MYCOTOXINS IN FOOD WITH PARTICULAR REFERENCE TO FUMONISIN B_1: THEIR HEALTH IMPACT ON A KRANSKOP RURAL COMMUNITY, KWAZULU NATAL

By

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DECLARATION

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this study was carried out in the department of Physiology, Medical School, University of Natal, under the supervision of Professor M.F. Dutton.

P K CHELULE

DEDICATION

To the Lord God Almighty,

From whom all blessings and knowledge come.

PUBLICATIONS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

AFB_1	aflatoxin B_1
AFM_1	aflatoxin M_1
ATA	alimentary toxic aleukia
CPA	Cyclopiazonic acid
C_{18}	Octadecyl
DON	Deoxynivalenol
ELEM	equine leucoencephalomalacia
FA_1	fumonisin A_1
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FB_4	fumonisin B_4
GC	gas chromatography
GIT	Gastrointestinal tract
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
IARC	International Agency for Research in Cancer
kg	Kilogram
MAFF	Ministry of Agriculture, Fisheries and Food
mg	Milligram
ml	Milliltre
mm	Millimetre
MRC	Medical Research Council
MS	mass spectrophotometry
m/z	mass to charge ratio
nm	nanometre
OC	oesophageal cancer
ppm	parts per million
ppb	parts per billion
SFE	supercritical fluid extraction
ST	sterigmatosystin
TB	tuberculosis
µg	microgram
µl	microlitre
χ^2	Chi Square Test
UV	ultra violet
ZEA	zearalenone

ABSTRACT

The use of the multi-mycotoxin screen based on dialysis to analyze foods and feeds for mycotoxins, is well documented. This study investigated the possibility of incorporating FB_1 into the screen. Maize meal (25g) was spiked with AFB_1, CPA, FB_1, ST and ZEA and extraction was done using acetonitrile/4% potassium chloride (90:10 v/v). The recoveries of the mycotoxins were 77.4, 61.5, 97.4, 79.8 and 98% respectively on analysis by HPLC. Fumonisin B_1 could not be completely incorporated into the screen due to its reaction with sodium hydrogen carbonate, which is a component in the method. Thus, FB_1 was determined in a separate portion of the extract.

The high cost of FB_1 standards which are often of inferior purity necessitated that FB_1 standards be locally produced in the laboratory using *Fusarium moniliforme* MRC 826, a good producer of FB_1. In this study, production of FB_1 was carried out using a stirred jar fermenter and patty cultures. The yields were 160mg/l and 6mg/g of FB_1 for the two methods respectively. Methyl esterification of tricaballylic acid moieties of FB_1 was done for effective clean-up. This was achieved by derivatizing FB_1, with diazomethane. It was found that other functional groups besides the tricaballylic acid moieties of FB_1 were undesirably methylated as well, which made cleanup by this method difficult as shown by electrospray mass spectrometric analysis. Attempts to de-methylate FB_1 methyl esters with esterase was not successful.

Analysis of human faecal samples was carried out with the view of developing a short term marker for assessing human exposure to FB_1. Faeces from rural (20) and urban (23) volunteers were analyzed by high performance liquid chromatography. The results showed that 35% of the rural samples and 9% of the urban volunteers had detectable amounts of FB_1 ranging from 0.600 to 19.56 mg/kg. There was a significant difference (p = 0.04) between the two population groups.

A study was carried out to assess the occurrence of FB_1 in a rural area of Tugela valley in Kranskop magisterial district of KwaZulu Natal. A questionnaire was administered to gather information on the family health and nutrition. Raw (stored) and processed foods and faeces, were collected for analysis of FB_1. A similar control study was carried out in the urban area of Durban Metro. Homes were mapped out using the GIS for easy follow-

up. Oesphageal cancer (OC) incidence from the local hospital and weather data for the study area were collected from South African Weather Bureau, Johannesburg.

The questionnaire results showed that the common diseases were mainly of respiratory origin (24% and 26%) from both rural and urban groups respectively. Food analysis (by HPLC) showed that the number of maize samples with FB_1 were higher in the rural area (31.9%) in comparison to the urban samples (6.1%). The level ranged from 0.092-22.225 mg/kg in food and 0.513-39 mg/kg in faeces. The mean concentration of FB_1 in the faeces and maize samples showed a similar significant difference of 0.014 between the two groups. However, these concentrations were much lower than those of high OC area in Transkei (117 mg/kg). There was no detection of FB_1 in fermented food products.

CHAPTER 1

INTRODUCTION

1.1 MYCOTOXICOSES... THE NEGLECTED DISEASES

"Of the innumerable diseases that affect man and domestic animals, the mycotoxicoses are perhaps the most unfamiliar and least investigated, presumably due to lack of proper techniques and interested scientists. This situation has prompted many to regard this elusive group of diseases hypothetical, when, in fact, there is abundant evidence to suspect mycotoxicoses as causal factors in disease of unknown aetiology" (Forgacs, 1962).

Mycotoxins are toxic substances produced by fungi (moulds), and are known to cause disease in animals and man. A more recent definition is "Mycotoxins are fungal metabolites which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death in man and animals, including birds" (Pitt, 1996). Diseases caused by mycotoxins are called mycotoxicoses. Historical incidences of human disease outbreaks e.g., ergot (Robbers, 1979) and animal poisonings e.g., equine leukoencephalomalacia (ELEM) in horses (Butler, 1902), have been recorded in the past. Unfortunately, the role of mycotoxins as the cause of human disease is poorly understood (Wild and Hall, 1996) and, therefore, are probably the most underestimated of the diseases that affect man and animals. It is exceedingly difficult to correlate human mycotoxicoses with the occurrence of a mycotoxin in food commodities, particularly when at chronic levels, as this does not prove that observed symptoms are directly linked to the presence of the toxin. Furthermore, it would appear that chronic diseases related to dietary factors, are multifaceted and result due to the convergence of several components, e.g, malnutrition, avitaminosis and alcohol abuse (Campbell, 1990).

The first challenge in the diagnosis and control of mycotoxicoses is to develop a method that is able to detect a wide range of mycotoxins in a sample. The method should be precise, sensitive and cheap. A multi-mycotoxin screen has been found to be useful in this regard (Patterson and Roberts 1979). This method, however, has drawbacks. It is not completely comprehensive as it was only designed to detect 20 mycotoxins. Other mycotoxins, such as

fumonisins, which were discovered much later, require the modification of multi-mycotoxin screen in order to detect them. Consequently, this is one of the problems addressed in this study.

Pure mycotoxin standards are required for optimization of analytical techniques and bioassays (e.g, cytotoxicity testing) necessary for establishing permissible limits in foods and feeds. This is particularly important in their quantitation, since any impurities from the culture material would give spurious results. Such mycotoxin standards are costly to produce, as the methods are lengthy, tedious and have to be customized for a product that has limited market. In several cases, e.g., fumonisin B_1 (FB_1), the required standard of purity is not attained in commercially available material. Therefore, one of the aims of this study is to produce pure FB_1 derived from the isolates of *Fusarium moniliforme*, by devising novel clean-up procedures.

Maize is a staple food for South African community, much of it locally produced with supplementation from the commercial sources during times of scarcity, such as drought. It is consumed in at least two daily meals, as the principle source of nutrition. When there is adequate harvest, it is stored and consumed until the next season. This is a major problem as the poor storage facilities encountered in rural areas, give rise to contamination with fungi and mycotoxins. Storage methods vary and depend upon tradition, size of harvest and available facilities (FAO, 1984; Aidoo, 1993; Dutton *et al.*, 1993) and this often plays a role in the type of mycotoxigenic fungi found (Gqaleni *et al.*, 1997). A factor which can affect the toxins found in maize, is the natural fermentations carried out in the home. Beer generally, has much higher levels of toxin because mouldy grain is specifically used for this purpose (Marasas, 1982). In the case of fermented maize porridges, mycotoxins may be introduced due to poor control of fermentation or removed by the action of the fermenting organisms (Gqaleni, *et al.*, 1998a).

Ingestion of mycotoxin contaminated food results in the body's biological response to this exposure. Since this response is specific to a particular toxin, it gives rise to a "biomarker" of exposure e.g., the formation of DNA adducts in the African population of KwaZulu Natal have been found to be more specific, informative and practical than other methods (Strickland

and Groopman, 1995) in the diagnosis of diseases associated with aflatoxins such as hepatocellular carcinoma and kwashiorkor. Measurement of sphingoid bases in physiological samples has been used as a biomarker to monitor fumonisin related diseases such as oesophageal cancer (Riley *et al.*, 1994b, Solfrizzo *et al.*, 1997, Shephard, 1997) but this method has not been very reliable due to technical problems in the recovery of the sphinghoid bases from samples. One of the goals of this study is to develop a biomarker method for analysing FB_1.

It is with these points in mind that this study on FB_1 was carried out in the Kranskop magisterial district of KwaZulu Natal. This area was chosen for the study, because the residents were approachable and willing to assist in the study. In addition, the area is semi-arid with unreliable rainfall pattern and large swings in temperature (Plate 1.1). This type of climate in addition to poor storage may encourage mycotoxin formation in crops, both in the field and in storage.

Plate 1.1 Panoramic view of Tugela valley villages in Kranskop

Fumonisin B_1 monitoring was done on cooked and uncooked food. This is because, although analysis of raw materials does give some information on the presence of mycotoxins in the diet, it does not allow for the effect of processing and cooking on the level of toxin ingested. The effect of preparation and cooking on mycotoxins in food in the rural areas has not been studied and, therefore, is not understood.

The urban population of KwaZulu Natal is not likely to be exposed to mycotoxins to the same extent as people in the rural areas since they obtain their maize from commercial sources. Unlike the home grown maize, commercial maize generally undergoes quality control before being sold into the market. Hence, the subjects and food materials from urban areas were incorporated into this study as controls for comparison purposes.

