflatoxin M_1 peak formed in the chromatogram using the CoBrA cell (Figure 50). The CoBrA cell which generates the derivatising agent bromine, enhances the fluorescence of the aflatoxins present in the matrix. It is clear from observing the two chromatograms, that there is an increase of aflatoxin florescence activity and high reproducibility of results when using a CoBrA cell.

In total 102 samples of farm gate milk samples were analysed from the winter and summer sampling sessions. The aflatoxin M_1 quantification values for the winter and summer sampling are shown in Table 12.

Table 12: A summary of the aflatoxin M_1 quantification values and statistical analysis results of the seasonal farm gate milk obtained from Vicam® immuno-affinity and high performance liquid chromatography coupled with UV detector and CoBrA cell.

Sample Winter	**Farm**	**Vicam® IAC (ppb)**	**HPLC (ppb)**	**Sample Summer**	**Farm**	**Vicam® IAC (ppb)**	**HPLC (ppb)**
1.1	1	0.4	0.04	1.1	1	0.4	0.03
1.2	1	0.6	**0.06**	1.2	1	0.22	**0.17**
1.3	1	0.4	0.05	1.3	1	0.2	**0.11**
1.4	1	0.4	**0.07**	1.4	1	1.0	**0.12**
1.5	1	0.2	**0.14**	1.5	1	0.22	**0.13**
2.1	8	0.4	0.04	2.1	2	0.6	0.02
2.2	8	0.2	**0.08**	2.2	2	1.0	**0.06**
2.3	8	0.4	**1.32**	2.3	2	0.4	**0.08**
2.4	8	0.4	**0.08**	2.4	2	0.6	**0.06**
2.5	8	0.2	**0.13**	2.5	2	0.22	**0.11**
3.1	9	0.6	**0.22**	3.1	3	0.4	0.03
3.2	9	0.4	**0.11**	3.2	3	0.8	**0.06**
3.3	9	0.8	**0.53**	3.3	3	0.6	0.02
3.4	9	0.8	**0.11**	3.4	3	0.6	0.05
3.5	9	0.4	**0.07**	3.5	3	0.2	0.04

Sample Winter	Farm	Vicam® IAC (ppb)	HPLC (ppb)	Sample Summer	Farm	Vicam® IAC (ppb)	HPLC (ppb)
5.1	6	0.4	0.05	4.1	4	0.6	**0.09**
5.2	6	0.2	**0.14**	4.2	4	0.2	**0.09**
5.3	6	0.6	**0.06**	4.3	4	0.6	**0.06**
5.4	6	0.2	**0.32**	4.4	4	0.6	**0.08**
5.5	6	1.0	**0.08**	4.5	4	0.2	**0.14**
6.1	7	1.0	**0.06**	5.1	5	0	**0.06**
6.2	7	0.4	0.04	5.2	5	0.4	**0.31**
6.3	7	0.2	0.04	5.3	5	1.6	0
6.4	7	0.6	0.05	5.4	5	1.6	0.04
6.5	7	0.2	**0.06**	5.5	5	0.2	**0.24**
7.1	3	0.8	0.03	6.1	6	0.4	0
7.2	3	1.0	**0.73**	6.2	6	1.2	**0.39**
8.1	2	1.6	**0.30**	6.3	6	0.24	0
8.2	2	0.4	**0.31**	6.4	6	0.2	0
8.3	2	0.8	**0.06**	6.5	6	3.6	0.05
8.4	2	0.4	**0.09**	n.a.	n.a	n.a	n.a
8.5	2	0.4	**0.17**	7.1	7	0.4	**0.11**
9.1	4	0.2	**0.23**	7.2	7	0.4	**0.09**
9.2	4	0.2	**0.23**	7.3	7	0.6	**0.15**
9.3	4	0.2	0.05	7.4	7	0.6	**0.10**
9.4	4	1.4	**0.06**	7.5	7	1.0	**0.30**
9.5	4	0.6	**0.11**	8.1	8	0.2	**0.74**
10.1	5	0.8	**0.07**	8.2	8	0.66	**0.81**
10.2	5	0.4	0.04	8.3	8	0.36	0.01
10.3	5	0.4	0.04	8.4	8	6.4	**1.54**
10.4	5	0.4	0.03	8.5	8	3.6	0.02
10.5	5	0.2	0.05	9.1	9	0.4	**0.06**
4	10	1.0	**0.07**	9.2	9	0.2	0.02
5	10	0.2	0.01	9.3	9	1.0	0.02
6	10	0.6	0.04	9.4	9	0.6	0.04
7	10	0.2	0.01	9.5	9	0.8	0.04
8	06/06	0.2	0.00	10.1	Dairy	3.6	0
9	07/06	0.4	0.01	10.2	Dairy	2.4	0
10	08/06	0.4	0.01	10.3	Dairy	0.6	0.04

Sample Winter	Farm	Vicam® IAC (ppb)	HPLC (ppb)	Sample Summer	Farm	Vicam® IAC (ppb)	HPLC (ppb)
11	09/06	0.4	0.01	10.4	Dairy	0.4	0
12	10/06	0.2	0.01	10.5	Dairy	0.4	0

Sampling season/ Method	n	Mean (ppb)	Range (ppb)	Std. Dev	SEM
Vicam® IAC (winter)	45	0.49	0.200-1.60	0.323	0.0511
HPLC (winter)	45	0.14	0.028-1.32	0.217	0.0343
Vicam® IAC (summer)	45	0.82	0.000-6.40	1.192	0.1880
HPLC (summer)	45	0.16	0.000-1.54	0.283	0.0448

Vicam® IAC: Immuno-affinity

HPLC: High Performance Liquid Chromatography

Std Dev: Standard Deviation

SEM: Standard Error of Mean

n.a: not analysed

The values which are highlighted in Table 12 exceed the legislated level of permitted aflatoxin M_1 contamination as prescribed by South African legislation.

There is a slight difference in the values of aflatoxin M_1 contamination reported during winter as compared to the summer results. The highest reported level of aflatoxin M_1 contamination in farm gate milk during the winter sampling was 1.32 ppb from farm 8 which is 26 times the permitted limit. This trend was repeated during summer when the highest level of aflatoxin M_1 contamination was also from farm 8.

The statistical evaluation was based on 90 comparative seasonal farm gate results. Based on the Vicam® immuno-affinity results reported above, statistically, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is *not* a statistically significant difference between the input groups (P = 0.097).

Based on the HPLC results reported statistically, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is *not* a statistically significant difference between the input groups (P = 0.746).

The mean value of aflatoxin M_1 based on the confirmatory result obtained by HPLC analysis indicates that the levels of aflatoxin M_1 contamination found in milk during winter and summer did not differ much, although relatively high rates of contamination were reported in the feed especially during summer.

The mean value of 0.14 (winter) and 0.16 (summer) of aflatoxin M_1 contamination found in the farm gate milk samples, based on the confirmatory result obtained by HPLC analysis, indicates that the milk exceeds the legislated permitted level of 0.05ppb of aflatoxin M_1 as allowed by South African law. The results of the winter farm gate milk are summarised in Figure 52.

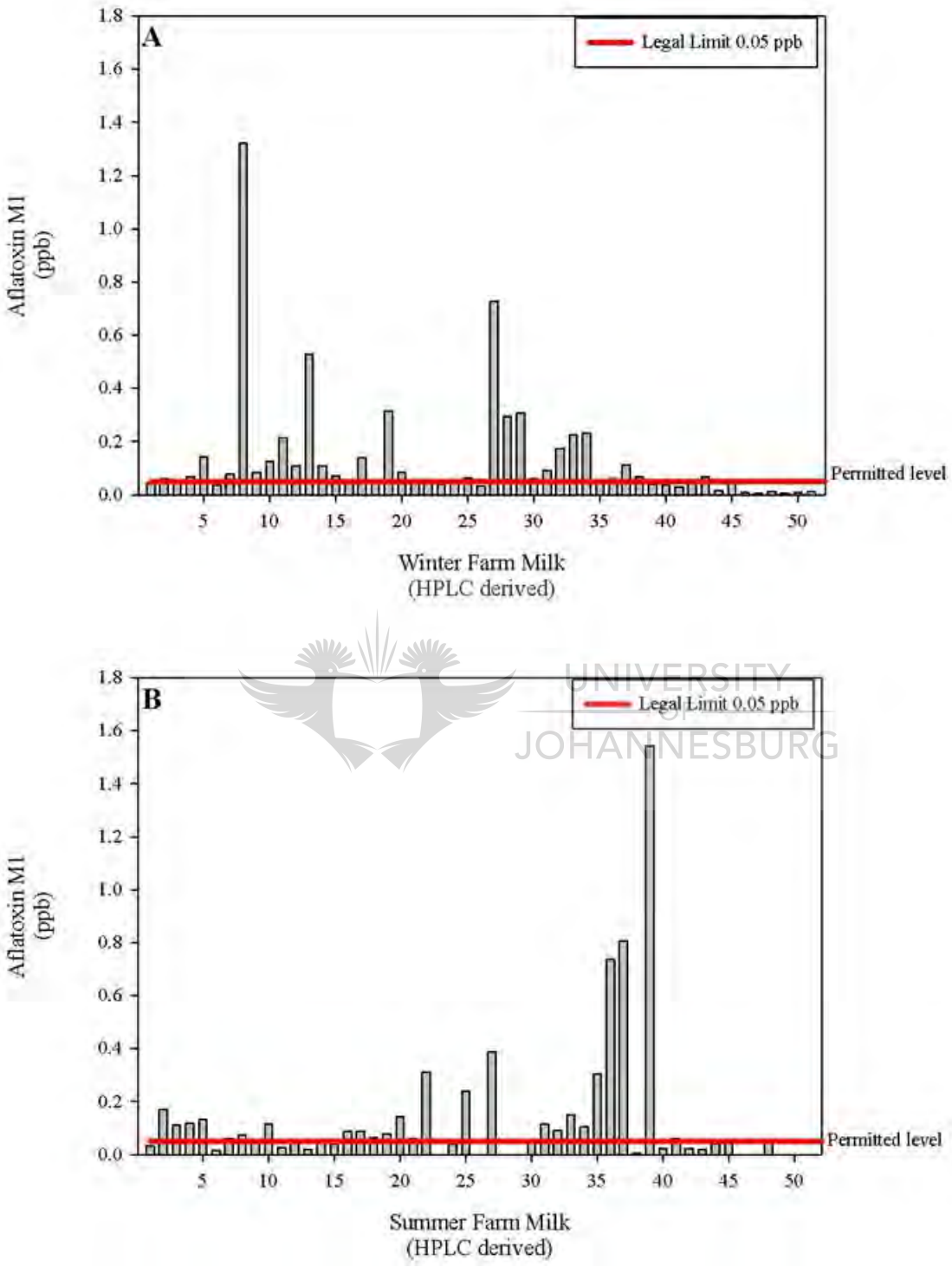


Figure 52: The quantification of aflatoxin M_1 (HPLC derived) in farm gate milk samples together with the permitted legislated level as reported in ***winter*** (A) and ***summer*** (B)

The high levels of contamination in the feed ingredients have definitely affected the farm gate milk, resulting in milk contaminated with aflatoxin M_1 with values exceeding the stipulated levels permitted by South African legislation. It must however be noted though that the contamination values obtained are well below the permitted levels allowed by CODEX of 0.5 ppb.

As seen in Figure 52, there is much similarity in the summer and winter mean values of aflatoxin M_1 contamination in farm gate milk samples analysed. It was found that from the 102 samples analysed, 53 (52%) of the samples exceeded the permitted aflatoxin M_1 contamination level of 0.05ppm. Whilst it is true that the effects of bulking up, is likely to reduce the level of contamination, there still is a serious concern about the high levels of aflatoxin B_1 contamination in the feed which acts as a precursor of aflatoxin M_1 in milk.

4.2.3.2 Retail Milk Surveillance Studies

There were 23 selected brands of commercially available milk analysed during this study. Pasteurised full fat, low fat and fat free variants of milk of the same brand were analysed. Selected brands of ultra high temperature processed milk and two brands of infant formulae were also analysed.

Table 13 shows a summary of the aflatoxin M_1 quantification values obtained from extraction with Vicam® immuno-affinity and confirmed by high performance liquid chromatography coupled with UV detector and CoBrA cell.

Table 13: A summary of the aflatoxin M_1 quantification values and statistical analysis results of seasonal retail milk, obtained from Vicam® immuno-affinity and high performance liquid chromatography coupled with UV detector and CoBrA cell.

Sample Winter	Descriptor Milk	Vicam® IAC (ppb)	HPLC (ppb)	Sample Summer	Descriptor Milk	Vicam® IAC (ppb)	HPLC (ppb)
1	Organic	0.8	0.05	1	FC	0.4	0.00
2	LF	3.2	**0.48**	2	LF	0.4	0.01
3	LF	1.2	**0.23**	3	LF	0.4	0.04
4	FC	0.8	**0.31**	4	FC	0.4	0.03
5	FC	1.2	**0.11**	5	FC	0.2	0.01
6	LF	0.8	**0.20**	6	LF	0.4	0.02
7	FC	0.8	**0.10**	7	FC	0.8	**0.11**
8	FC	1.2	**0.22**	8	LF	0.8	0.03
9	LF	1.2	**0.27**	9	FC	0.2	0.02
10	LF	0.8	**0.44**	10	FC	0.2	0.00
11	FC	2.8	**0.19**	11	FC	0.4	0.01
12	2% fat	2.0	**0.04**	12	2% fat	0.8	0.01
13	FC	1.2	**0.07**	13	FC	0.2	0.03
14	FC	1.6	**0.05**	14	FC	1.2	0.02
15	FC	1.2	**0.11**	15	FC	0.4	0.01
16	Fat free	1.6	**0.13**	16	FC	0.2	0.04
17	FC	1.2	**0.07**	17	FC	1.2	0.01
18	Infant formula	1.6	0.02	18	LF	0.4	**0.06**
19	Infant formula	1.2	0.02	19	Fat free	0.4	0.01
20	Fat free	1.2	**0.10**	20	LF	0.4	0
21	FC	1.0	**3.07**	21	LF	0.4	0.01
22	2% fat	0.4	**0.07**	22	Infant formula	3.2	0
23	FC	0.8	**0.06**	23	Infant formula	0.4	0.04

Sampling season/ Method	n	Mean (ppb)	Range (ppb)	Std. Dev	SEM
Vicam® IAC (winter)	23	1.29	0.40-3.20	0.64	0.13
HPLC (winter)	23	0.27	0.02-3.07	0.62	0.13
Vicam® IAC (summer)	23	0.60	0.20-3.20	0.63	0.13
HPLC (summer)	23	0.02	0.00-0.11	0.02	0.0

LF: low fat milk

FC: full cream milk

Vicam® IAC: Immunoaffinity

HPLC: High Performance Liquid Chromatography

Std Dev: Standard Deviation

SEM: Standard Error of Mean

The values which are highlighted in Table 13 exceed the legislated level of permitted aflatoxin M_1 contamination as prescribed by South African legislation. Further, Table 13 shows that from

46 samples of the selected retail brands analysed, 44 (96%) of the samples analysed, show some level of contamination by aflatoxin M_1.

The statistical evaluation was based on 23 comparative seasonal retail milk results. Based on the Vicam® IAC results statistically, the difference in the mean values of the two groups is greater than would be expected by chance; there *is* a statistically significant difference between the input groups (P = <0.001). Based on the HPLC results statistically, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is *not* a statistically significant difference between the input groups (P = 0.055).

The results of the seasonal surveillance studies from the statistical analysis are graphically represented in Figure 53 and it is clear that the contamination is higher in winter in comparison to summer, by almost a ten-fold increase.

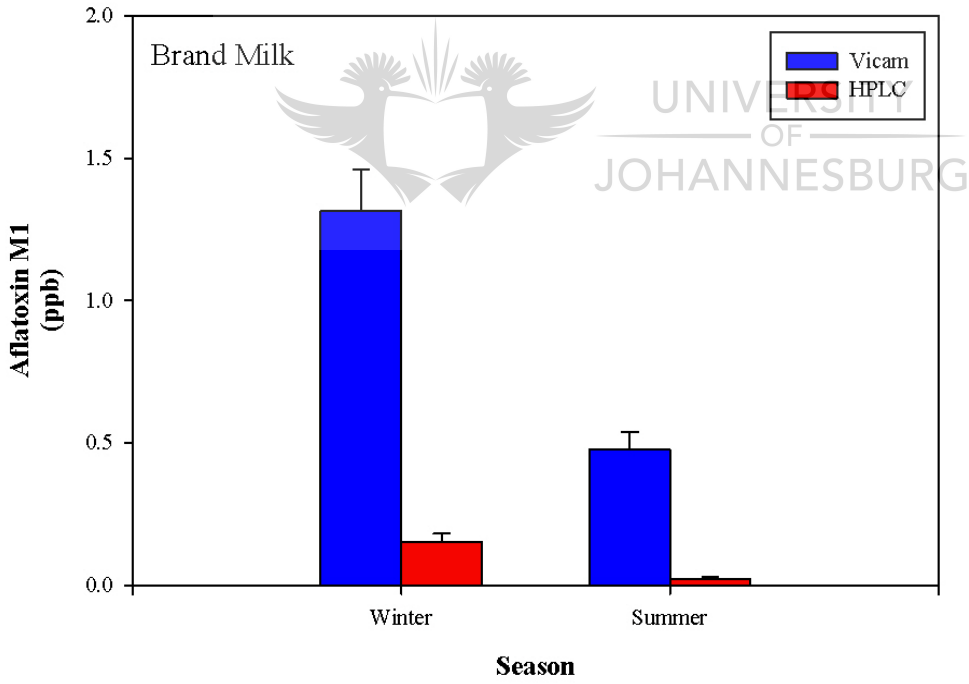


Figure 53: The average contamination levels of aflatoxin M_1 during ***winter*** and ***summer*** in selected retail milk available in South Africa

A comparison of the same branded retail milk levels of aflatoxin M_1 contamination during winter and summer is depicted in Figure 54.

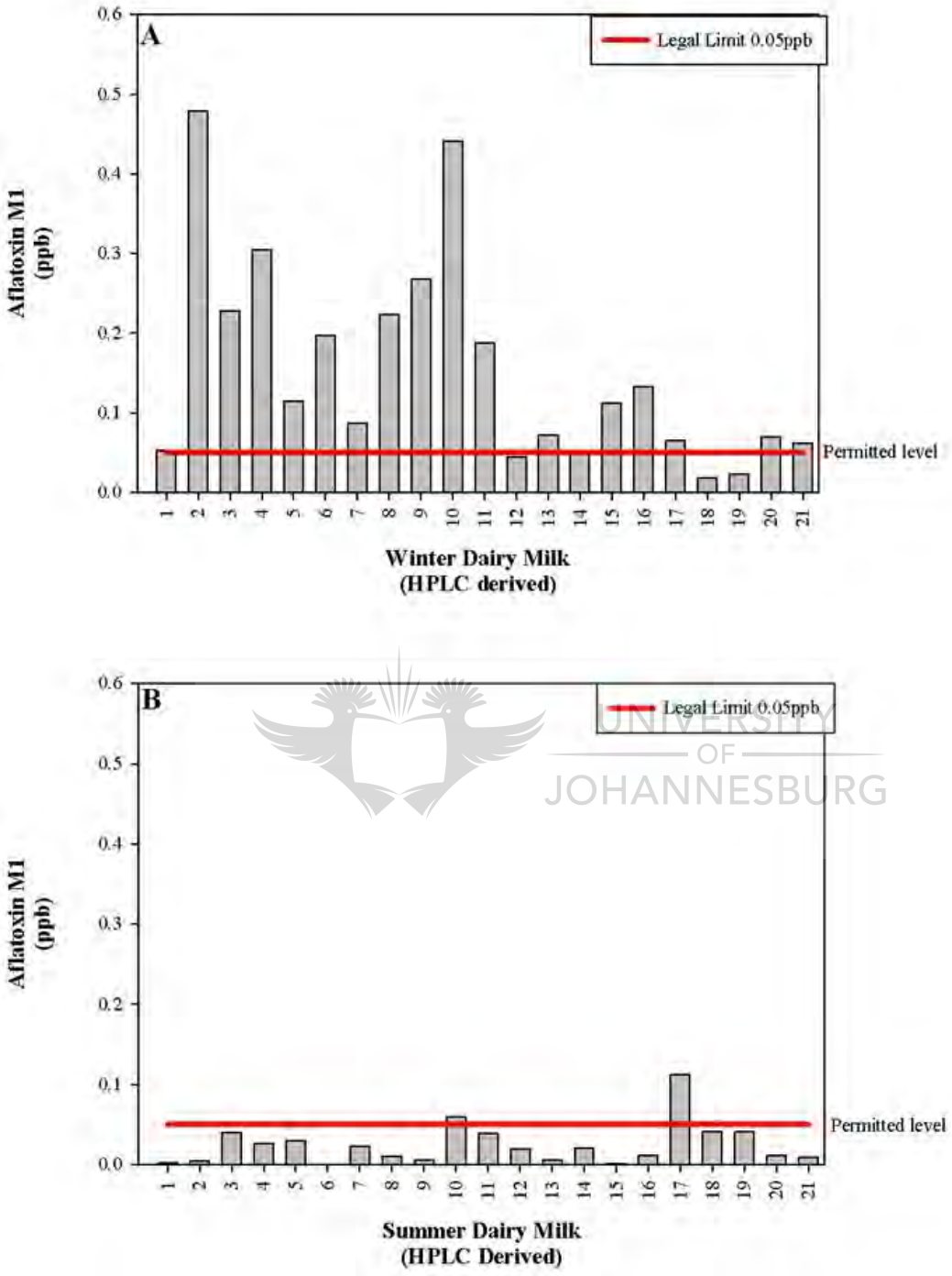


Figure 54: The quantification of aflatoxin M_1(HPLC derived) in retail milk sample together with the permitted legislated level as reported in ***winter*** (A) and ***summer*** (B)

During the winter surveillance study, 23 (100%) of samples tested positive for aflatoxin M_1 contamination whilst 21 (91%) tested positive during summer. Furthermore 18 (78%) of samples of commercial branded in South Africa analysed during the winter, was found to exceed the legislated permitted level of 0.05ppb of aflatoxin M_1. Only 5 (22%) of samples analysed were within the South African prescribed legislated level of aflatoxin M_1.

During the summer surveillance study, only 2 (9%) of samples of commercial branded milk analysed was found to exceed legislated permitted level of 0.05ppb of aflatoxin M_1. The remainder of the samples, 21 (91%) of milk was within the South African prescribed legislated level of aflatoxin M_1.

Upon comparison of Figures 54 (A) and (B) it is clear that the level of aflatoxin M_1 contamination is far greater during the South African winter season than in the summer season, and this was further confirmed by the statistical analysis in Table 13.

4.2.3.3 Various selected methods used to analyse for Aflatoxin M_1

Selected milk samples from the summer surveillance study were analysed using three various methods to determine if a correlation amongst the methods could be determined.

The results of the summer surveillance study are shown in Table 14. In this case, Vicam® immuno-affinity (IAC) and Biotage® solid phase extraction (SPE) were used as the extraction method with final confirmation by HPLC, and Ridascreen® ELISA was used as the third method.

Table 14: A summary of the aflatoxin M_1 quantification values and statistical analysis results of the summer retail milk obtained from the various methods of clean–up and final analysis by high performance liquid chromatography

Sample Summer	Descriptor	Vicam® IAC HPLC (ppb)*	Biotage® SPE HPLC (ppb)*	Ridascreen® ELISA (ppb)*
1	FC	0.00	0.02	**0.06**
2	LF	0.01	**0.15**	**0.11**
3	LF	0.04	**0.13**	**1.38**
4	FC	0.03	**0.12**	**1.05**
5	FC	0.01	0.05	**1.56**
6	LF	0.02	**0.09**	**0.13**
7	FC	**0.11**	**0.08**	**0.89**
8	LF	0.03	**0.09**	**2.05**
9	FC	0.02	**0.16**	**0.10**
10	FC	0.00	**0.18**	0.03
11	FC	0.01	**0.24**	**0.55**
12	2% fat	0.01	**0.13**	**0.71**
13	FC	0.03	**0.12**	0.01
14	FC	0.02	0.02	0.04
15	FC	0.01	**0.07**	**0.45**
16	FC	0.04	0.02	**0.19**
17	FC	0.01	0.03	**0.31**
18	LF	**0.06**	**0.09**	**0.10**
19	Fat free	0.01	0.01	0.00
20	LF	0.00	**0.15**	0.05

Method	n	Mean(ppb)	Range (ppb)	Std. Dev	Std. Error
ELISA	20	0.48	0.00-2.05	0.60	0.134
Biotage® SPE	20	0.09	0.01-0.23	0.06	0.013
Vicam® IAC	20	0.02	0.00-0.11	0.02	0.005

FC: full cream milk LF: low fat milk

Vicam ®IAC: Immuno-affinity column HPLC: High Performance Liquid Chromatography

Biotage® SPE: Solid Phase Extraction Ridascreen® ELISA: Enzyme Linked Immuno- Assay

*ppb: for purpose of comparison, the ELISA results in this table are shown in ppb values only.