1.2 AIMS OF THE PROJECT

- To adapt a multi-mycotoxin screening technique to include fumonisin analysis.
- To prepare and purify fumonisin B_1 standard from *Fusarium moniliforme* cultures.
- To analyse faecal samples as a short term biomarker for individual exposure especially to FB_1.
- To estimate the levels of mycotoxins, (with particular reference to fumonisin B_1), in various food commodities consumed in a rural area of the Tugela Valley near Kranskop, KwaZulu Natal and to correlate environmental factors such as rainfall, temperature and storage with the proliferation of mycotoxigenic fungi.

CHAPTER 2

LITERATURE REVIEW

2.1 WHAT ARE MYCOTOXINS?

Mycotoxins are secondary metabolic products of certain filamentous fungi (moulds), which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death in man and animals, including birds (Pitt, 1996).

2.1.1 Fungi

Filamentous fungi, commonly known as "moulds" are widespread heterotrophic microorganisms, with a vegetative body consisting of a filamentous branching thallus of hyphae. A large intertwined mass of hyphae is known as the mycelium. They invade and colonize their substrate by means of hyphal structures. Many of the pathogenic fungi reproduce asexually by means of spores often called conidia or sporangiospores (Beneke and Rogers, 1996). Unicellular type of species which reproduce by budding are known as yeasts. Pathogenic fungi can infect live plant or animal tissue causing mycoses.

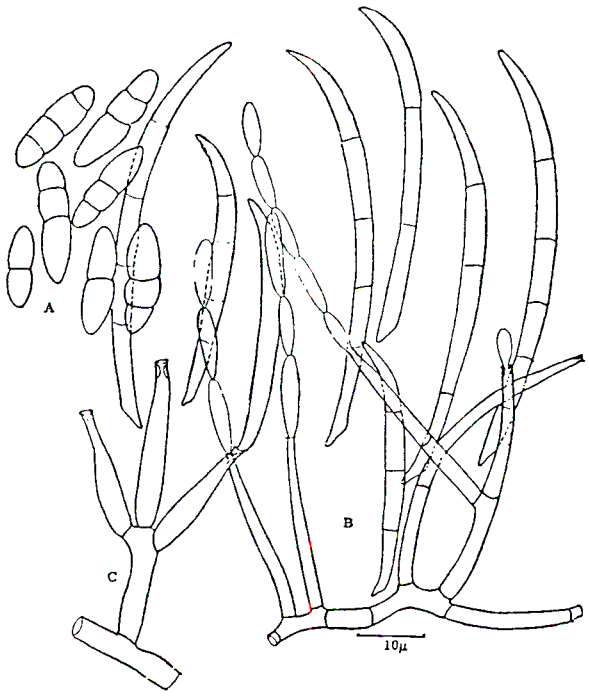


Figure 2.1 Types of spores in *Fusarium moniliforme*. A, ascospores; B, microconidia and conidiophores; C, macroconidia and conidiophores (Adapted from Beneke and Rogers, 1996)

Filamentous fungi develop adequately at room temperature, between 24 and 28°C with a moisture content of approximately 20% (Lacey, 1989). Examples of common food spoilers include *A. fumigatus, Cladosporium* spp., *Rhizopus oryzae, R. stolonifer* and those that infect food occasionally producing mycotoxins include, *Alternaria* spp., *Aspergillus flavus, A. parasiticus, Fusarium graminearum, F. moniliforme, F. subglutinans* and *Penicillium commune*. Among these, *F. moniliforme* has been found to be the most dominant fungus infecting maize worldwide and especially in South Africa (Rava *et al.,* 1996) (Figure 2.1). Although it is known to infect crops mainly in the field, *F. moniliforme* can thrive under favourable storage conditions with high humidity to produce mycotoxins.

2.1.2 Fungal Secondary Metabolites

The fungal growth cycle is divided into two phases, trophophase and idiophase (Bu'Lock, 1967). Trophophase is a phase where normal cell growth occurs (primary metabolism) while idiophase is a domancy stage where spores are produced (secondary metabolism). Secondary metabolism is a phase found in micro-organisms (mainly fungi) and plants but not in animals. In this latter stage of secondary metabolism, secondary metabolites, among them mycotoxins, are formed (Dutton, 1988).

Individual secondary metabolites are very limited in distribution (only produced by a limited number of microbial species) and have no role in the growth of the producing organism (Demain, 1992). However, some have been known to be poisonous to other microorganisms, and have been utilised by man therapeutically as antibiotics e.g., cyclopenins produced by *Penicillium commune*. Secondary metabolites may also have a useful role in assisting the producing organism compete with other fungi for limited space and other resources, such as food, in an ecological environment (Wicklow, 1981 and Betina, 1989).

2.1.3 Mycotoxins

The definition of mycotoxins has been given in Section 2.1 above. Mycotoxins are produced by various filamentous fungi infecting foods, feeds, or raw materials used in their manufacture; making them potentially dangerous to human health. They may enter the body through ingestion, inhalation and absorption through the skin, in the case of some

trichothecenes, which when introduced in low concentrations to animals and man can result in a toxic response, a mycotoxicosis. At least 300 different mycotoxins have been identified with only about 20 being of a major concern in crops used for human consumption and animal feeds (Smith and Solomons 1994). Some of the mycotoxins, which have been shown to contaminate foods and feeds more frequently than others, are discussed below.

2.1.3.1 Aflatoxins

Aflatoxins are a group of related mycotoxins mainly produced by *A. flavus* and *A. parasiticus*. The basic skeleton of the aflatoxins is condensed bisfuranocoumarin-lactone/cyclopentenone ring system (Figure 2.2). Aflatoxins are soluble in organic solvents like chloroform and methanol but is insoluble in water. They are very stable at high temperatures with little or no destruction under ordinary cooking conditions or during pasteurization. There are four main types: Aflatoxin B_1(AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2). The "B" types of aflatoxins produce blue fluorescence while the "G" type produce green fluorescent spots under ultra violet light. *Aspergillus flavus* produces AFB_1 and AFB_2 while *A. parasiticus* produces these in addition to the compounds named above. Two other significant aflatoxin derivatives, M_1 (AFM_1) and M_2 (AFM_2) ("M" from milk) are often found in milk of lactating animals fed with aflatoxin contaminated feed. The fluorescent activity of aflatoxins is an important physico-chemical property in their analysis. Of the known mycotoxins, AFB_1 is the most studied, most toxic and commonly found in staple foods and animal feeds. It has also been found to be airborne (in respirable grain dust), causing a potential risk to the workers in grain production and processing industries (Ghosh et al., 1997). Dietary AFB_1 has been linked to human hepatocellular carcinoma (HCC) (Ngindu *et al.*, 1982, Van Rensburg, 1985 and Peers *et al.*, 1987). The carcinogenicity of AFB_1 is achieved when it is metabolized by liver enzymes, cytochromes P-450, into AFB_1-8,9-epoxide which then binds to proteins and DNA bases (Coulombe, 1993). Measurement of protein adducts in blood or urine has allowed direct determination of the actual AFB_1 exposure in populations, which is an improvement over random dietary assays and imprecise dietary recall surveys (Strickland and Groopman, 1995). Aflatoxin B_1 has also been associated with the aetiology of kwashiorkor (Hendrickse, 1982).

2.1.3.2 Sterigmatocystin

Sterigmatocystin is a xanthone derivative (Figure 2.2) that resembles aflatoxin in chemical structure but with lesser toxicity (one tenth that of AFB_1) and it is one of the intermediates in aflatoxin biosynthetic pathway. It is thought to be carcinogenic since it causes liver cancer in rats (Purchase and Van Der Watt, 1970) and is produced by several isolates of *A. glaucus* and. *A. vesicolor*. Sterigmatocystin is not widely occurring, although low levels have been detected in wheat and cheese (Scott, 1985).

2.1.3.3 Trichothecenes

Trichothecenes are sesquiterpene epoxides produced predominantly by *F avenaceum, F. equiseti, F. graminearum, F. moniliforme* and *Fusarium sporotrichioides*. They were first discovered by scientists searching for antibiotics (Brian and McGowan, 1946). They share a tricyclic nucleus (12,13,-epoxy-Δ^9-trichothecene) which contains an epoxide at C-12 and C-13 and are essential for their toxicity (Desjardins *et al.*, 1993). Hydrogenation of C-9, 10 double bond results in a slight decrease in toxicity while opening of the 12,13-epoxide abolishes their biological activity (Ueno, 1977) (Figure 2.2). The main examples are T-2 toxin, nivalenol, deoxynivalenol (DON), diacetoxyscirpenol (DAS). T-2 toxin and nivalenol are the most toxic followed by DON which is the most common contaminant of maize worldwide. T-2 toxin was implicated in the outbreak of alimentary toxic aleukia (ATA) (Table 2.2). Trichothecenes have been known to inhibit all steps of protein synthesis in intact ribosomes (Cundliffe and Davis, 1977) alters immune parameters (Coulombe, Jr., 1993) and have been associated with the process of apoptosis (cell death). Deoxynivalenol (DON) has been associated with feed refusal in animals.

2.1.3.4 Zearalenone

Zearalenone (ZEA) is a mycotoxin with oestrogenic activity similar to that of β-oestradiol as it has the characteristic aromatic ring (Figure 2.2). Its name is derived from *Zea mays* (maize) the substrate from where it was first isolated. It is a white crystalline material with molecular weight of 318. It is soluble in organic solvents like chloroform, alcohols, acetonitrile, dichloromethane, ethyl acetate and strong alkalis but insoluble in water. The most important fungal producers of ZEA are *F. graminearum* and *F. semitectum*. It is co-produced with

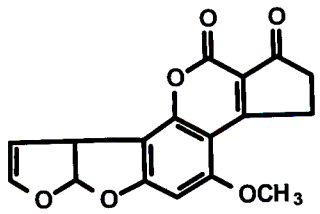
deoxynivalenol (DON) by *F. graminearum*. It has limited evidence of carcinogenic activity in animals and has been named 'mycoestrogen' because it has oestrogenic effects such as abortion, decline in ovulation, conception rates in females and feminization in males (Newberne, 1987). These abnormalities were observed in animals eating food contaminated with ZEA. Zearalenone is a possible carcinogen (IARC, 1993) and has been implicated in human cervical and breast cancer.