The results reported by Vicam® IAC and Ridascreen® ELISA indicate that 95 % of milk analysed were found to be contaminated with aflatoxin M_1, whilst Biotage® SPE reported that 100% of samples analysed were found to be contaminated by aflatoxin M_1. Some levels of AFM_1 contamination reported by ELISA were extraordinary high, recording values of between 1.05-2.05 ppb.

The results reported from Vicam® IAC indicate that 2 (10%) samples from the selected 20 retail milk were found to exceed legislation of 0.05ppb whilst results reported from Biotage® SPE indicate that 14 (70%) samples exceed the legislation, however the results reported from Ridascreen® ELISA report that 15 (75%) of samples exceed legislation.

Selected milk samples which were not contaminated with mycotoxins were used for mycotoxin recovery using mycotoxins standards. The samples were spiked with known levels of mycotoxin standards and analysed with the other samples as per the documented methodology.
The results are shown in percentage recoveries of standard toxins, in Table 15 below.

Table 15: Mycotoxin recovery from samples which were spiked with mycotoxin standards with using Vicam® fluorometry assay and solid phase extraction

Aflatoxin standard	**Spiked concentration/g in milk**	**Means recovery (µg)**	**Percentage (%)**
Aflatoxin M_1 Vicam® IAC	100ng/10ml milk*	98 (±1.3)	98
Aflatoxin M_1 Biotage® SPE	100ng/10ml milk*	65(±(5.4)	68

Table 15 shows a summary of the mean recoveries obtained from the milk samples spiked with aflatoxin standards using high performance liquid chromatography. The recovery rate was adequately sufficient to continue with the analysis of the milk samples using the HPLC coupled with UV detector and CoBrA cell.

Based on statistical information obtained, the differences in the mean values among the treatment groups are greater than would be expected by chance; there *is* a statistically significant difference (P = <0.001).
In Figure 55, a graphical correlation is shown between the samples which were extracted using *Vicam® immuno-affinity column* and confirmed using HPLC analysis, with samples which were extracted using *Biotage® solid phase extraction* and confirmed using HPLC analysis.

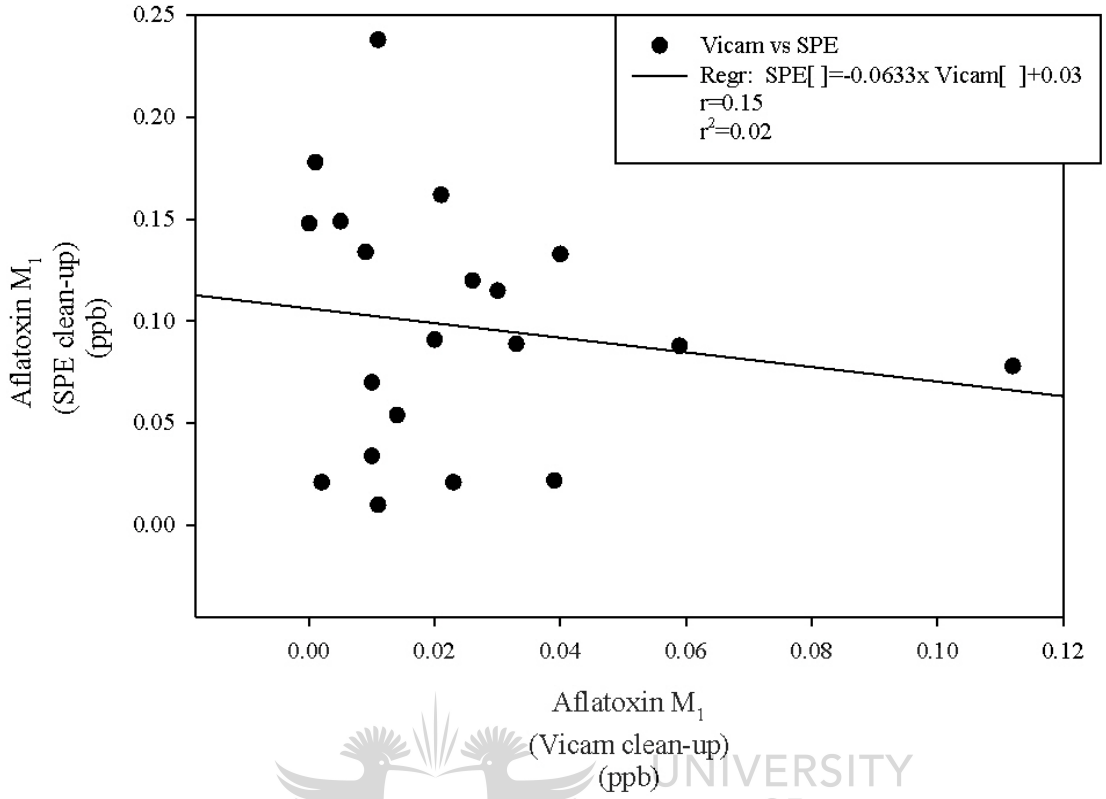
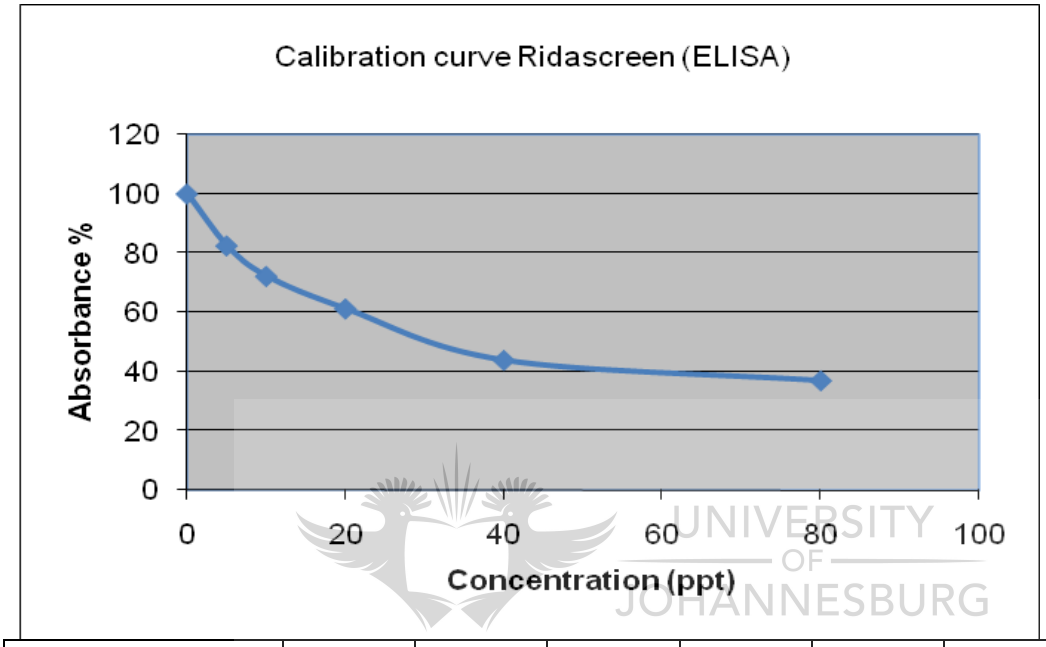


Figure 55: The quantification of aflatoxin M_1 in the ***summer*** retail milk samples showed in a correlation graph using Vicam® IAC and Biotage® SPE

This correlation was performed on 20 samples. The value of the correlation co-efficient is -0.151 thus, a weak negative correlation exists as the value is close to **0**. The 'P' value is 0.526 which means that the change that the correlation is false is high because 'p' value approaches 1. There was no thus ***no correlation*** between the data obtained.

A similar validation of Ridascreen® ELISA as used by Mohamadi-Sani *et al.* (2010), was performed prior to analysis of the samples to ensure data quality. Validation was carried out through the determination of recoveries and the mean variation co-efficient for fresh milk spiked with different concentrations of AFM_1 (10, 20, 40 and 80ng/L). The calibration curve for ELISA is shown in Figure 56. The absorbance values obtained for the standards and the samples were divided by the absorbance value of the first standard (zero standards) and multiplied by 100 (percentage maximum absorbance). Therefore, the zero standard is made to equal 100% and the

absorbance values are quoted in percentages as shown in Figure 56. The values calculated for the standards were entered in a system of co-ordinates on semi-logarithmic paper against the AFM_1 concentration in parts per trillion (ppt). The absorbance for the standards may decrease during the shelf life of the kit, so the general shape of the calibration curve recorded will remain similar, whilst the slope might change slightly. (The reporting of results from ELISA is usually done in units of ppt so the calibration curve and ELISA results graph will be displayed in ppt units).



Standard	Std 1	Std 2	Std 3	Std 4	Std 5	STD 6
Conc (ppt)	0.0	5	10	20	40	80
% Absorbance	100	82	72	61	44	37

Figure 56: Calibration curve obtained from the Ridascreen® ELISA aflatoxin M_1 test kit together with the reference values

A regression value of: $y = - 0.704 + 84.26$

$R^2 = 0.791$

This value shows good agreement (R^2= 0791) indicating that the matrix effect on the ELISA assay could result in a slight over-estimation of the aflatoxin content as shown in Table 16.

Table 16: A summary of the aflatoxin M_1 quantification values of the standards and summer retail milk obtained from ELISA

Sample Identification	Percentage absorbance	Concentration of AFM_1 (ppt)
Standard 1	100	0
Standard 2	82	5.0
Standard 3	72	10.0
Standard 4	61	20.0
Standard 5	44	40.0
Standard 6	37	80.0
Sample 1	91	2.5
Sample 2	85	4.5
Sample 3	40	**55.0**
Sample 4	43	**42.0**
Sample 5	39	**62.5**
Sample 6	83	5.0
Sample 7	46	**35.5**
Sample 8	35	**82.0**
Sample 9	86	4.0
Sample 10	97	1.0
Sample 11	59	**22.0**
Sample 12	52	**28.5**
Sample 13	99	0.5
Sample 14	95	1.5
Sample 15	63	**18**
Sample 16	76	**7.5**
Sample 17	69	12.5
Sample 18	88	4.0
Sample 19	108	0.0
Sample 20	93	2.0

The results shown in the Table 16, report that 95% of summer selected retail milk samples show some level of contamination by aflatoxin M_1. The percentage absorbance and AFM_1 concentration values are shown in Figure 57.

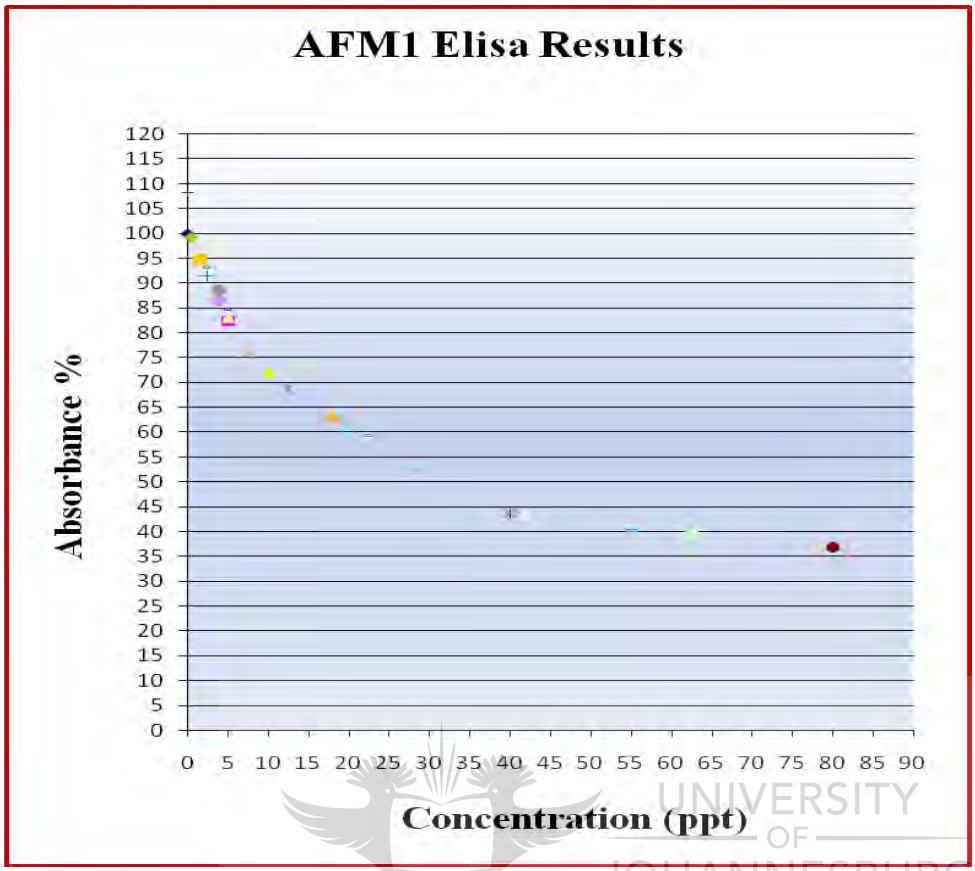


Figure 57: The quantification of aflatoxin M_1 in the ***summer*** retail milk obtained by using ELISA

The Ridascreen® ELISA method reported values of between 0 and 82 ppt concentration levels of aflatoxin M_1 contamination in the selected summer retail milk. As mentioned prior the results reported by Ridascreen® ELISA indicate that 75% of selected retail milk sampled during summer was found to exceed the South African permitted legislative of 0.05 ppb.

The statistical correlation performed on 20 samples to determine the correlation co-efficient between *Biotage® SPE* and *Ridascreen® ELISA* results was valued at 0.0459, thus a weak correlation exists. There was no thus ***no correlation*** between the data obtained.

The statistical correlation performed on 20 samples to determine the correlation co-efficient between *Ridascreen® ELISA* and *Vicam® IAC* was reported to be 0.269, thus a weak correlation exists. There was no thus ***no correlation*** between the data obtained.

The p value was 0.251 further confirming and concluding that there are no significant relationships between any pair of variables in the correlation table (P >0.050) thus ***there is a significant statistical difference which exists amongst the various methods used to analyse for aflatoxin M_1 contamination in milk during this study.***

4.2.3.4 Lateral Flow Devices

It was not economically feasible due to budget constraints to test all 20 summer surveillance samples using the Charm® system. However, the few samples which were tested were comparable in qualitative results when compared to the results obtained from Vicam® IAC on the same set of samples.

It is important to note that the qualitative interpretation of the concentration is relative to the EU MRL of 50 ppt. So a result less than 40 ppt is a **negative result.** A result greater than or equal to 40 ppt is a **positive result.** A summary of the comparative AFM_1 values is shown in Table 17.

Table 17: A summary of the aflatoxin M_1 quantification values of the summer retail milk obtained from Charm® and clean up by Vicam® immuno-affinity with final confirmation by high performance liquid chromatography.

Sample identification	**Charm® (ppt)**	**Vicam® IAC HPLC (ppt)**
Sample 3 (23.11.2010)	43 positive	40ppt
Sample 4 (23.11.2010)	**57 positive**	**30ppt**
Sample 5 (23.11.2010)	24 negative	10ppt
Sample 11 (23.11.2010)	23 negative	10ppt
Sample 13 (23.11.2010)	0 negative	30ppt
Sample 14 (23.11.2010)	23 negative	20ppt

Vicam® IAC: Immuno -affinity

HPLC: High Performance Liquid Chromatography

Based on the information supplied in Table 17, only 1 sample (sample 4) from a total of 6 samples seems to be inconsistent when compared to the confirmatory results of high performance liquid chromatography analysis. Thus it is clear that this on-site method is very efficient for use as on-site testing unit, however, it is important to note that confirmation by high performance liquid chromatography is required for the final determination of results.

The supplier of the Idexx Snap® system allowed the use of both the equipment and 20 test strips for the analysis at no charge and the results are recorded in Table 18. As mentioned earlier the Snap® system detects aflatoxin M_1 at or below 0.5ppb.

Table 18: A summary of the aflatoxin M_1 quantification values of the summer retail milk obtained from Snap® and Vicam® immuno-affinity with final confirmation by high performance liquid chromatography

Sample Identification	**Snap® ratio & result**	**Vicam® IAC HPLC (ppb)**
1	0.87 negative	0.002
2	0.83 negative	0.005
3	0.83 negative	0.04
4	0.71 negative	0.026
5	0.71 negative	0.014
6	0.67 negative	0.02
7	0.86 negative	0.112
8	0.73 negative	0.033
9	**1.37 positive**	**0.021**
10	0.59 negative	0.001
11	0.61 negative	0.011
12	0.89 negative	0.009
13	0.60 negative	0.03
14	0.45 negative	0.023
15	0.50 negative	0.01
16	0.86 negative	0.039
17	0.31 negative	0.01
18	0.26 negative	0.059
19	0.68 negative	0.011

IAC: Immuno-affinity column

HPLC: High Performance Liquid Chromatography

Based on the information supplied in Table 18, only one sample (sample 9) from a total of 19 samples seems to be inconsistent when compared to the confirmatory results of high performance liquid chromatography analysis, thus it is clear that this method / apparatus is very efficient as use as on-site testing unit.

5. DISCUSSION

New research findings into which food crops may be used to feed livestock occurs often, so there are almost no restrictions as to which food ingredients may be used for animal feed. However as cattle consume a wide array of feed ingredients they are also exposed to a wider array of mycotoxins, than are mono-gastric animals (Whitlow *et al.,* 2010).

Smith & Moss (1985), confirm that it is important to screen commodities for fungal contamination as a complementary exercise to mycotoxins detection. There are three genera of mycotoxin producing filamentous fungi which are commonly found in food commodities namely *Aspergillus, Fusarium* and *Penicillium* (Boutrif & Canet, 1998).

Silage is used by farmers as cattle feed especially during the winter when natural forage is scarce. During the winter sampling of feed ingredients, a silage pit which was not adequately packed and covered, was found to be contaminated with fungal spoilage as shown in Figure 58. Microbiological analysis of the contaminated silage sample and fungal identification by the Faculty of Biological and Agricultural Sciences of the University of Pretoria indicated that *A. fumigatus, F. oxysporum and O. truncatum* were present in the silage sample. Both *A. fumigatus* and *F. oxysporum* are known to produce various mycotoxins.

Figure 58: A photograph showing mould contaminated silage sampled during winter

Distiller's grains or brewer's meal is a popular feed ingredient in South Africa especially since sorghum is a staple food and is also used in the production of a locally fermented rural beer, *umqombothi.* During the summer sampling of feed ingredients, temperatures of 38ºC

were experienced and at one particular farm, brewer's meal was found stored under a plastic sheet in the hot sun. Upon sampling the material which was very warm, mould growth was evident as shown in Figure 59, accompanied by a very unpleasant fermenting odour. This contaminated feed ingredient was being blended in the feed for the dairy cow and beef cattle.

Figure 59: A photograph of mouldy brewer's meal sampled during summer

The results of the microbiological analysis reported in this study is further supported by authors Bezuidenhout *et al.* (1988); Dutton & Kinsey (1995); and Marasas *et al.* (1972, 1988) who reported that agricultural commodities and feeds in South Africa tend to be contaminated with the three common genera of *Aspergillus*, *Fusarium* and *Penicillium.*

Certain authors such as Cullen & Newberne (1993); Eaton *et al.* (1993) & Shapira *et al.* (1996) have reported that foods and feeds especially in warm climates are susceptible to invasion by aflatoxigenic *Aspergillus* species as well as the subsequent production of aflatoxins during pre-harvesting, processing, storage and transport. The temperatures experienced in countries on the African continent are sufficiently hot and humid to support the growth of aflatoxigenic fungi.

Mycotoxin analysis

Feed

The prolonged storage of feed ingredients especially if coupled with ecological factors such as high temperature and high relative humidity, provide the ideal conditions for fungi to develop. The type of feed ingredients used and the seasonal effects from the country of origin of feed, are all factors which have an effect on the levels of aflatoxin contamination in feed (Dashti *et al*. 2009). The amount of aflatoxin B_1 present in contaminated feed is usually between 1% and 6% , however values as high as 6% has been reported too (Pittet,1998).

The statistical analysis results obtained from the feed ingredients analysed, indicate a serious issue regarding the heavy contamination of the feed ingredients with regards aflatoxins B_1 contamination. With mean values of 138.452 ppb recorded for the winter results and 230.919 ppb obtained for the summer results, it is clear that aflatoxin B_1 contamination in cereals, legumes and oil seeds are of a serious concern posing a major health hazard to both the South African public and livestock. Needless to say, these aflatoxin B_1 quantification values far exceed the permitted legislation levels of 5 ppb as permitted by Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947(Act No.36 of 1947).

During the summer sampling, the temperatures recorded in the areas being sampled ranged between 35°C and 38 ºC. The marginally higher mean value in summer may be as a direct result of these extreme temperatures. Most of the storage sheds used on dairy farms as shown earlier in this review may not be adequately enclosed, ventilated and protected against the harsh elements of nature. Seasonal rains can initiate mould growth in feed ingredients which are not stored under-cover, and fungal spores may be carried by wind settling on feed ingredients resulting in possible mycotoxin development. Also, the respiration of feed ingredients tends to occur due to the increased environmental temperatures, thus creating hot spots which may very well support the growth of storage fungi, which may produce a range of mycotoxins.

Even though the mean value of the feed is reported to be marginally higher in summer than in winter, the range of contamination should be taken into account too. Certain animal feed ingredients contained very low amounts of aflatoxin B_1, whereas others ingredients were found to be heavily contaminated. As the farmer blends a range of ingredients during the

preparation of the total mixed ration (TMR), it may be that the ingredient which is the most contaminated, for example cottonseed, be used sparingly thus contributing to an overall reduced level of aflatoxin B_1 in the final mix. Or the farmer may use a greater proportion of an ingredient such as maize which is known to be frequently contaminated by aflatoxin B_1.

The aflatoxin B_1 contamination values of cotton seed during this study was found to range between 0 and 741.3 ppb, whilst the sunflower and sunflower oil cake contamination levels ranged between 0 and 1348 ppb. Furthermore, it was also found that soya and soya bean oil cake were contaminated with aflatoxin B_1 at levels ranging between 0 and 265.9 ppb. Mellon & Cotty (1998) have outlined an explanation as to why cottonseed and in general, oil seed and by-products of the refining process such as sunflower and soya bean oil cake are usually found to be heavily contaminated with aflatoxin B_1. These authors reported that aflatoxin stimulation by storage proteins in the presence of carbohydrate may occur in the developing cottonseed, as lipids present in the developing cotyledons (location of storage reserves) may provide an easily accessible carbon source. Mature cottonseed contains up to 10% raffinose by weight, this storage tri-saccharide may also drive aflatoxin production when associated with storage protein as in cottonseed. The overall close proximity of storage proteins to accessible carbon resources (in the form of carbohydrate or oils) may explain the copious production of aflatoxin in developing oilseeds under certain conditions. This could explain the extraordinary high levels of aflatoxin B_1 found in cottonseed, soya and sunflower oil seed products during this study.

The extreme climatic conditions we are currently experiencing in South Africa, is likely to result in a reduction in the amount of natural forage available. This lack of natural forage will result in the more frequent use of prepared animal feeds, which is usually in the form of silage, compound feeds or a combination of grains and legumes which have all been found to be sources of aflatoxin B_1 contamination in this study.