2.1.3.5 Cyclopiazonic acid

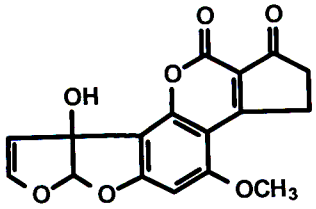
Cyclopiazonic acid (CPA) is an indole-tetramic acid (Figure 2.2), white crystalline compound soluble in solvents like methanol, chloroform and dichloromethane but not in water. It is mainly produced by several isolates of *A. flavus, P. commune* and *P. griseofulvum.* Cyclopiazonic acid has been associated with kodua poisoning in humans (Rao and Husain, 1985), and has been shown to play a role in necrosis of liver or gastrointestinal tissue and necrotic changes in the skeletal muscle, kidney and also prevents calcium uptake by the sarcoplasmic reticulum by inhibiting Ca^{2+}-ATPase (Geoger *et al.,* 1988). It is usually co-produced with aflatoxins (Gallager *et al.,* 1978) and have similar occurrence and distribution.

2.1.3.6 Fumonisins

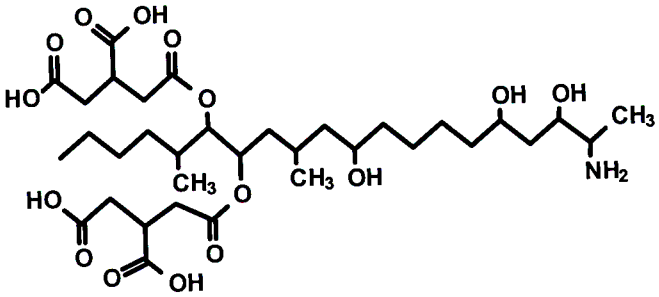
Fumonisins are mycotoxins predominantly produced by *F. moniliforme, F. proliferatum, F. anthophilum, F.subglutinans, F. nygamai, F. napiforme* and *Alternaria alternata f. sp. lycopersici. Fusarium moniliforme* and *F. proliferatum* are frequent in crops and 90% of them are chief fumonisin producers (Bacon and Nelson, 1994). Six fumonisins have been reported: FA_1, FA_2, FB_1, FB_2, FB_3 and FB_4. Fumonisin B_3 was reported by Plattner *et al.* (1992). Fumonisins A_1 and A_2 are N-methyl derivatives of FB_1, FB_2. Only FB_1 and FB_2 appear to be of toxicological significance as FA_1, FA_2, FB_3 and FB_4 occur at extremely low concentrations under natural conditions (Bezuidenhout *et al.,* 1988). Fumonisin B_1 and B_2 were first reported in South Africa by Bezuidenhout *et al.* (1988) and Gelderblom *et al.* (1988) and were associated with the aetiology of oesophageal cancer. Fumonisins are structurally related toxins characterized as 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane, esterified at both C-14 and C-15 with propane-1,2,3-tricarboxylic acid (TCA) (Figure 2.2).



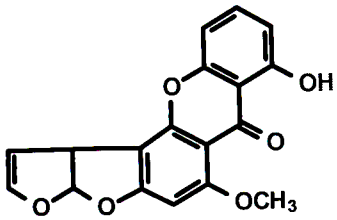
Aflatoxin B_1



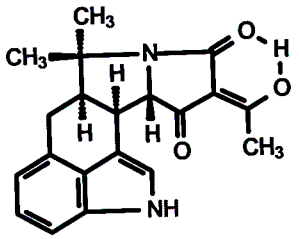
Aflatoxin M_1



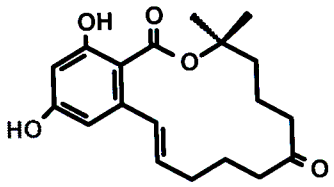
Fumonisin B_1



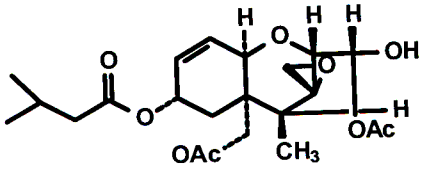
Sterigmatocystin



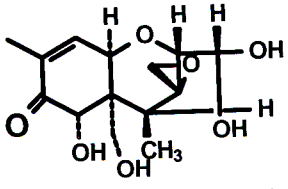
Cyclopiazonic Acid



Zearalenone



T-2 Toxin



Deoxynivalenol

Figure 2.2 Structure of commonly occurring mycotoxins in food

They are highly polar compounds and are soluble in polar solvents e.g., water, methanol and acetonitrile but insoluble in organic solvents. Fumonisins are hydrolyzed by strong acids, alkalis and heat to produce TCA and the corresponding aminopentol (Jackson *et al.*, 1996).

The major toxic effects of FB_1 are based solely on its disruptive effects on sphingolipid biosynthesis, interference with cellular signal transduction pathways and accumulation of sphingoid bases leading to cell membrane damage as seen, for example, in kidney cells (Yoo *et al.*,1992; 1996). The chemical structure of the backbone of the fumonisin compounds is similar to that of the cellular sphingholipids (Figure 2.3). The specific site of FB_1 action appears to be the enzymes sphinganine and sphingosine-N-acyltransferases (Figure 2.4). Similarities between fumonisins and the sphingoid bases allow them to be recognized as substrate for the enzyme N-acyltransferase (Wang *et al.*, 1991). Inhibition of this metabolic pathway results in depletion of complex sphingolipids, increased intracellular concentrations of free sphinganine and sphingosine (Wang *et al.*, 1991) (Figure 2.4). Accumulation of sphinghanine, a compound that is toxic to many cells, may account for the evidence of varied effects such as toxicity and induction of apoptosis in the liver, kidney of rats, mice and rabbits (Howard *et al.*, 1996; Gelderblom *et al.*, 1996; Wang *et al.*, 1996 and Bucci *et al.*, 1998).

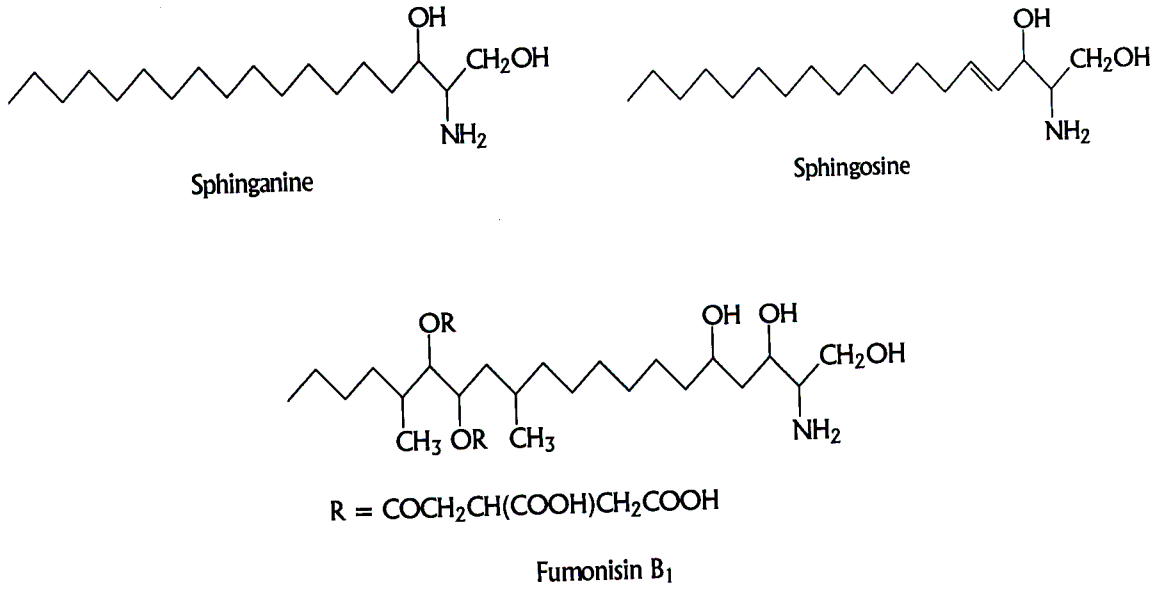


Figure 2.3. Structures of sphinganine, sphingosine and fumonisin B_1

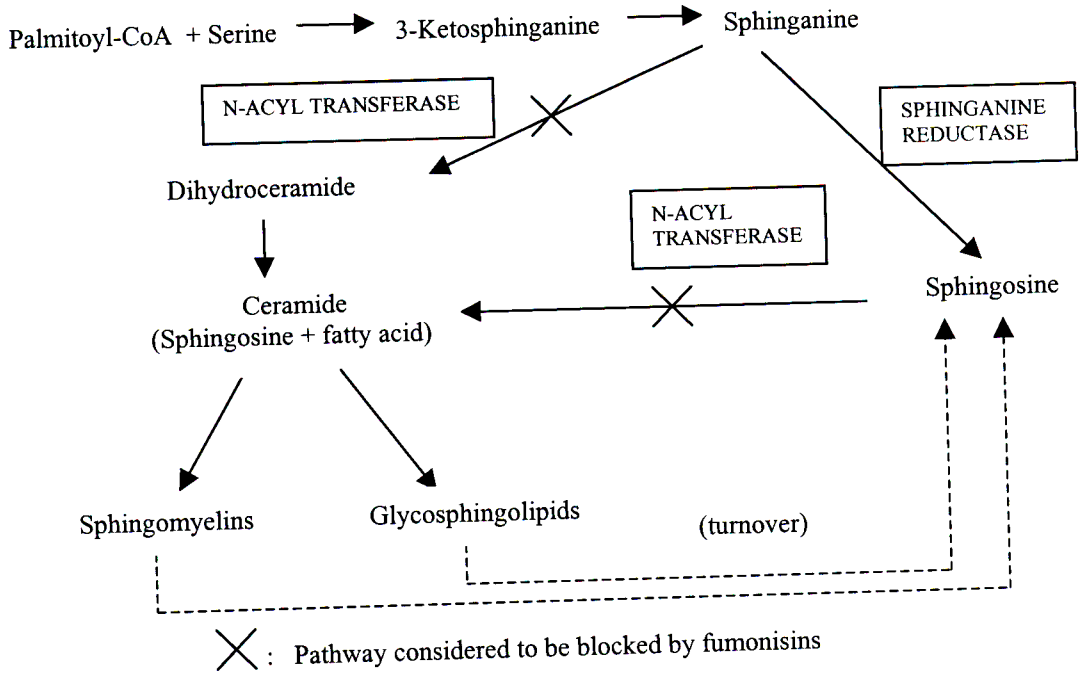


Figure 2.4. Mechanism of inhibition of sphingolipid biosynthesis by fumonisins (Diaz and Boermans, 1994)

Fumonisins have also been reported in black oat feed from Brazil (Sydenham *et al.*, 1992a), and forage grass in New Zealand (Mirocha *et al.*, 1992). Although sorghum is a commonly consumed food product in many communities, it is rarely contaminated since they are infected with *F. moniliforme* type "F" (Klittich and Leslie, 1992), the least producers of fumonisins. Type "A" which usually infect maize, are the main producers of fumonisins. Animal health is affected by levels of above 5.0μg/g (Bullerman, 1996). In USA, maize meal and flour have been found to be contaminated with FB_1 at levels of 0.5-2.05μg/g with much lower levels in maize based cooked products (Bullerman, 1996). Maize in the high oesophageal cancer (OC) area of Transkei, South Africa, had levels of 10.2 μg/g in good maize to 140.2 μg/g in mouldy maize (Rheeder *et al.*, 1992). Maize samples collected from the households in high OC areas of China, contained 18-155 μg/g of FB_1 (Chu and Li, 1994).

2.2 PRODUCTION OF MYCOTOXINS

Factors which affect fungal infection of crops in the field and food in storage have been well discussed (Hesseltine, 1974) and are summarised in Table 2.1. Field fungi develop and

Table 2.1. Factors which determine plant fungal infection (Hesseltine, 1974). (+ for determinants, and – for non-determinants)

	In the Field	At Harvest	In Storage
Physical Factors			
Rapidity of drying	-	+	+
Re-wetting	-	+	+
Relative humidity	+	+	+
Temperature	+	+	+
Mechanical damage	+	+	+
Blending of grain	-	+	+
Hot spots	-	-	+
Time	+	+	+
Chemical Factors			
Carbon dioxide	-	-	+
Oxygen	-	-	+
Nature of Substrate	+	-	+
Mineral Nutrition	+	-	+
Chemical Treatment	-	-	+
Biological Factors			
Plant stress	+	-	+
Invertebrate vectors	+	-	+
Fungus infection	+	-	+
Plant varietal differences	+	-	+
Spore Load	+	+	+
Microbiological ecosystem	+	-	+

storage also depends on the ratio of oxygen (O_2) and carbon dioxide (CO_2) concentration in the intergranular atmosphere. Oilseeds like sunflower and groundnut which have high unsaturated fatty acid levels, (Chulz, 1991) have been known to encourage aflatoxin production. Harvesting in the rainy season results in high humidity required for the

proliferation of these organisms. Interaction of mycotoxigenic fungi with other moulds and bacteria can lead to inhibition (Moss and Badii, 1982) or promotion (Fabbri, 1980) of mycotoxin production. Fungicide applications can result in the accumulation of more mycotoxins in the crop by affecting fungal metabolism (Miller, 1995). Strict moisture control of the grain is the best method of preventing mycotoxin contamination in storage.

2.2.2 Effects of Food Processing on Mycotoxins

It is important to know the stability of mycotoxins in contaminated food and how long they would retain their biological activity, if realistic regulatory levels have to be set. While certain treatments have been found to reduce concentrations of specific mycotoxins, no single method has been developed that is equally effective against the wide variety of mycotoxins that may be present in particular contaminated grain. Under normal circumstances, mycotoxins are, generally, heat stable and thermal processing of food does not seem to appreciably reduce their levels. However, three categories of decontamination methods are recognised: physical, chemical and biological (Charmley and Prelusky, 1996). The aflatoxin content of peanuts was lowered by roasting to below 50% (Stoloff, 1977). Chemical decontamination of AFB_1 include ammoniation (Park *et al.*, 1988), and use of hydrated sodium calcium aluminosilicate (Colvin *et al.*, 1989). Use of propionic acid in combination with fermentation on AFB_1 caused up to 81.9% reduction (Amra *et al.*, 1996). Zearalenone and deoxynivalenol were substantially reduced by 95% and 87% respectively when contaminated maize was soaked in 0.1M sodium carbonate solution for 72 hours (Smith and Solomons, 1994).

Studies of the effects of biological, chemical and physical treatments on the reduction of fumonisin levels in food have shown variable results. Most maize-based foods are thermally processed before eating but fumonisins have been shown to be fairly heat stable compounds (Dupuy *et al.*, 1993, Scott and Lawrence, 1994, Alberts *et al.*, 1990). Generally, loss of FB_1 and FB_2 was more rapid and extensive in alkaline or acidic environments than at neutral pH. Effects of thermal processing fumonisin B_1 and B_2 in an aqueous buffer showed that the rate and extent of their decomposition increased with processing temperature and time depending on the pH of the processing medium (Jackson *et al.*, 1996). It was found that thermal processing of foods caused a firmer binding of the toxin to the food matrix rather than its

chemical decomposition, hence decreasing its extraction efficiency (Bordson *et al.*, 1993, Scott and Lawrence, 1994). While no losses were seen at 100°C, more than 80% of FB_1 was lost at temperatures over 175°C after 60 min of processing. The products of thermal processing were found to be mainly hydrolyzed and partially hydrolyzed FB_1 (Jackson *et al.*, 1996) which are more toxic than the parent compounds, since the aminopentol backbone is responsible for the toxicity of FB_1 (Hendrich *et al.*, 1993). However, when FB_1 is heated with reducing sugars such as fructose, an adduct is formed which, although it increases FB_1 absorption in the gastrointestinal tract, masked the primary amine group, the main site of FB_1 toxicity (Hopmans *et al.*, 1997). This could be a practical approach to FB_1 detoxification. Although the findings show that some fumonisins are lost during thermal processing, it is doubtful whether rural cooking attains temperatures notable for the toxin degradation (over 150°C) because this is done as aqueous mixtures with short processing times.

Washing of maize contaminated with FB_1 in water did not change the level of toxin, whereas nixtamalization [$Ca(OH)_2$ in combination with hydrogen peroxide and sodium carbonate in cooking] reduced FB_1 but produced hydrolysed FB_1 (aminopentol). Ammoniation was found to have little effect on FB_1 (Norred *et al.*, 1991) while fermentation of contaminated maize into ethanol resulted in limited amount of FB_1 degradation (Bothast *et al.*, 1992, Scott *et al.*, 1995). However distilled ethanol had no FBs as they are not volatile, instead, most of FB_1 was recovered in distillers' grains, thin stillage and distillers' soluble fractions. It was also found that FBs were stable when added to yeast-free wort and kept for 8 days at 25°C (Scott *et al.*, 1995).

2.2.3 Storage and Preservation

In order to lessen the contamination of food crops by mycotoxins, especially in storage, cereals, seeds and nuts require drying soon after harvesting to lower the moisture content thus inhibiting fungal proliferation. This is usually achieved traditionally by exposure to the sun and wind. Improved drying is by use of solar dryers. In maize growing areas of the tropics, proper storage facilities are lacking and various traditional methods used include: hanging the unshelled maize or sorghum in poles near the kitchen-fire, underground pits, farm bins, sacks, metal and plastic drums, and sacks (Aidoo, 1993). It has been shown that grain stored in

drums with water activity (a_w) above of 0.9, heated spontaneously with the growth of micro-organisms, while the temperature attained by drier grain was lower (Hill and Lacey, 1983). When the drums were sealed to prevent air exchange, heat generation was prevented. And the oxygen concentration decreased to about 10% while carbon dioxide increased to above 20%. However, when the sealed drums when kept outside the house, fluctuations on the effect of night and day temperatures were reported. Post-harvest reduction of fumonisin production is also possible by maintaining low oxygen tension (storage under modified atmospheric packaging, MAP) and low kernel moisture content of below 22% (Le Bars *et al.*, 1992).

2.3 ANALYSIS AND DETECTION

2.3.1 Introduction

Mycotoxins are diverse in their chemical nature, therefore, posing a great challenge to their collective analysis. There are two possible approaches for the detection and determination of mycotoxins: biological and chemical. Chemical assays are preferred at the moment, because they are more easily quantifiable and more sensitive than bioassays, which are more useful in screening and detecting unknown mycotoxins. Mycotoxins are usually analyzed separately, each toxin being screened using its corresponding technique. There has been difficulty in designing a single procedure for collective screening of diverse mycotoxins in one sample and which can also be used to screen a wide range of foods and feeds. Multimycotoxin screening techniques, first developed in 1970's, were used to detect several mycotoxins in foods and feedstuffs (Stoloff *et al*, 1971). They are based on a general flow pattern: sampling, sample preparation, extraction, clean-up, separation, detection, quantitation and confirmation (van Egmond and Paulsch, 1986). Multimycotoxin screens have the advantage of screening for important mycotoxins in one sample, hence were convenient in addition to their rapidity of application. The detection limit of the technique may be higher than that of individual analytical methods, but it is more practical to apply it in developing countries because it is cheap and does not need a high level of personnel training.

2.3.2 Multi-mycotoxin Screen

2.3.2.1 Sampling

It is known that there is not a normal distribution of mycotoxins in a batch of contaminated sample. Contamination of food products is likely to occur in isolated "pockets" of mycotoxins. This maybe due to localized fungal infection in the field under unfavourable conditions of stress or in storage with isolated areas of high temperature (hot spots) and moisture (Hesseltine, 1974). Thus, there is need to obtain a representative sample from the product under investigation. A large sample size (up to 45 kg in some cases) is taken, mixed well and sub-sampled to the required size. This is necessary where samples of large particle size like peanuts and maize are involved; however for small particulate samples like sorghum and wheat, smaller evenly mixed samples may do. The sub-samples taken from a lot should be ground to reduce the particle size, mixed to increase homogenicity. Guidelines on proper sampling for mycotoxin analysis have been published (Park and Pohland,1989) while other food safety agencies have set their own sampling plans (Jewers *et al.*, 1989).