Dashti *et al.* (2009) reported that aflatoxins contamination was evident in lucerne pallets sampled in Kuwait. In this instance, the aflatoxin B_1 concentrations of contamination recorded ranged between 4.0 and 22.9 ppb. However a mouldy sample of lucerne which was sampled at one of the larger farms reported an aflatoxin B_1 contamination value over 900ppb, so in this instance the fungal contamination was accompanied by aflatoxin production.

The mouldy brewer's grains as shown earlier in Figure 59 was reported to contain only 8.71 ppb of aflatoxin B_1, whilst another sample which did not show any signs of visible fungal growth was found to be more severely contaminated with a value of 452.84 ppb aflatoxin B_1.

The silage sourced from the farm where the mould contamination was evident in the silage pit as shown in Figure 58, was a major source of aflatoxin B_1 contamination reporting a value of 145.7 ppb. Silage sourced from farm 7 did not show any signs of mould contamination, however the level of aflatoxin B_1 was found to be extraordinary high at 421.95 ppb. Most of the silage sampled were maize based, however even cowpea silage from farm 9 was found to be contaminated with aflatoxin B_1 at a quantification value of 14.6 ppb.

The effects of contamination with regards to lucerne, brewer's grains and silage as discussed above is explored by the authors Smith & Moss (1985), who highlight that although mould growth may be present in a sample it does not confer the presence of mycotoxins and whilst no growth of mould may be visible, it does not mean that mycotoxins are absent. This situation poses an even greater threat to global food safety with regards mycotoxins contaminated foodstuff, especially in developing countries where there is a lack of infrastructure and technical skill to support the strict quality control and quality assurance practices.

A clinical trial involving the feeding of cows with mould contaminated silage showed that the animal experienced a reduced filling of the rumen, poor feed conversion and mild diarrhoea. Furthermore, it was also noted that reduced milk production occurred followed by an increased incidence of sub-clinical mastitis with raised somatic cell counts, which contributed to further milk losses (Hadley *et al.* 2006; Wenz *et al*. 2007).

A level of 179.2 ppb of aflatoxin B_1 contamination was found in maize chop from a smaller farm, where the maize was stored in an on-site silo, and 159.4 ppb aflatoxin B_1 was reported in maize from a larger farm, where the maize was also stored in an on-site silo. It is possible that the contamination by aflatoxins could be accelerated due to improper ventilation issues as none of these silos had aeration units to cool the grains. According to Fink-Gremmels (2008), the increasing use of maize and maize by-products which are contaminated with aflatoxins in ruminant diets tends to induce a subclinical chronic impairment of liver function and hence may contribute to hepatic lipodosis following Negative Energy Balance (NEB). It is reported that even low amounts of aflatoxins are able to affect the cellular and humoral

immune system resulting in an increased susceptibility to infectious diseases of exposed animals (Marin *et al.*, 2002).

Based on the Vicam® IAC results, statistically, the difference in the mean values of the two groups of the winter feed results and the summer feed results, is greater than would be expected by chance; there *is* a statistically significant difference between the input groups (P = <0.001). A summary of the statistical analysis on the feed samples indicate that based on the final confirmatory results obtained from the high performance liquid chromatography analysis, statistically, the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is *not* a statistically significant difference between the input groups (P = 0.395).

The sampling of winter feed occurred during the cooler month of May, so the temperatures and relative humidity were fairly low in comparison to the extreme temperatures experienced during the summer sampling. In this study, there may be a double barrel effect, where feed ingredients sampled during the summer may have been subjected to initial contamination by aflatoxins in storage during the previous summer season. As a result of inappropriate storage during the hot humid climatic conditions as experienced during the summer sampling, this effect may thus be further exacerbated. The big difference in mean values between the seasons is a clear indication of improper and inadequate ventilation of the storage of feed ingredients, which supports the development of aflatoxin contamination.

The results obtained using thin layer chromatography (TLC) was not very clear in demonstrating the presence of aflatoxins in the respective feed samples. One of the explanations for this may be that because a very small amount of extract is used for the plating, this then equates to a very minimal amount of aflatoxin compound actually present in the extract. The aflatoxins present in the extract can be recovered for instrumental analysis, but there may be insufficient for a further reaction on a TLC plate. Also, although optical and spectroscopic methods can be used to sensitively detect the sample spots in terms of their visible color, UV absorption, and fluorescence excitation spectra, these methods are not comprehensive and such detections are restricted by the chemical or optical properties of the analytes. Even if the values of R_f and the spectroscopic characteristics of the sample and standard are compatible, the capability to determine the molecular structure of the analyte by these detection techniques remain low (Cheng *et al.*, 2011).

The differences in the level of contamination of the feeds destined for dairy cattle should be a factor to consider as the same batch of raw material may present varied amounts of mycotoxin contamination. The contamination of feed ingredients is also likely to vary according to the area where it was sampled, certain climatic differences as well as prevailing temperature. As discussed earlier the raw materials which are used for feed or in the production of feed, are grown in various countries so the source of supply will always differ, and so will the level of contamination by aflatoxins.

These heavily contaminated feeds impact on the health of the dairy cow affecting milk production and milk quality. According to author Fink-Gremmels (2008) it is generally assumed that within the rumen, the protozoal population has the highest capacity to detoxify ingested mycotoxins, but this may vary between different chemical classes of mycotoxins. Also, the contribution of bacteria and other rumen organisms might have been under-estimated as generally only the overall capacity to degrade a given mycotoxin has been tested.

Fink-Gremmels (2008) further documents that in controlled experiments animals are challenged with one individual or defined group of toxins and generally show a high tolerance to contaminated feeds. In contrast, under field conditions dairy cows are exposed to complex mixtures of toxins originating from roughages and concentrates. The detoxifying capacity of rumen micro-flora then becomes exhausted and the unchanged mycotoxins are absorbed via the duodenum creating an unexpectedly high internal challenge, which results in aflatoxin M_1 residues being found in milk.

Binder *et al*. (2007) reported that the following practices may assist to reduce the effects of mycotoxin contamination in feed and feed ingredients. The blending of contaminated feed with non contaminated feed, re-routing contaminated grain to less susceptible animal species, and the addition of feed additives based on adsorption and enzymatic modes of action are all potential strategies to reduce mycotoxin induced performance impairment.

Farm gate milk analysis

Stoloff (1977) reported that aflatoxin M_1 was detected in milk between 12 to 24 hours following the first ingestion of feed contaminated with aflatoxins B_1. Certain authors have

also reported that through the process of elimination and degradation of toxins by rumen micro-flora, reduced quantities of aflatoxin M_1 may be found in milk (Sassahara *et al.,* 2005).

According to the statistical results obtained from the analysis of the farm gate milk samples, the mean value of aflatoxin M_1 contamination in winter was recorded at 0.14 ppb and 0.16 ppb aflatoxin M_1 was the reported mean value for the summer sampled farm gate milk. The range of aflatoxin M_1 contamination during winter was recorded to be between 0.028 and 1.321 ppb whilst the summer range reported values between 0 and 1.544 ppb.

The highest reported level of aflatoxin B_1 contamination in farm gate milk was 1.321 ppb from farm 8 during the winter sampling which is 26 times the permitted limit. It should be noted that the silage sampled from the same farm reported a value of 145.71 ppb aflatoxin B_1 as shown in Table 11, so in this case a bio-tracer analogy.

However, the effect of diluting and bulking milk during transport and prior to processing at the dairy facility resulted in the contamination level being lowered, as clearly shown in the aflatoxin M_1 quantification level recorded in Table 12 for the dairy processing facility. Values between 0 and 0.043 ppb aflatoxin M_1 were reported during the summer sampling and values between 0.06 and 0.07 ppb aflatoxin M_1 were reported during the winter sampling as shown in Table 13 (sample no 22 and 23).

A summary of the statistical results of the seasonal farm gate milk based on the Vicam® IAC reported for the winter and summer farm gate milk indicate that statistically the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is *not* a statistically significant difference between the input groups ($P = 0.097$). This result is mirrored when the high performance liquid chromatography results are summarised, indicating that there is *not* a statistically significant difference between the input groups ($P = 0.746$).

It should be noted that the summer sampled farm gate milk mean values, matched the trend of the higher mean values of the summer sampled feed with regards aflatoxin B_1 contamination. However if one aims to apply a bio- tracer approach, it is questionable as to why such a huge difference is apparent between the seasonal feed sample aflatoxin B_1 quantification values, versus the very small quantification values of aflatoxin M_1 especially during the summer period.

Further explanations may be offered in studies which have been undertaken to observe the effects of consumption of aflatoxin B_1 contaminated feed and formation of aflatoxin M_1 residues in milk. There seem to be many variables involved, which influences the carry–over i.e. the percentage of aflatoxin B_1 in feed that passes to milk as aflatoxin M_1 (Prandini *et al*., 2007).

The following has been reported by Masoera *et al.* (2007) and van Egmond (1989a):

- It seems that a high individual variability exists among the different species of milk producing animals with regards to carry-over (Battacone *et al.,* 2003).
- The difference in rumen degradation activity is a factor which affects carry-over (Westlake *et al*., 1989).
- The difference in rumen bio-transformation to aflatoxicol and other metabolites has a direct effect on the carry-over rate (Auerbach *et al*., 1998).
- The differences in the induction of the enzymatic aflatoxin B_1 oxidation system (Steiner *et al.,* 1990).
- van Egmond (1989a) also reported that at the beginning of lactation, the carry-over is between 3.3 and 3.5 times greater.
- It has also been suggested that an increase in aflatoxin M_1 occurs due to *Staphylococcus* udder infection (Veldman *et al*., 1992).
- Mammary alveolar cell membrane health influences carry-over as reported by Lafont *et al.,* (1983).
- It has been found that mastitis increases the number of somatic cells in milk, thus altering milk composition (Walstra & Jenness, 1984) and may affect the carry-over rate by increasing membrane permeability.
- It seems that cows ingesting a quantity of aflatoxin B_1 greater than 40µg/head/day produces milk with aflatoxin M_1 concentrations greater than 0.05µg/kg (van Egmond 1989a)

All of these factors may have therefore had an influence over the concentration of aflatoxin M_1 in the summer farm gate milk.

It could also be that maybe during the first sampling phase, the farmers became more aware of mycotoxins and the negative effects it presents on milk production and the health of the dairy cow. This may have motivated research and trials of adsorbents or other mycotoxin inhibiting agents, which may very well become a regular part of the animal's diet a few months before the second round of sampling occurred in summer.

Furthermore, according to Caggioni & Pietri (1999), van Egmond (1989a) and Veldman *et al*. (1992) the quantity of aflatoxin M_1 in milk decreases to almost zero within 2-3 days when a diet free of aflatoxin contamination is fed to the dairy cow. Also, as more natural forage becomes available during the warmer temperatures as experienced during spring and the onset of summer, it is very possible that the cows could have been fed on natural forage during or just prior to the summer sampling. This could further explain the low levels of aflatoxin M_1 residues found in milk.

A report by Rosi *et al.* (2007) indicated that the amounts of aflatoxin M_1 contamination present in milk samples which were frozen, thawed and analysed after 2 days showed lowered values of between 2% and 20% in comparison to corresponding fresh samples.

As mentioned earlier in the methodology, farmers were asked to sample milk over a five day period and freeze immediately. Analysis of farm gate milk occurred a few days later following a time lapse of approximately 10 days after the first sampling of milk. Is it possible that aflatoxin M_1 residues secreted in milk may be found in varied concentrations during the milking process? This would mean that a specific sampling protocol will need to apply to milk which is potentially contaminated with aflatoxin M_1 as this would certainly impact the quantification values obtained. Further research is however required in this field.

Surveillance study

Regular surveillance surveys are one of the most effective ways to determine the extent of contamination levels of aflatoxin contamination in food and feed commodities. There does not seem to be any reports available from the relevant regulatory authorities within South Africa, regarding any monitoring programs of aflatoxin contamination in milk or feed.

During the surveillance sampling, at least fourteen brands of milk were analysed. Each brand could consist of three different fat compositions, be of organic origin, or be subjected to ultra high temperature processing. All the samples were however homogenised and pasteurised and were of bovine origin only. At least 21 samples of commercial milk was analysed as shown in Figure 54.