2.3.2.2 Extraction

This step involves extraction of the sample to isolate the analyte from the matrix components. Several methods based on two principles, have been used to achieve this; either by use of supercritical fluid extraction (SFE) (Kalinoski *et al.*, 1986, Wu *et al.*, 1995, Selim *et al.*, 1996, Smith, 1996) or appropriate conventional solvents to solubilize the toxins from the matrix by shaking for at least 60 minutes. After shaking, the solid components are separated by cold centrifugation (4°C) or by filtration (Sydenham *et al.*, 1992b). Different matrices adsorb and retain analytes to different extent hence solubilization of the toxin from the matrix is varied. This requires a subtle choice of extracting solvent in order to obtain a near full recovery of toxins.

Solvent systems utilised to extract various mycotoxins have been recorded (Betina, (1989). Organic solvents used previously, such as chloroform, and dichloromethane were not suitable, as they would not extract polar toxins and could not easily penetrate the matrices with moisture. To overcome this, water miscible solvents were used such as methanol and acetonitrile. Studies done by Gelderblom *et al.* (1992a), showed that acetonitrile/water mixture

(90:10 v/v) was the best solvent for extracting most mycotoxins from the food matrices in preference to various proportions of methanol in water.

Mycotoxins are also co-extracted with general lipids and plant pigments, which interfere with their separation by thin layer chromatography and lowering their recovery. This has been overcome partly by defatting the extracts with special solvents like hexane and iso-octane (2,2,4-trimethylpentane). Acidic toxins are able to react with sodium hydroxide (Chang-Yen and Bidasee, 1990) and sodium hydrogen carbonate (Patterson and Roberts, 1979) to form salts and which remain in the aqueous fraction, while the non-acidic (neutral toxins) go into the organic phase. This physico-chemical property is useful in identifying the two classes of mycotoxins in the sample and has been used routinely to monitor mycotoxin levels in foods and feeds by bodies like the Ministry of Agriculture, Fisheries and Food (MAFF, 1992) in UK and South African Maize Board. Currently, the technique is being reviewed to improve its sensitivity and ability to accommodate more recently discovered mycotoxins, e.g., the fumonisins (Gelderblom *et al.*, 1988) and moniliformin, which are polar mycotoxins not amenable to analysis by the currently used methods.

2.3.2.3 Clean-up

Many mycotoxins are normally present in very low levels and require concentration to make their detection possible. Co-extracted impurities may interfere in the final detection hence clean-up is necessary before concentration (van Egmond and Paulsch, 1986). Several techniques have been used in the past, including thin layer chromatography, liquid-liquid partition, open columns packed with materials such as silica gel, florisil, sephadex, charcoal and aluminium oxide (Romer, 1986). Separation was attainable by adsorption onto the matrix of the packing. These methods are time consuming during column packing and rather tedious during their application (Scudamore, 1998). Glass mini-columns were developed much later but had the same applicability as the big columns (van Egmond and Paulsch, 1986). Solid phase extraction (SPE) has become a rapid efficient method of clean-up of late (Scott, 1993). They are usually available in small disposable columns or cartridges, which usually contain plain silica, C_{18} or strong anion exchange bonded phases. They are more popular and convenient to work with, than the conventional chromatography and liquid-liquid partition.

Gel permeation chromatography (GPC) is a technique, recently used with a high degree of success, to clean-up 14 mycotoxins (Scudamore and Hetmanski, 1992). However, this technique may not be used to detect mycotoxins like fumonisin and moniliformin, which are completely different physico-chemically from those previously cleaned up using the method.

Other methods of clean-up have so far been employed including immunoaffinity chromatography. Immunoaffinity columns (IACs) are widely used for clean-up and isolation of mycotoxins extracted from foods and biological fluids, particularly aflatoxins, ochratoxin A, and fumonisins. The columns are coated with antibodies specific for a given mycotoxin to a specially activated solid-phase support and packing the support suspended in aqueous buffer solution into a cartridge (Scott and Truckess, 1997). Although the latter method is sensitive and specific, it is not suitable for collective analysis, as it is selective. Difficulty in developing antibodies against certain mycotoxins, in addition the high cost of IACs has limited the scope of method application.

Further improvement of multimycotoxin technique was done by introducing the dialysis membrane to improve the clean-up of samples (Roberts and Patterson, 1975, Patterson and Roberts, 1979). The membrane clean-up was effective in separating diffusible low molecular weight mycotoxins from the large molecular weight substances, like fats and pigments, which are retained in the sac, while the mycotoxins diffuse into the aqueous acetone phase. The dialysis method was successfully used by Dutton and Kinsey (1995) and Dutton and Westlake, (1985), to clean-up mycotoxins from grain sent to the University of Natal for routine analysis. Although it does not rid all impurities from the sample, it can be applied to clean up a wide spectrum of mycotoxins and in addition, it has been shown to be better in mycotoxin recoveries than GPC (Early, 1995).

2.3.2.4 Thin Layer Chromatography

Over the years, thin layer chromatography (TLC), has been widely used as a procedure of choice for identifying mycotoxins. It involves spotting of the sample on silica, cellulose, alumina or polyamide coated plate and developing it in suitable mobile phase solvents (Appendix 2). Thin layer plates are usually available as 20 x 20 cm aluminium of plastic

backed plates; however, 10 x 10 cm plates, cut from the former size are better to use, as they save time and cuts down on cost. A two-dimensional development is preferred to one directional method as the former gives a better separation of different mycotoxins in a mixture and their separation from other co-extracted substances. In effect, the development of the TLC plate in one direction is a "clean-up" step while development in the second direction is the actual separation-detection step. In two-dimensional TLC, the sample extract is spotted at the corner of the plate and two developments are carried out successively parallel to the two sides of the plate using two different developing solvents. The choice of solvents depends very much upon the polarities and the solubilities of the compounds to be separated from the mixture. The developed spots are then visualised under ultra violet light (UV) or spraying with the appropriate reagents to form coloured complexes. It is important that the spots, which appear, are immediately dotted around with pencil and recorded as to colour and R_f value. After notation of retardation factors (R_fs), the spots are then compared with those of the known standards from which quantitation and identification can be deduced. The R_f values are useful in identification while the appearance and intensity of the spots can be used to estimate the concentration of the unknown sample extract.

2.3.2.5 High Performance liquid Chromatography

The goal of high performance liquid chromatography (HPLC) is to separate components of a sample within a reasonable period of time into separate bands of peaks as they migrate through the column. This is achieved by using high pressure to drive the analyte in solution through a packed chromatographic column, causing separation of the analyte under test and can therefore be identified and quantified using appropriate methods. The major components of a HPLC system include: the mobile phase reservoir, the pump, sample injector, separating column, detector and recorder (Figure 2.5, Plate 2.2).

Plate 2.2 HPLC Spectra System 2000

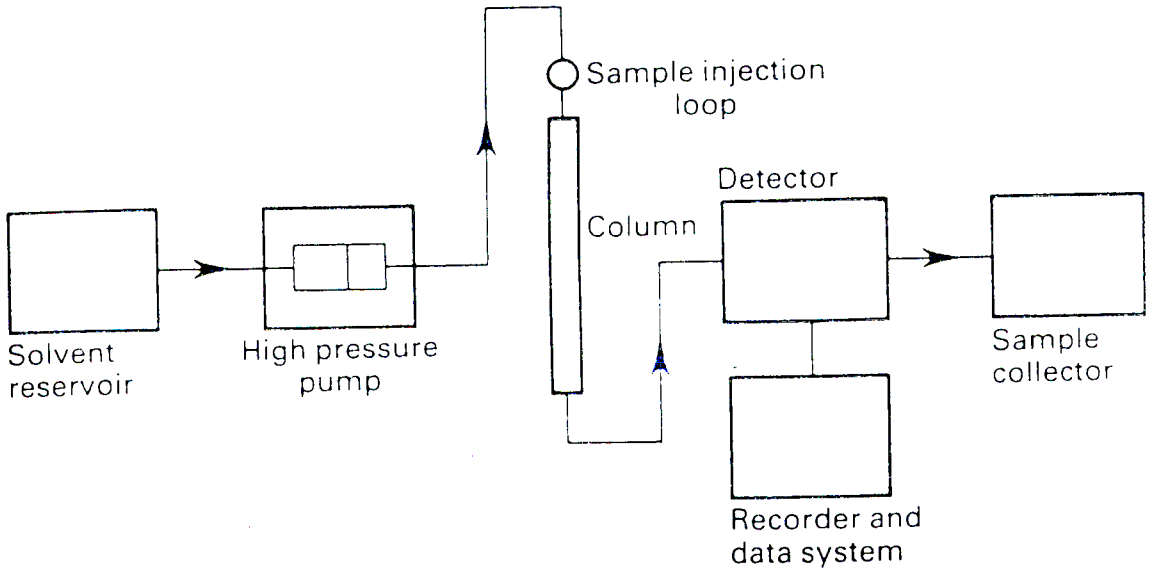


Figure 2.5 The diagram of a HPLC system (Wilson and Walker, 1994)

The identification and quantitation of the analyte depends mainly on the performance of the column, type of mobile phase used and the physico-chemical nature of the analyte.

The success of HPLC is measured by its ability to separate completely (resolve) one analyte from a mixture of similar compounds. Peak resolution (R_s) is related to the properties of the peaks such that:

$Rs = 2(t_{RB} - t_{RA})/ W_A + W_B$

Where t_{RA} and t_{RB} are the retention times compounds of compounds A and B respectively, and W_A and W_B are the base peaks for A and B respectively. The Rs values of 1.0 corresponding to 98% are usually adequate for quantitative analysis (Wilson and Walker, 1994).

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for the solute to achieve complete equilibration between the mobile and stationary phases. Each zone is called a theoretical plate and its length in the column is called the plate height (H), which has dimensions of length. The more efficient the column , the greater the number of theoretical plates that are involved. The number of theoretical plates (N) involved in the elution of a particular analyte is given by:

$$N=16(t_R/w)^2$$

The plate number can be increased by increasing the column length (L) to a limit since length increases with peak broadening. The plate height (height equivalent to a theoretical plate, HETP) is a parameter which is useful for comparative purposes such as operating the column under different conditions. It is expressed as

$$HETP=L/N=H$$

The maximum number of peaks that can be separated by a specific chromatographic system is called peak capacity (n) and is expressed as:

$$N=1+\sqrt{N}/16(\mathrm{In}V_\omega/V_\alpha)$$

Where V_ω and V_α, are the first and the last peaks respectively. Peak capacity can be increased by performing a gradient elution. The other major functions necessary for good resolution in a chromatographic system include: Selectivity, the ability of the system to discriminate between structurally related compounds, and efficiency, a measure of the diffusion effects that occur in the column to cause peak broadening and overlap.

2.3.2.6 Capillary Electrophoresis

Capillary electrophoresis (CE) is a family of related techniques that employ narrow-bore (20-200μm i.d) capillaries to perform high efficiency separation of both large and small molecules. These separations are facilitated by use of high voltages (100-500V/cm), which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary buffer solutions. The heat that is generated is efficiently dissipated by large surface area to volume ratio of the capillary. High electrical fields results in short analysis times, high efficiency and resolution. Theoretical considerations of CE is given by the following equation:

$$t=L^2/\mu V$$

where t is the migration time for a solute, L is the length of the tube, μ is the electrophoretic mobility of the solute, and V is the applied voltage. The separation efficiency in terms of the total number of theoretical plates, N, is given by:

$$N=\mu V/2D$$

Where D is the solute's diffusion coefficient. Sample application is usually by high voltage injection where 5-30μl solution is introduced into the capillary. A high voltage (up to 20 kV) is then put across the capillary tube and component molecules in the injected sample migrate at different rates along the length of the tube. Since the flow is very strong,, the rate of endoosmotic flow usually being greater than the electrophoretic velocity of the analytes, all ions, regardless of charge sign, and neutral species are carried towards the cathode. Cations migrate fastest while anions migrate slowest because of their attraction to the anode. As the

separated molecules approach the cathode, they pass through a viewing window, where they are detected by a UV monitor that transmits a signal to a recorder, integrator or computer. Run times are usually between 10 and 30 minutes. A typical capillary eletropherograph is shown (Figure 2.6).

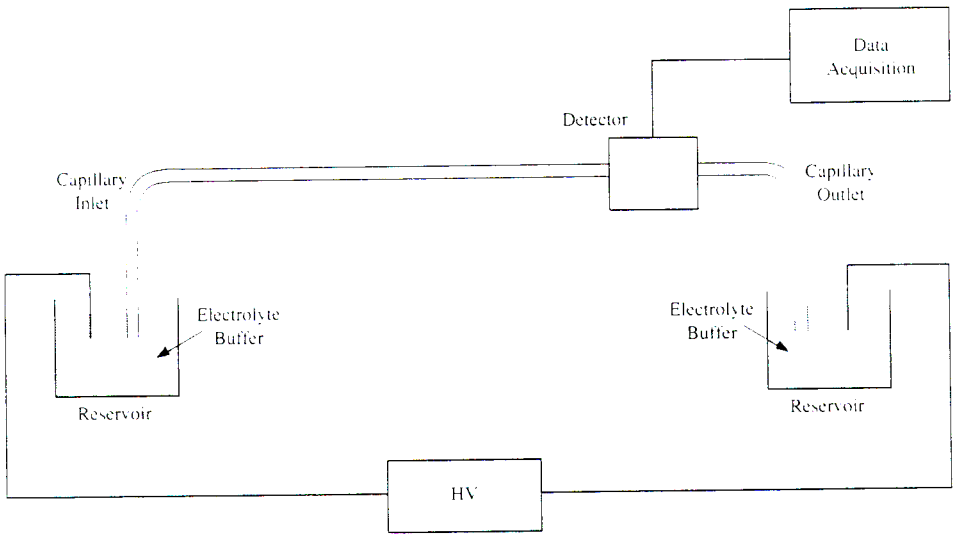


Figure 2.6 A diagram of a capillary electropherograph

2.3.3 Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay is a newly developed technique has been shown to be useful tools for the screening cereals for mycotoxins (Chu 1992, Pestka, 1994). ELISA exploits the specificity and sensitivity of antibodies which are produced when they are immunized with a specific antigen e.g., mycotoxins. The version used to detect mycotoxins is known as competitive ELISA.The technique is either in wet or dry (strip) format. The strip format is mainly suitable for field testing (Schneider *et al.*, 1991). The wet format involves use of polystyrene multi-well plate coated with the specific antibodies for each mycotoxin. In principle, wells in the polystyrene multi-well plate are coated with the specific antibodies. After a period of incubation, the plate wells are washed to remove unbound antibodies and any remaining protein binding sites blocked. To each well is then added the target analyte, which has been bound chemically to an enzyme conjugate such as horseradish peroxidase. Extract

samples or standards of known toxin concentration are also added to the wells. Any free toxin in the sample or standard competes with the conjugate for the antibody binding sites. The more free toxin then the greater the competition and less conjugate manages to bind with the antibody. After the incubation period, the wells are washed again and the enzyme substrate is added. Breakdown of the substrate results in a colour development, the intensity of which is proportional to the amount of enzyme present. The lighter the colour, the more free toxin was present in the sample and *vice versa*. Visual interpretation can be done by comparing the colour development in the sample well with that of a toxin of known concentration.

Use of ELISA test kits to screen commonly occurring mycotoxins in cereals has been reviewed by Holmes, (1996). Monoclonal antibodies have been prepared against the fumonisins (Azcona-Olivera *et al.*, 1992) from mice immunized with fumonisin B_1-cholera toxin conjugate. The antibodies produced were used to devise a direct competitive ELISA method for FB_1 analysis. Comparative assessement of fumonisin in grain-based foods showed that although there was a positive correlation between FB_1 detected by HPLC and ELISA, the latter yielded higher results than HPLC particularly in more contaminated samples (Pestka *et al.*, 1994). Differences in sample preparation among the methods may have resulted in the different observations. Presence of structurally related compounds in the extracts that are detectable by ELISA but not the other two methods could also contribute to the discrepancy in the results. Immunoassays (ELISA) essentially represent a rapid, reliable, simple, relatively inexpensive and field-adaptable alternative to conventional chromatographic and colorimetric methods.

2.3.4 Fumonisins

Human and animal exposure to fumonisins is known to be hazardous. In order to assess fumonisin levels in foods and feeds, a precise, sensitive and accurate method for its screening is necessary. Fumonisins in foods are extracted with polar solvents, the two most popular being methanol/water (3:1 v/v) and acetonitrile:water (50:50 v/v). Comparative studies on these two mixtures showed that acetonitrile:water is more efficient in extracting FB_1 from maize products than methanol:water and yields an extract that is cleaner and less cloudy (Bennet and Richard, 1994). It was found that cooked maize-containing foods caused a firmer

binding of the toxin to the food matrix rather than its chemical decomposition, hence decreasing its extraction efficiency (Bordson *et al,* 1993, Scott and Lawrence, 1994). However, when the pH of the extracting solvent (50% acetonitrile) was adjusted to 2.7 with 0.1M HCl (to overcome any buffering capacity), remarkable recoveries of FBs were achieved in comparison to the solvent with unadjusted pH. For example, a corn pasta sample which gave 90ng/g FB_1 when extracted with plain 50% aqueous acetonitrile, gave 246 ng/g FB_1 when extracting solvent was adjusted to pH 2.7 (Murphy *et al.,* 1996). Food additives and metal ions are likely to affect the recoveries of fumonisins. When ferrous sulphate heptahydrate was added to 200mg/g maize meal, it completely inhibited the recovery of fumonisins (Scott and Lawrence, 1994).

Clean-up of FBs is usually done on C_{18} or Strong Anion Exchange (SAX) cartridges. Strong anion exchange columns are more consistent and efficient than C_{18} cartridges in isolating fumonisins from crude extracts (Bennet and Richard , 1994) resulting in cleaner fractions with lower detection limits. Effectiveness of the SAX columns is enhanced by adjusting the pH of the sample extracts between 5.8 and 6.5 for better separation (Sydenham *et al.,* 1992b). A more recent immunoaffinity column method of clean-up, though sensitive and specific is only valuable for isolating small amounts of fumonisins from the tissue or plasma of animals exposed to fumonisins as it gets saturated easily in high concentrations (Bennet and Richard, 1994, Truckess *et al.,* 1995). Commercial clean-up immunoaffinity columns are now available (Abouzied and Pestka, 1994). Solid phase C_{18} cartridges have also been used, but are less effective in comparison to purification on SAX media (Stockenstrom *et al.*, 1994, Sydenham, and Thiel, 1992). However C_{18}s are preferred when cleaning up faecal sample extracts (Shephard *et al.,* 1994).

Thin layer chromatography is the least expensive method for fumonisin detection in maize samples (Gelderblom *et al.*, 1988). This method is mainly used for screening and cannot detect low levels of fumonisins below 500µg/g (ppm) (Sydenham and Thiel, 1992). Thin layer chromatography involves the use of silica gel glass or aluminium backed plates where 20µl of clean extract is spotted and developed in two dimensions, in two different solvent systems (Dutton, 1993). After drying, the spots were visualized using p-anisaldehyde spray and heated

at 120°C for 2-3 minutes. A purple colour with the same R_f value as that of the standard is a positive confirmation for fumonisins.