The mean value of aflatoxin M_1 as based on the confirmatory result obtained by HPLC analysis indicates that the quantification value was higher at 0.278 ppb during the winter sampling period, in comparison to the summer sampling period which reported a contamination of 0.022 ppb. These seasonal differences in aflatoxin M_1 contamination concentrations are comparable with the findings of various authors such as Tajkarimi *et al*. (2007); Dashti *et al.* (2009); Bachner *et al.* (1990) and Blanco *et al.* (1988) who have reported that seasonal effect influences aflatoxin M_1 occurrence, with a higher incidence of contamination during cold seasons compared to hot seasons. Ruangwises & Ruangwises (2010) have also reported that the average concentration of aflatoxin M_1 in pasteurised milk samples collected in Thailand was significantly higher in winter, than during the summer.

A summary of the statistical results indicate that based on the Vicam® IAC results, statistically the difference in the mean values of the two groups is greater than would be expected by chance; there *is* a statistically significant difference between the input groups (P = <0.001). However based on the high performance liquid chromatography results, statistically the difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability and there *is not* a statistically significant difference between the input groups (P = 0.055).

The occurrence of aflatoxin M_1 in pasteurised milk and dairy products has also been reported in Argentina (López *et al.,* 2003), Brazil (Oliveira *et al*., 2006) India (Rastogi *et al*., 2004), Iran (Alborzi *et al.,* 2006, Kamkar, 2005), Japan (Nakajima *et al*., 2004), Korea (Kim *et al.,* 2000), Kuwait (Srivastava *et al*., 2001) and Turkey (Bakirci, 2001; Gürbay *et al.,* 2006). It must be noted as discussed earlier in this review, that the effects of various thermal treatments used to increase the shelf life of milk such as ultra high temperature (UHT) processing, pasturisation and sterilisation do not destroy aflatoxin M_1 (Galvano *et al*., 1996). During the seasonal surveillance studies aflatoxin M_1 contamination was found in powdered infant formulae too, meaning that the toxin may be resistant to the effects of spray drying.

The high performance liquid chromatography results of the winter surveillance study indicate that that all 23 (100%) of commercial milk analysed was found to be contaminated with aflatoxin M_1. The HPLC results of the summer surveillance study indicate that 21 (91%) of samples were found to be contaminated with aflatoxin M_1 even if at extremely low concentrations.

Interestingly no brand in particular was completely free of aflatoxin M_1 contamination. However, the one brand of organic milk analysed was within the South African permitted level of 0.05ppb aflatoxin M_1 contamination during both sampling seasons. Even though one brand may not be sufficient for distinction, the principles of organic farming may hold the key to producing milk with limited aflatoxin contamination.

According to Neeson & Banks (2004), organic farming can be defined as a system of sustainable farming that produces agricultural products without the need for artificial pesticides or fertilisers. This system follows a set of well defined published standards that constitute an organic quality assurance system. So if a farmer and its produce are to be certified 'organic' the farmer's methods of production and storage must comply with standards for organic farming with regular quality audits by representatives of the National Standard for Organic and Biodynamic Produce. Interestingly, some Certifier's Standards detail procedures as to how grains should be stored.

The correct harvesting strategies, storage design and layout, grain storage management practices, monitoring pest incidence, controlled atmosphere storage, heating and cooling treatments and inert atmosphere vacuum packaging are all organic-compatible practices useful for maintaining the quality of stored grain (Neeson & Banks, 2004). So clearly if the farm producing organic milk has to adhere to detailed quality practices including the appropriate storage of grain, this will have a direct positive influence over the quality of milk produced, as demonstrated in the low levels of contamination found in organic milk analysed during this study.

This trend in compliance with legislation is further demonstrated with the two brands of infant formulae analysed during both sampling seasons. This compliance with legislation may be possibly due to the importing of spray dried milk powder from countries within the European Union.

Methodology

Regarding the methodology used and the comparison of the various methods used to analyse for aflatoxin M_1, it is clear that upon comparison of the mean values of the IAC to the HPLC values, the Vicam® IAC extraction results were far higher than the confirmatory results by HPLC.

An explanation as to why the aflatoxin M_1 quantification values of the seasonal farm gate milk especially the summer sampled milk may be higher than actually reported in this study, maybe explained as follows. Rosi *et al*. (2007) reported that the processes of homogenisation and pasteurisation require a higher rate of centrifugation since these sanitation treatments reduce the size of fat globules and make them more difficult to separate.

Considering that minimal amounts of fat (0.4-0.6%) in milk can block an immune-affinity column, it must also be noted that centrifugation for prolonged periods of time will precipitate some of the caseins to which the aflatoxins are bound, thus lowering the final detectable values (Rosi *et al*., 2007). During the extraction process whilst using both Vicam® IAC and Biotage® SPE, blockages by the fat globules present in the centrifuged milk samples was found to occur frequently, even though the milk had been filtered twice using a micro-fibre filter.

However the excellent performance of IAC clean up with the subsequent HPLC- flourescence determination of aflatoxins even at very low levels such as 0.1ng/g has been demonstrated in a collaborative trial carried out at an International level (Stroka *et al*., 2001), so this official method of the AOAC 2000.08 seems to be the best suited especially so, if used in conjunction with CoBrA cell for derivitisation.

There was *no correlation* in the results obtained from the extraction of aflatoxin M_1 from milk using Vicam® IAC, Biotage® SPE and Ridascreen® ELISA during this study. Based on the statistical information obtained, the differences in the mean values among the various methods are greater than would be expected by chance and there *was a statistically significant difference* ($P = <0.001$) amongst the results received. Extraction by IAC reported a mean aflatoxin M_1 value of 0.023, whilst the SPE value was 0.097 and the highest mean value of 0.488 was obtained from ELISA.

The Standard Error of Mean showed a greater difference between ELISA (0.601) and the other two methods where the SEM values were 0.023 and 0.061 for IAC and SPE respectively. The standard deviation obtained for ELISA was 0.601, whilst the IAC value was 0.023 and the SPE value was 0.061.

Roos *et al*. (1997) compared the results of analysis using IAC and SPE columns on maize and found that there was *no significant difference* in the results obtained. The authors reported that the method for IAC was easier to use, less solvent was required and greater sample throughput was obtained. As the matrix of maize differs substantially from that of bovine milk, it may be that the high concentration of fat in milk has possibly hindered the correlation of results achieved during this study.

Most reports in literature refer to ELISA being used as a screening test to determine if the level of aflatoxin M_1 present in milk is within the stipulated legislation or if there is cause for concern. Rosi *et al.* (2007) documented that ELISA may be considered a reliable analytical method to discriminate between safe raw materials and those that should be excluded due to high contamination levels. It is also possible to obtain results fairly quickly when testing a large number of samples using the ELISA method.

However, as reported by Rosi *et al*. (2007) immune-chemical assays can suffer from the so called 'matrix effect or matrix interference' due to non specific interaction of antibodies with substances different from the analyte, giving rise to under or over estimation. For this reason, samples presumed positive or suspect after ELISA, must be confirmed by HPLC coupled with fluorescence detection. It was further reported by Anklam *et al.* (2002) reported that the possibility of false positive results due to cross reactions and that of false negatives are cited as definite drawbacks of the ELISA method.

Rosi *et al.* (2007) in their article titled "Aflatoxin M_1 in milk: Reliability of the Immuno-enzymatic assay" concluded that the purpose of the ELISA assay is to provide the quickest and simplest method of reliable results with good sensitivity, high precision and optimal recovery of the determination of aflatoxin M_1 in milk.

The Charm® and Snap® lateral flow devices were the two additional methods tested during the course of this study. These point of care (POC) testing units are easy to use, rapid to test and specific. These units would thus be suited for use in quality assurance laboratories where a

lack of technical skill and finances are an issue in third world countries such as Africa, where the global demand for agricultural products such as groundnut necessitates export which is also important for revenue generation for such developing countries. The purpose of including these lateral flow devices in this study is because their introduction to the South African market is relatively new and these test units may form an important component in the quality system checklist of farmers and milk producers alike, as they eliminate the need for tedious expensive laboratory testing as they provide a on- site test result.

The Charm® method can also be used to test for a wide range of mycotoxins which may be present in grain, cereals and feed ingredients. Due to budgetary constraints, feed sampled during this study was not analysed using this system unfortunately. Both Charm® and Snap® systems showed excellent potential for the qualitative measurement of aflatoxin M_1. These units are suitable for application at the farm level and dairy processing facility, however it is necessary that final confirmation through the use of high performance liquid chromatography be applied before large quantities of milk is discarded or rejected.

In conclusion, the over or underestimation of mycotoxins when using rapid tests is still an issue, often attributed to the cross reactivity of the antibody to closely related fungal metabolites and/or to the matrix itself. Different matrices have been shown to have different effects on the test result, as has been previously shown not only for rapid tests but also for standard chromatographic mycotoxins analysis (Haubl, 2006).

It has to be assured that the analytical methods used by official food control laboratories produce reliable results. In–house methods must be regularly controlled by means of certified reference materials (CRM's) and appropriate validated confirmatory methods in case screening methods are applied as part of a cost effective measurement and prevention strategy. This will ensure that reliable data are generated for risk assessment (Anklam *et al.,* 2002).

All the microbiological and mycotoxin analysis conducted during this study was performed at the FEHRG laboratory at the University of Johannesburg. This laboratory currently does not have any formal quality accreditations by the South African National Accreditation System.

Aflatoxin Intervention Strategies

Interventions to reduce aflatoxin-induced illness can be roughly grouped into three categories: Agricultural, dietary, and clinical. *Agricultural* interventions are methods or technologies that can be applied either in the field also referred to as pre-harvest factors or during the drying, storage and transportation of grains (cereals and oilseeds) collectively referred to as post-harvest factors, which contribute to reducing the levels of aflatoxins in food. Agricultural interventions can thus be considered "primary" interventions, because they directly reduce aflatoxin in food. *Dietary* and *clinical* interventions may be considered "secondary" interventions. They cannot reduce actual aflatoxin levels in food, but they can reduce aflatoxin-related illness; either by reducing aflatoxin's bio-availability in the body (e.g., through enterosorption) or by ameliorating aflatoxin-induced damage (Wu & Khlangwiset, 2010).

Pre- harvest control has involved the use of agronomic practices which minimise mycotoxins accumulation in the field. These include proper irrigation, pesticide application, the use of resistant or adapted hybrids, tillage type and proper fertilisation. It seems that breeding for mycotoxin resistant hybrids has been only partially successful and fungicides have shown little efficacy in controlling pre-harvest aflatoxins contamination in maize (Duncan *et al*. 1994).

As *A. flavus* is one the most frequent crop contaminants, it makes sense to devise pre-harvest methods to eliminate this fungus. It has been reported that populations of *A.flavus* in agricultural fields are composed of strains which vary widely in aflatoxin–producing ability and this ability is unrelated to a strain's potential to infect and colonise host tissues (Cotty, 1989; 1990). Thus it is possible that these naturally occurring atoxigenic strains which are active under the same environmental conditions as the toxigenic strains, may be able to out-compete toxigenic strains during the infection of developing crops and thereby prevent aflatoxin contamination (Cotty, 1989; 1990).

It has been reported that the application of an atoxigenic strain of *A.flavus* reduced pre-harvest aflatoxin contamination by between 80% and 95%, whilst also reducing post-harvest aflatoxin contamination, through the introduction of a new toxigenic strain coupled with the strains which were already resident on the kernel during pre-harvest. This suggests that

atoxigenic strains of *A. flavus* may have the potential to be used as biological control agents directed at reducing both pre-harvest and post-harvest aflatoxins (Brown *et al*. 1991)

Pitt & Hocking (2006) describe the following five criteria for the choice of atoxigenic *Aspergillus* strains. The strains should:

- be unable to produce toxins
- be unlikely to revert or incapable of reverting to toxicity; i.e., they should be genetically stable
- be competitive with naturally occurring toxigenic strains under field conditions
- be naturally occurring rather than mutated or genetically modified
- produced and applied in such a way as to ensure operational safety.

The African Agricultural Technology Foundation (AATF) and European Union MycoRed project are currently funding a trial in Nigeria, where farmers have participated in the bio-control of aflatoxins using Aflasafe™ a fungus based, bio-control product suited for maize. It has been reported that the maize been grown is free of disease by *Aspergillus* (Science-In-Africa, 2009).