A more accurate and sensitive method commonly used is high performance liquid chromatography (HPLC). Unlike Aflatoxins, fumonisins do not fluoresce, nor absorb UV nor visible light. They require derivatisation with fluorescing substances to enable their detection in a fluorimetric detector in conjuction with the HPLC (Figure 2.7). O-phthaldialdehyde (OPA) has been the most common derivatising agent and it allowed detection limits of less than 50 ng/g. (Sydenham *et al.,* 1992b). However, the OPA derivative is less stable when compared to a more recent introduced derivatizing agent, naphthalene dicarboxaldehayde (NDA) in the presence of cyanide (CN-) which is highly fluorescent and stable beyond 24 hours (Bennet and Richard, 1994). OPA-fumonisin derivative progressively decays following periods in excess of 4 minutes, which necessitates precisely timed derivatization and HPLC operation. Although NDA derivatives are very sensitive and stable enough to utilize autoinjectors, their derivatization procedure is a rather long reaction (15 mins), a high temperature (60°C) and the use of an extremely toxic reagent (sodium cyanide) (Bennett and Richard, 1994). Furthermore, NDA measures FB_1 only while it is necessary to determine FB_2 also (Thiel *et al.,* 1992). Use of OPA in fumonisin analysis is therefore, preferred instead of NDA.

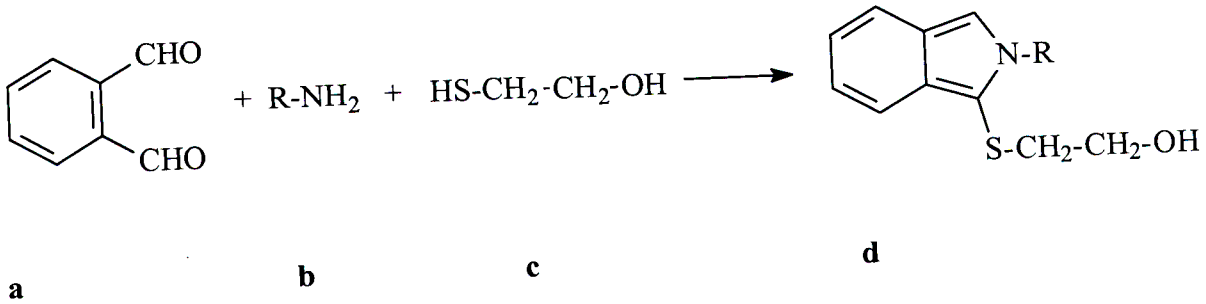


Figure 2.7. Schematic diagram of derivatization reaction: a, *o*-pthaldialdehyde; b, fumonisin; c, 2-mercaptoethanol; d, OPA-fumonisin derivative.

Analysis of FBs by capillary electrophoresis (CE), has been carried out (Korfmacher *et al.,* 1991; Maragos *et al.,* 1996) with a detection limit of 50ng/g. Although the results compare favourably with those of HPLC, CE method is tedious and time consuming, taking up to 2.5

hours to derivatize the sample. Direct mass spectrometry (MS) with or without coupling with gas chromatography (GC) (Plattner *et al.*, 1994) or with HPLC (Smith and Thakur, 1994) has also been used to analyze FBs. The GC-MS method involves volatilization of FBs as tri-fluoro dervatives before analysis.

Several techniques have been used to evaluate and characterize FB_1 including liquid secondary ion mass spectrometry (LSIMS) (Bezuidenhout *et al.*, 1988), thermospray, (TSMS) fast-atom bombardment (FABMS) and electrospray mass spectrometry (ESMS) (Korfmacher *et al.*, 1991 and Josephs, 1996).

2.3.5 Biomarker Analysis

Human exposure to mycotoxins can be determined by analyzing samples of food commodities. If a food commodity is contaminated, exposure is assumed based on human consumption of the commodity. This approach is subject to errors, because dietary composition varies with individuals while commodities are unevenly contaminated and some people may not actually ingest any contaminated commodity at all. Food processing may also have a modifying effect on the level of mycotoxin in the commodity. The use of chemical specific biomarkers to mycotoxins is therefore preferred as a method of assessing individual exposure to mycotoxins (Wild *et al.*, 1990, Groopman and Kensler, 1993, Strickland and Groopman, 1995). This exposure assessment at individual level is direct and valid, however, while positive results do prove exposure, negative results only indicate that the biomarkers are not present, that is, the biomarkers may have been excreted or exposure did not occur.

Biomarkers are classified into three major categories: markers of exposure, reflecting dose of toxic agents; markers of effect, indicating a biological response to an exposure and markers of susceptibility, indicating the individual sensitivity to a particular toxic agent. Analysis of aflatoxin-N7-guanine adducts in urine and albumin adducts in sera has been carried out to assess the role of aflatoxin in liver cancer (Wild *et al.*, 1990, Groopman *et al.*, 1993). Measurement of sphingoid bases, sphinganine (Sa) and sphingosine (So) which accumulate in blood due to the action of the FB_1, has been used as a biomarker for human exposure to fumonisins (Riley *et al.*, 1994b, Shephard *et al.*, 1997) but its disadvantage seems to be poor

and erratic recoveries of the sphingoid bases from physiological fluids and tissues. Fumonisin B_1 analysis in the faeces of non-human primates and other animals has been carried out previously (Shephard *et al.*, 1994b, Prelusky *et al.*, 1994, Smith and Thakur, 1996). This latter approach holds promise for use as a short term biomarker of exposure to FB_1 in humans.

2.4 THE ROLE OF FUNGI AND MYCOTOXINS IN HUMAN HEALTH

2.4.1 Mycotoxicoses

The major effects of mycotoxins on animals and man can largely be classified into acute primary, chronic primary and secondary mycotoxin diseases (Smith *et al.*, 1996). In acute mycotoxicoses, severe illness is observed and in many cases, like in ergot poisoning for example, results in death of the individual. Specific symptoms are often observed with gross injury to the tissues involved. Ingestion of gross mycotoxin contaminated food and feeds results in this condition.

Chronic mycotoxicosis is caused by a long time ingestion of low levels of mycotoxins with long term additive effects such as, reduced reproductive efficiency, poor growth and often with no clearly defined immediate visible changes. Secondary mycotoxin diseases are much well less defined involving long-term small dose exposure to some mycotoxins. They usually impair with the immune system weakening the host's resistance to infectious diseases (Smith *et al.*, 1996).

2.4.2 Occurrence

History has recorded several human and animal diseases thought to be caused by mycotoxin-contaminated foods and feeds. The outbreaks of ergot alkaloid poisoning in Europe throughout the Middle Ages are a good example. This is the oldest and best-known human mycotoxicosis, which plagued man in the middle ages. This, came to be known as St. Anthony's fire (Barger, 1931; Bove, 1970). Some of the specific observable acute disease symptoms include hepatitis, spontaneous abortion, haemorrhage, nephritis and necrosis of oral and gastrointestinal mucosa (Smith *et al.*, 1995). Alimentary toxic aleukia (ATA) disease was first reported in Russia in 1913 (Joffe, 1963) and was associated with the consumption of grain contaminated with *Fusarium spp* which had over-wintered in snow-covered fields. This disease came to be

associated with T-2 toxin (Table 2.2). Balkan endemic nephropathy (BEN) was first discovered in 1950's in Bulgaria as a chronic disease of unknown aetiology. The outbreak of the disease is correlated with higher rainfall in summer and high humidity in autumn in endemic areas (Larsen, 1928) and ochratotoxin A (OTA) is believed to be the causative agent. Kashin Beck disease (KBD) disease name is derived from two Russian Scientists, Kashin and Beck who studied it in 1860s (Joffe, 1986). It has been found to exist in Taiwan, Korea, China, Japan and Holland (Joffe, 1986). The likely mycotoxin involved has been suggested to be fusarochromanone (Wright *et al.,* 1987). Aflatoxins were discovered in 1960s, have been associated with hepatocellular carcinoma in many areas (Ngindu *et al.,* 1982, Van Rensburg, 1985 and Peers *et al.,* 1987). In Japan, a large amount of mouldy rice was consumed at the end of the second world war and this gave rise to yellow rice disease which had similar symptoms to vitamin B_1 deficiency (Saito *et al.,* 1971).

Oesophageal cancer (OC) has been found to be most prevalent in parts of southern Africa, China, and northeastern Iran (Kmet and Mahboubi, 1972; Marasas *et al.*, 1988a; Chu and Li, 1994). It was found to be the most common cause of cancer death among the Africans, both rural and urban and among Coloured males in many parts of southern Africa (Rose, 1982). In Transkei- 50-200 cases per 100,000 were diagnosed positive annually. Several aetiological possibilities, including nitrosamines, nutritional deficiencies, fermented and moldy foods and the inhalation of polycyclic aromatic hydrocarbons (PAH) present in tobacco smoke have been considered, but none has been convincingly linked to the high rates of oesophageal cancer in China (Yang, 1980). In Africa, high-risk areas are always associated with the consumption of maize usually contaminated with fumonisins (Marasas *et al.,* 1988a). Fewer cancer cases were reported in areas with mineral rich soils contrary to the areas with mineral deficient soils (Marais, 1962). Nutritional and climatic stress on food plants alters their chemical composition and increases their susceptibility to infection by fungi such as *Fusarium moniliforme* (the main fumonisin producing fungus). Concentration of essential minerals in household water was also found to be lower in high than in low OC areas. High nitrate content in drinking water was also noted in high OC areas (Rose, 1982). Dietary deficiencies such as trace elements (Zinc, molybdenum, and manganese), vitamins A, C, and B_2 were observed in high OC areas. Intake of traditionally brewed alcohol was common in

high than in low OC (Marasas *et al.,* 1988a). Alcohol is thought to be a co-carcinogen promoter as it inhibits salivation and thus encouraging local accumulation of carcinogens in oesophageal mucosa (Rose, 1982). Traditionally brewed beer from high OC areas of Transkei was made from molded maize, which therefore increased the chances of fumonisin contamination. The prevalence of OC in high rate areas was associated with oesophageal cytological abnormalities (mild to advanced changes) as seen in brush biopsies; and high infection of home grown maize by *F. moniliforme* (Marasas *et al.,* 1988a). The range of FB_1 contamination in maize was 0-55mg/kg in low OC areas and 50-117mg/kg in high OC areas in Transkei (Rheeder *et al.,* 1992). Although it has been shown statistically that a relationship exists between *F. moniliforme* and human OC exists, the ability of the fungus to cause the disease has not been demonstrated experimentally (Marasas *et al.,* 1988a). In view of these findings, the possible role of fumonisins in the aetiology of oesophageal cancer merits further investigation.