Other post-harvest approaches for management of mycotoxin contamination include the ammoniation of corn and cottonseed to destroy aflatoxins, the analysis for mycotoxins and the possible dilution of contaminated with non-contaminated material, if permitted (Trail *et al*. 1995b).

Storage technology such as the storage eco-system discussed earlier in this review is of paramount importance, in the control chain to minimise or prevent contamination by aflatoxins. The accumulation of grains in a silo immobilises large dead volumes of air making it a good thermal insulator. The temperature in the middle of the silo remains close to harvest temperature while grains close to the silo wall remain cooler. Wet spots may occur in micro-climate conditions which will favour fungal growth. As a result of fungal activities the temperature inside the contaminated spot will increase rapidly, and thus temperature recorders distributed at different heights in the silo is a very efficient way to detect any microbial activity (Jouany, 2007). The use of modified atmosphere gases and sulphur dioxide

in the storage of packaged products which are susceptible to mould has been researched, but the cost involved is the limiting factor (Neeson & Banks, 2004).

Combined cooling and drying operations associated with ventilation systems is important, as is the rotation of grains at regular intervals. The construction of silos should also include the addition of adequate ventilation units to promote the aeration of grains during storage. The purpose of an aeration system is to preserve dry, stored grain by cooling the grain and preventing moisture migration. Aeration helps to conserve the quality of certain grains without pesticide residues, fungal odours, or germination damage (Canadian Agriculture, 2001). Supplementary refrigeration is sometimes used to reduce the relative humidity of the input air if the air is too humid.

Cool, dry storage conditions with sufficient pest control facilities and general good hygiene will further limit mycotoxin formation during post harvest storage. The recent use of mineral dusts based on diatomaceous earth are useful in organic practice pest control, as these products adhere to and absorb the waxy coatings of insects causing death by dehydration rather than chemical means. (Neeson & Banks, 2004). These natural approaches to pest control is likely to be accepted for use by the consumer more favourably, as it means that less chemicals are present in the final food product. It must be noted that atoxigenic strains are considered bio-pesticides and thus the use of atoxigenic strains must comply with pesticide law (Cotty & Mellon, 2006).

Grains, cereals and oil seeds do not necessarily go directly from farmer to food processor or consumer. This means that there are many entities involved in the transport of grain as well many different modes of transport involving travel by road, rail and sea. The storage of this grain during transport is important as it may create the ideal condition for mycotoxin development especially for these susceptible commodities. It is important that grain handlers are familiar with the required hygiene and pest control practices as well as the importance of keeping grains dry and cool.

Turner *et al.* (2005) reported findings of a post-harvest intervention package used to reduce aflatoxin in groundnuts, tested in Guinea. The package consisted of six components: education on hand-sorting nuts, natural-fiber mats for drying the nuts, education on proper sun drying, natural-fibre bags for storage, wooden pallets on which to store bags, and

insecticides applied on the floor of the storage facility under the wooden pallets. After five months of conducting this study, it was reported that individuals who had received the post-harvest intervention package had on average 57.2% lower aflatoxin-albumin concentrations in the blood compared with individuals in the control group.

As mentioned earlier in this review, the genetically modified maize referred to as Bt corn is currently being grown in South Africa. Although there are certain negative connotations surrounding genetically modified foodstuffs, this corn is superior in that it has superior pest protection properties and thus lowering possible contamination by mycotoxins.

Whilst training and education about mycotoxins is important, it is even more important that the persons involved actually believe and buy into the suggested concepts to ensure the success of controlled experiments and trials. Seed suppliers and farmers should be working hand in hand to ensure disease free crops are achieved so that the contamination by mycotoxins is kept to a minimum.

Economics of trade

Earlier in the literature review, the permitted legislative limits imposed on certain foods was questioned as to whether they may be too restrictive, thus possibly posing as deliberate barriers of trade. Regarding the trade of groundnuts from Africa, Otsuki *et al.* (2001a; 2001b) reported that the strict permitted level of 8 ppb aflatoxin B_1 contamination in groundnuts as prescribed by the European Union (EU) would not significantly lower the health risk to consumers, but the loss of this export business for Africa, will have a serious financial impact amounting to millions of dollars annually. However the World Bank (2008) concluded that for most countries in sub Saharan Africa, the stringency of EU standards has neither served as a significant barrier to trade or as a significant catalyst for proactive action.

It was reported that the suppliers tend to be unreliable with regards the consistent supply of good quality products. Furthermore, upgrades at the production and harvest levels are required to improve basic quality characteristics and ensure consistency in the quality of supply and product. It seems that the Rapid Alert system for Food and Feed (RASFF) reported that nearly 80% of African consignments intercepted by the European Union authorities between 2004 and 2006 would have failed even the less strict Codex standard of 15ppb aflatoxin B_1 contamination.

The World Bank however also highlighted that considerable research and efforts are in effect to prevent and reduce aflatoxin contamination in groundnut during production, storage and trade in sub Saharan Africa, in order to achieve compliance with the very stringent standards. However, certain challenges still remain in public- private sector collaboration efforts to ensure the cost effectiveness of adopted approaches.

During this study, the possible country of origin of the feed ingredients was investigated through information supplied by the Department of Agriculture and Department of Forestry and Fisheries. Currently most of the soya bean seems to be imported from Argentina, whilst there is some evidence of local production too. As South Africa produced one of its largest maize crops during 2009/2010, it is possible that the maize sampled during this study was locally produced. It seems that most of the cottonseed is imported from a country within the African continent. The sunflower oil cake is obtained from the major oil refineries in the country, whilst the source of the seed may be imported or locally produced.

In an attempt to ensure a secure safe global supply of food there in an increasing need for all involved in the supply chain, from the agricultural producers through to food manufacturers, to enforce stringent quality control systems to ensure safe to consume food is available to consumers worldwide. There is an increased need for transparency, trust and accountability in the relationships of all involved in the supply food chain. The threat of terrorism, global trade and food safety issues all play a role in the importance of developing proper food safety processing systems, which involve compliance to global regulatory standards, thus ensuring global food trade can occur with accurate traceability programs. These effects coupled with the negative impact of food toxins and outbreaks of food poisoning as reported in recent years, has prompted the need for the tightening of food processing legislation globally. This has resulted in regulatory bodies worldwide insisting on the implementation of certain quality systems by both suppliers and manufacturers of food ingredients and food products. Should the raw materials or final products be found not conform to the legislated specifications, then no further commercial transactions can progress. Figure 60 below shows an evolution of the European Union conformity assessment system for groundnuts which has evolved over the years.

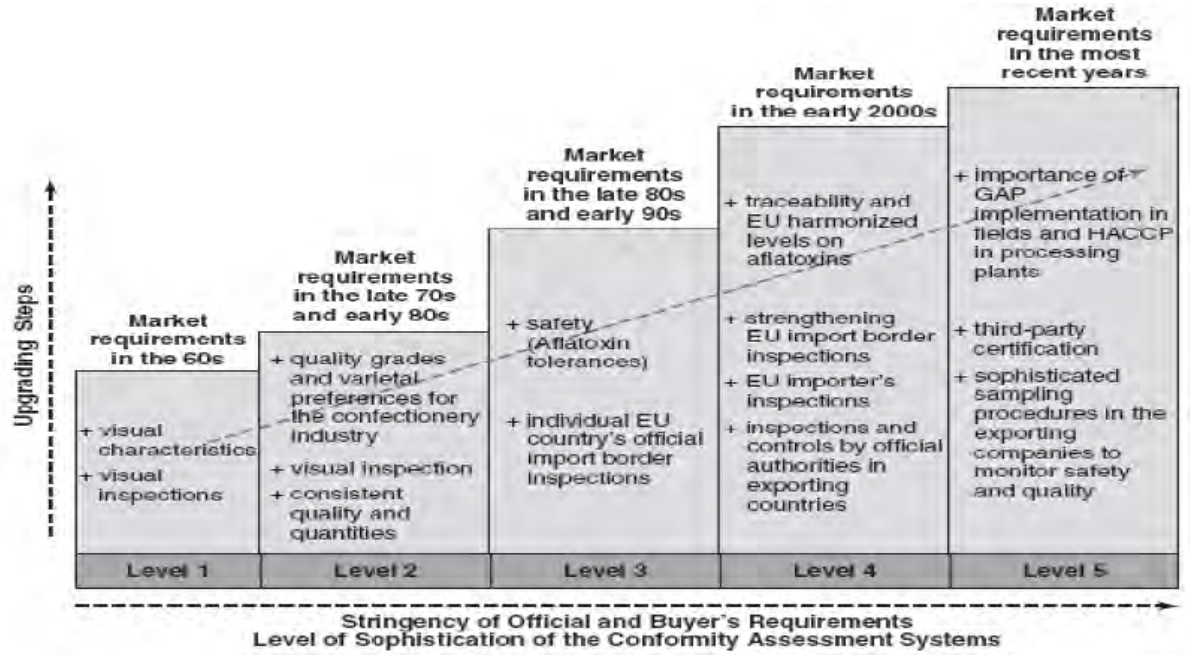


Figure 60: A graphical representation of the evolution of European Union market requirements and associated conformity assessment systems for groundnuts (World Bank, 2008).

Food processing standards and conformance to enforced regulatory laws has prompted the implementation of various global quality systems and standards, such as the Hazard Analysis Critical Control Program (HACCP) which have increased over the past few decades and is an important component of risk control and management. Regular audits by accredited third party quality control representatives, has become a food industry norm globally.

Risk Management

Risk management is the most effective method to prevent unforeseen outbreaks of aflatoxicosis and have an understanding of the level of aflatoxin contamination within a country.

Regular surveillance surveys are one of the most effective ways to determine the extent of contamination of aflatoxins in feed and should be conducted by Governments and local authorities to ensure food and feed ingredients entering our ports are within the locally prescribed legislated limits and that commodities such as milk and animal products are screened regularly to ascertain the level of aflatoxin contamination.

Feed suppliers should have a traceability program in place to identify contaminated sources of supply with the application of Good Agricultural Practices (GAP) such as crop rotation, the use of crop resistant varieties, effective insect control, maintained irrigation, as well as timed planting and regular weeding can reduce mycotoxins. Harvesting should be such as to avoid damage to commodities and to remove any extraneous matter whilst maintaining sufficiently low water activity levels. Fungicides have been tested on micro-flora to reduce the size of soil-borne pathogens (Elmholt *et al*. 1993). However the soil saprophyte microbial biomass is affected by the use of fungicides and therefore there are limitations to this application. Natural essential oils such as clove and cinnamon is also been researched for their effectiveness as anti-fungal and anti-bacterial agents in soil (Magan & Aldred, 2007).

The procurement, production and manufacture of animal feed and feed ingredients should be carefully considered and a HACCP plan should be the minimum quality assurance tool used by feed manufacturers. Raw materials should be delivered with a Certificate of Analysis (COA) and Product Specification document, which details detailing the source of the product as well as certain technical data such as the physical, chemical and microbiological attributes of the product. Accredited laboratory reports detailing the type of mycotoxins screened and the concentration present at the time of sampling should accompany shipments of raw materials. If raw materials were analysed prior to being shipped from a country across the seas, the product should be re-sampled and screened to ensure compliance with the destination country's legislation, regarding the allowable levels of permitted mycotoxin contamination. If farmers and feed ingredient procurement officers adopt a more stringent approach the situation will be greatly improved.

A Good Manufacturing Practices (GMP) program should be developed as this is a pre-requisite for the Hazard Analysis Critical Control Program (HACCP). The HACCP system is a very effective program assisting in the identification and analysis of the potential hazards which may occur during each step of the processing and supply of food production.

The HACCP concept with focus on fungal toxins is outlined below (adapted from FAO, 2001):

1. Hazard Analysis: identify where potential mould contamination could occur, assess the risks and describe preventative measures like requesting Certificates of Analysis

from suppliers with every shipment of cereal or grain or compound feed detailing level of aflatoxin B_1 level of contamination present.

2. Critical Control Points (CCP): define material or processes that need to be monitored for fungal contamination, analyse incoming raw materials using physical, chemical and microbiological quality tests.

3. Critical limits: determine the maximum tolerable toxin levels which are acceptable within an operation.

4. Monitoring procedures: establish procedures for monitoring CCP's. Example: implementation of frequent quality control checks to involve regular aflatoxin screening of feed ingredients.

5. Corrective actions: establish procedures for corrective actions should a deviation from a critical limit occur. Example: develop strategies for detoxification etc.

6. Verification procedures: establish procedures for verification to confirm effectiveness of HACCP plan. Example: regular internal audits and sampling plans.

7. Documentation and record keeping: set up documentation of all procedures and records.

Hazard Analysis Critical Control Programmes have been useful in managing risks associated with potential contamination of food products with pathogenic microorganisms and chemical toxicants. Food safety programs routinely utilise information about the factors leading to the contamination to establish preventative and control procedures thus providing the consumer with safe food (FAO, 2001).

The possible HACCP application stages in agricultural commodities, food products and animal feedstuffs outlined in Table 19.

Table 19: A possible HACCP principle application during the various stages in agricultural commodities, food products and animal feedstuffs (adapted from FAO, 1999)

Stage	Commodity	Hazard	Corrective Action
Pre- harvest	Cereals, grains, oil seeds, nuts, fruit	Mould formation with possible mycotoxins formation	Utilise crop resistant varieties, enforce effective insect control programs, maintain adequate irrigation schedules, perform good tillage, crop rotation, weed control practices
Harvesting	Cereals, grains, oil seeds, nuts, fruit	Increase in mycotoxins formation	Harvest at appropriate time; maintain at lower temperature, remove extraneous material, dry rapidly to below 10% moisture
Post- harvest, storage	Cereals, grains, oil seeds, nuts, fruit	Increase and/or occurrence of mycotoxins	Protect stored product from moisture, insects, environmental factors and store products on clean dry surface.
Post -harvest, processing and manufacturing	Cereals, grains, oil seeds, nuts, fruit	Mycotoxins carry over or contamination	Testing of all ingredients added, monitor processing/ manufacturing operation to maintain high quality product, follow good manufacturing practices
Animal feeding	Dairy meat and poultry products	Transfer of mycotoxins to dairy, meat and poultry products.	Monitor mycotoxins level in feed ingredients and test for mycotoxin residues

It is important that an internal risk assessment is developed within an operation to determine the extent of contamination and methods should be devised to reduce or rid contamination by aflatoxin or at the very least get the situation under control. All feed suppliers and feed manufacturers should have a HACCP program in which the HACCP concept is documented as the one shown in Table 20.

Table 20: HACCP Plan Worksheet – Maize Based Animal Feed (FAO, 2001).

The implementation of internationally accredited quality systems also means that large amounts of finance are required to get the desired integrated systems in operation. These costs affect the pricing of the final product which in turn gets passed to the consumer. The current global recession means that the average consumer generally has less disposable income. The effects are generally felt most by the low Living Style Measures (LSM) consumer and the unemployed, who is most likely to be nourished on a staple and very basic diet of maize and other cereals and legumes which are generally the most affected commodities with regards mycotoxins contamination.

The dairy industry in South Africa consists of approximately 400 milk producers who in turn employ 60 000 farm employees and create indirect employment for an additional 40 000 persons through milk processing and milling functions (DAFF, 2010).

In an article titled 'Low prices forcing milk producers out' (I-Net Bridge, 2011), The Milk Producer's Association (MPO) announced that 21 milk producers from the Overberg region left the dairy industry since the beginning of 2011. A further 12 producers will be forced to leave the industry if producer prices are not increased. This in turn will result in major geographical shifts in milk production, which will impact on the capital investment of milk buyers. The cost to produce milk in South Africa is more expensive in comparison to New Zealand, the EU and USA as farmers from these countries are granted subsidies

The continued survival of the South African Dairy industry is dependent on the support of Government and the major dairy product suppliers involved in the retail trade of milk and milk derived products. Price adjustments of producer prices paid for milk and financial support in the form of subsidies to farmers are long overdue and require urgent attention, in order to ensure that as a country we able to provide sufficient milk for local consumption.

As seen with the declining global trade of groundnuts from Africa, there is much to do in the way of education and training regarding the safe processing of agricultural products on the African continent, especially sub Saharan Africa. Training should be initiated regarding good farming practices, appropriate storage conditions and the negative effects of mycotoxins.

One of the biggest hurdles in accomplishing these objectives remains the manner in which socio-economic issues are addressed, as the lack of farmers and communities' involvement have a direct effect on the success of implementation of proposed quality systems.

The negative health implications brought on by continuous mycotoxin exposure means that the overall productivity within an economy declines owing to frequent absenteeism in the work place and early deaths. A poor economy of a country means that the poor get poorer and often consume whatever staple food is available. A cyclical effect is thus in operation as farmers who are uneducated re mycotoxins, allow low cost contaminated food into the public food chain as seen in Kenya in 2004, which either results in quick deaths of exposed persons or the continued exposure of mycotoxin contaminated foods to rural communities.

The future of improved quality agricultural commodities remains an important issue which needs to be tackled by Governments, big business corporations especially the seed and agricultural suppliers as well as the relevant regulatory bodies concerned. These efforts are necessary to reduce the negative health implications associated with aflatoxins, especially on the African continent where approximately 250 000 hepato-cellular deaths occur annually (Miller & Marasas, 2002) due to the regular and over consumption of aflatoxin contaminated foods.

6. CONCLUSION AND RECOMMENDATIONS

The presence of mycotoxins in food is often over-looked in Africa due to public ignorance about their existence, lack of regulatory mechanisms, the dumping of food products and the introduction of contaminated commodities into the human food chain during chronic food shortage due to drought, wars, political and economic stability (MRC, 2006).

As most cereals, legumes and oil seeds are imported into South Africa from various international countries, these commodities are exposed to long periods of time in inadequately ventilated vessels. The respiration process of agricultural commodities increases the surrounding humidity within the transport vessel, thus creating the ideal conditions for fungal infection and possible mycotoxin formation.

These conditions are further exacerbated if feed manufacturers and end product users do not follow proper storage practices post-purchase or if incorrect sorting and production techniques are employed which increases the concentration of contamination by aflatoxins. Furthermore, the global trade of agricultural commodities such as cottonseed especially from neighbouring countries presents an even greater challenge as it is unlikely that Good Agricultural Practices (GAP), correct storage conditions or the relevant quality assurance tests have occurred during the production of the commodity.

The warm climatic conditions in Africa, has always been conducive to supporting fungal contamination of agricultural products. This is clearly shown in the high levels of aflatoxin B_1 contamination found in the feed samples analysed during this study which far exceeded the maximum permitted levels of contamination as stipulated by South African legislation. However the extreme environmental conditions experienced recently owing to the effects of global warming will impact negatively on the metabolism of animals such as the dairy cow, and is likely to accelerate fungal spoilage of susceptible food commodities. The effects of extreme weather patterns on agricultural commodities are already influencing both the price and availability of food and feed ingredients globally. The steep electricity and fuel costs coupled with the increased price of feeds and farm supplies mean that farmers carry the largest risk in the value chain.

These financial pressures may force dairy and cattle farmers to feed their livestock poor quality feed materials such as production waste or materials which have been rejected owing to failed quality specifications by food or feed manufacturers, which generally tend to be available at lower prices. Also in an effort to reduce the cost of transport and benefit from bulk buying, the farmer may choose to order larger quantities of feed which tend to be stored under inappropriate conditions for extended periods of time, further exaggerating the effects of possible fungal contamination and mycotoxin development.

As discussed earlier the development of quality systems such as Hazard Analysis Critical Control Program (HACCP), involving GAP and Good Manufacturing Practices (GMP) all involve some capital outlay which the average South African dairy farmer can probably not afford currently. The recent advent of on-site mycotoxin testing units in the form of lateral flow devices, may prove to be an important tool for port authorities, feed manufacturers, farmers and dairy processors for the initial screening of commodities, but these come with a substantial price tag too. The reality is that local farmers of agricultural products are forced to compete with imported products which tend to be better priced, possibly due to the subsidies afforded to those farmers from other countries, thus compromising the survival of the agricultural sector in South Africa.

The wide selection of imported products available at local retailers at a lower price than locally produced products is a clear indicator of the situation. Government should look to provide certain incentives, rebates and subsidies to local dairy farmers to ensure survival and stability of the South African dairy industry. This in turn could assist the local economy with job creation and as well as generating revenue from local and export trade.

The lack of resources and limited finances owing to the low producer prices paid for milk could mean that the average dairy farmer is consumed with concern just managing the daily expenses of a dairy farm, and thus the onerous liability of implementing quality systems may not be as important. However, the recent introduction of the Consumer Protection Act, 2008 (Act No. 68 of 2008) means that the responsibility and legal liability of safe food processing and supply extends to all involved in the supply chain, from farmers through to retailers. So it is necessary that all involved in food and feed manufacture be quality conscious in their efforts.

The results obtained in this study indicate that aflatoxin contamination in South Africa is problematic, especially amongst ingredients such as cereals, legumes and oil seeds. This contamination leads to a whole spectrum of food groups being affected such meat and meat products, milk and milk products, cereals and cereal based products, legumes which include peas, beans and pulses which in turn means that a very wide portion of foods available to man is contaminated with possibly at least one type of mycotoxin.

The high levels of contamination of aflatoxin M_1 found in the farm gate milk, following the consumption of heavily contaminated feed with aflatoxin B_1 by the dairy cow further highlights the application of the bio-tracer analogy which was one of the objectives of this study. As milk and milk products are important sources of calcium and are generally popular dietary choices of both mother and child, the contamination of these products by a carcinogenic toxin, such has aflatoxin M_1 during the early vulnerable stages of development is concerning. It is even more alarming that human breast milk may be contaminated with this toxin too, following the consumption of foods contaminated with aflatoxin B_1.

The results obtained for the seasonal surveillance studies are comparable to reports of other authors from various countries indicating that it may be safer to consume more milk in summer, as the levels of aflatoxin M_1 contamination seem to be lower. However it is likely that milk consumption may be higher in winter generally, as most warm beverages such as hot chocolate and malt containing beverages, are milk based and tend to be drank mostly during the cold months of winter, which further exacerbates the exposure of humans to this carcinogenic toxin.

However if an assumption may be based on the results obtained for the organic milk analysis during both seasons, could it be possible that organic milk is of a superior quality owing to the low level of contamination reported? One could consider that this low level of aflatoxin M_1 contamination is possibly a direct result of correct storage conditions proposed by the Organic Certifying Body, involved on the farm producing this milk.

The initial reaction of most persons with the knowledge of aflatoxin M_1 would be to consider a dairy milk substitute such as oat, soya or rice milk. However it must be remembered that the sources of these substitutes are cereal or oil seed based, which are also considered to be commodities frequently affected by aflatoxin B_1 contamination. The controlled variation of an individual's diet which may be achieved through alternating the sources of grains, cereals

and animal proteins, may probably be the most effective way to minimise the exposure to aflatoxins.

There are still considerable amounts of research required in the field of aflatoxin M_1 stability data, suggested sampling protocols and actual bio-tracer effects to better understand the scientific make-up of this toxin. However it is clear that the findings of most countries mentioned in this review, have reported the contamination of milk and milk products by this resistant, carcinogenic toxin.

Hopefully the Department of Health and Department of Agriculture consider the results of this study and thus better assess the level of aflatoxin contamination in agricultural products entering our food chain, as well as the dairy feed industry. Further it may be a useful suggestion to the relevant regulatory bodies, to promote the more lenient legislation of 0.5ppb aflatoxin M_1 contamination level in milk, thus mirroring the CODEX legislation as opposed to the current more stringent European Union level of 0.05 ppb. A more reasonable legislation of 20 ppb aflatoxin B_1 may be suggested for dairy feed as permitted by the Food Drug Administration, USA The purpose of extending these prescribed limits does not imply that less accountability and efforts should be employed in promoting the reduction of aflatoxin contamination in South Africa though.

South African food and feed manufacturers should ensure that frequent mycotoxin screening of agricultural commodities become an essential component of their organisation's quality control program to ensure that the food and feed being produced is safe from mycotoxin contamination or that the levels of contamination are within the prescribed limits, at the very least. However the documented negative health effects experienced by humans and animals due to the continued exposure to mycotoxins, has become a far bigger issue than just that of a mere quality test. The level of mycotoxin exposure and its effects needs to be treated as the precursor of a global epidemic, which requires the enforced commitment and involvement of Government as well as food and feed businesses on a social and ethically level too, especially in Africa.

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