Occurrence of FB_1 in animal feeds has also been associated with animal mycotoxicoses. In USA, 126mg/kg were associated with equine leukoencephalomalacia (ELEM) and outbreaks of porcine pulmonary oedema (PPE) in pigs (Harrison *et al.,* 1990). Long term feeding of rats with at a concentration of 50mg/kg FB_1 was shown to be carcinogenic and caused primary hepatocellular carcinoma (Gelderblom *et al.,* 1991). Nephrotoxicity has been reported in several animal species (Bucci et al., 1998).

2.4.3 Risk, Legislation and Control

In order to reduce the human health risk by fungi, safety measures need to be established in order to reduce food contamination and to set maximum permissible levels in food for human consumption (FAO, 1995; Van Egmond and Dekker, 1995). Risk assessment (exposure assessment and hazard assessment) is used to determine the specific mycotoxin level that would result in negligible risks or a reasonable certainty of no harm to food consumer(Kuiper-Goodman, 1995). It is calculated from the exposure (probable daily intake or PDI) and hazard assessment (tolerable daily intake or TDI). Exposure assessment is calculated from the level of the toxin in food intake or from biomarker/direct measurement in humans fluids and tissues

(Kuiper-Goodman, 1994), while TDI is the maximum amount of toxin which can be ingested without causing expected toxic effects in experimental animals.

Table 2.2 Some mycotoxins produced commonly by fungi that infect food and their associated human mycotoxicoses.

MYCOTOXIN	FUNGAL SPECIES	MYCOTOXICOSIS	REFERENCE
Aflatoxin	*A. flavus* *A. parasiticus*	Liver Cancer, Reyes syndrome	Linsell and Peers, 1977
Ergot alkaloids	*Claviceps purpurea*	Ergotism	Robbers, 1979
Deoxynivalenol	*Fusarium* spp.	Intestinal haemorrhage, diarrhoea	Bhat *et al.*, 1997
Ochratoxin A	*Penicillium* spp.	Nephrotoxicosis	Kuiper-Goodman and Scott, 1989
Zearalenone	*Fusarium* spp.	Breast/cervical cancer	IARC, 1993
T-2 Toxin	*Fusarium* spp.	Alimentary Toxic Aleukia	Marasas *et al.*, 1984
Cyclopiazonic acid	*Penicillium* spp.	Kodua poisoning	Rao and Husain, 1985
Moniliformin	*Fusarium* spp.	Onyalai	Rabie *et al.*, 1975
Fumonisin B_1	*Fusarium* spp.	Oesophageal Cancer	Van Rensburg, 1985
Islanditoxin	*Penicillium* spp.	Yellow Rice	Saito *et al.*, 1971

Since the discovery of the aflatoxins in the 1960's, regulations have been established in many countries to protect the consumer from harmful effects of mycotoxins that may contaminate foodstuffs (Van Egmond and Dekker, 1995). Various factors influence the establishment of limits for certain mycotoxins such as, availability of survey data, toxicological data, analytical methodology, and knowledge about the distribution of mycotoxins in contaminated commodities. There is no consistent rationale for setting limits or for enforcement control. Many countries in the world have set their own limits and marketing strategies. International inquiries on existing mycotoxin regulation in foodstuffs and animal foodstuffs have been

carried out several times in the 1980's and the details about tolerances, legal bases, responsible authorities, official protocols of analysis and sampling have been published (Schuller *et al.*, 1981, Van Egmond, 1989b, Van Egmond and Dekker, 1995, FAO, 1995). Food and Agricultural Organisation of the United Nations (FAO) has been assisting a number of developing countries with regard to implementing preventive programmes on food contamination by mycotoxins (Boutrif, 1995). In order to achieve realistic legislation, there is need to harmonise regulations with co-operation of industry, consumers, scientific sector and regulatory agencies.

Risk assessment of mycotoxins in humans is difficult to establish because the risk data available are based on animal experiments. Most of the available regulations and tolerances in all countries concern aflatoxins since they are the earliest noted. Regulatory levels are varied depending ranges from 20 and 300pbb for human food and animal feed respectively (Park and Pohland, 1986, FAO, 1995). However, these levels contradict those of Barnes (1966) who stated that "No level (in human food) is considered safe for aflatoxin B_1" because there is no safe dose of a carcinogen. There is no conclusive evidence based on animal studies that humans will respond in the same manner as animals to the same dosage of toxin. However, Barnes (1966) suggested that the toxicity figures based on monkey studies (0.01ppm AFB_1) could be applied.

Risk assessment of exposure to fumonisins can be monitored by analysing foods for FB_1 (Sydenham *et al.*, 1992b, 1996) or by measuring their biomarkers (sphingoid bases) in blood and urine. The first permissible levels of FBs in commercial maize-based foods were set at 1mg/kg in Switzerland (Zoller *et al.*, 1994). At this moment, it is difficult to predict a safe levels based on animal studies since FB_1 affects several targets different from those of humans (Kuiper-Goodman, 1995). However, to extrapolate animal acceptable daily intake (ADI) levels of FB_1 to that of humans, a tenfold safety factor has to be used in order to obtain the lowest credible level of FB_1 in food. Further epidemiological studies are required, in order to precisely define the role of FB_1 in Transkei and other areas where oesophageal cancer is high and where maize is consumed.

2.4.4 Limiting Contamination

Since *Fusarium moniliforme*, which produces FBs, infects live plants in the farm, prevention can be achieved through selectively using cultivars that are resistant to infection, control of weeds and use of fertilisers in order to grow healthy plants resistant to stress. Stressed plants are vulnerable to infection. Harvesting during dry season and using sealed storage facilities are also recommended (Section 2.2).

2.4.5 Situation in Rural Areas of Southern Africa

Contamination of food commodities is a global problem. The countries of Southern Africa have not been spared. Rural mycotoxin surveys done in Swaziland implicated the aflatoxins in aetiology of liver cancer (Peers *et al.*, 1987). In rural areas of South Africa, storage methods are varied and depend on tradition, size of harvest and available facilities (FAO, 1984; Aidoo, 1993; Dutton *et al.*, 1993). Climatic conditions and storage were cited for the high contamination rates and have often played a role in the growth of various mycotoxigenic fungi found (Gqaleni *et al.*, 1997). *F. moniliforme* has been found to be the most dominant fungus infecting maize in southern Africa (Rava *et al.*, 1996). Screening of rural samples in Kangwane rural area showed that aflatoxin, deoxynivalenol, fumonisin, and zearalenone were frequent (Dutton *et al.*, 1993) and were the main mycotoxins contaminating stored maize and peanuts. Fungal microflora of *Fusarium, Aspergillus*, *Penicillium*, *Altenaria* and *Diplodia* were the major ones isolated from maize. Incidence of various disease conditions observed in several local hospitals in southern Africa are suggestive of mycotoxicoses including oesophageal cancer, hepatocellular carcinoma, gynaecomastia, kwashiorkor, idiopathic congestive cardiopathy (Campbell, 1990). Some of these conditions were observed on some of the patients attending King Edward VIII hospital in Durban, South Africa (Dutton *et al.*, 1993).

2.4.6 Fumonisins and Health in South Africa

Fumonisins have been linked to OC in some of the Eastern Cape areas of South Africa (Rheeder *et al.*, 1992). Research into the cause and epidemiology of (OC) in Transkei, show that it is related to consumption of fumonisin contaminated maize, which is a staple diet in these communities. The disease affects males more than females in the ratio of 2:1 (Rose,

1982, Jaskiewicz *et al.,* 1987). Much of the research work on oesophageal cancer has been done in Transkei, Eastern Cape, South Africa. No data has been forthcoming from other areas of South Africa with similar climate, agricultural practices and storage. Maize samples from Transkei were homegrown, harvested and consumed by the rural people (Marasas *et al.,* 1988a). It would be of interest to know epidemiologically the extent and level of food contamination by FB_1 in other areas of South Africa.

CHAPTER 3

MULTI-MYCOTOXIN SCREEN: INCORPORATION OF FUMONISIN B_1

3.1 INTRODUCTION

Over 300 mycotoxins have been identified (Steyn *et al.*, 1991) and some are known to play an important role in food-borne diseases of humans and animals. They are usually present in a variety of foods and feeds at significant levels and frequency, creating a food safety concern. Development of reliable and affordable analytical techniques to screen for these toxins in foods, feeds, animal tissues and fluids is therefore necessary, in order to safeguard human health worldwide. Knowledge of mycotoxin levels in foods and feeds and their extent of exposure (to humans and animals) can be used to set minimum safe limits and enforce mycotoxin regulations in food (Van Egmond, 1989, Stoloff *et al.*, 1991).

Mycotoxins are diverse group of compounds with different physico-chemical properties making it difficult for them to be analyzed collectively using one technique. Currently, methods available for analysis are single assays, designed to screen individual mycotoxins. Although they may be sensitive and specific, they may not be cost effective nor readily available to developing countries, their populations of which, are the major victims of mycotoxicoses. In order to redress these shortcomings, multi-mycotoxin techniques have been developed (Section 2.3) but now need further modification so that other commonly occurring mycotoxins may be included. Since their discovery (Gelderblom *et al.*, 1988), fumonisins have always been analyzed separately and to date have not been incorporated into a multi-mycotoxin screen (Figure 3.1). The main drawback lies in their high polarity, which hinders their extraction by non-polar organic solvents such as dichloromethane and chloroform. This study is aimed at investigating ways of incorporating FB_1 (a commonly occurring mycotoxin in maize and maize-based foods), into the multimycotoxin screen.

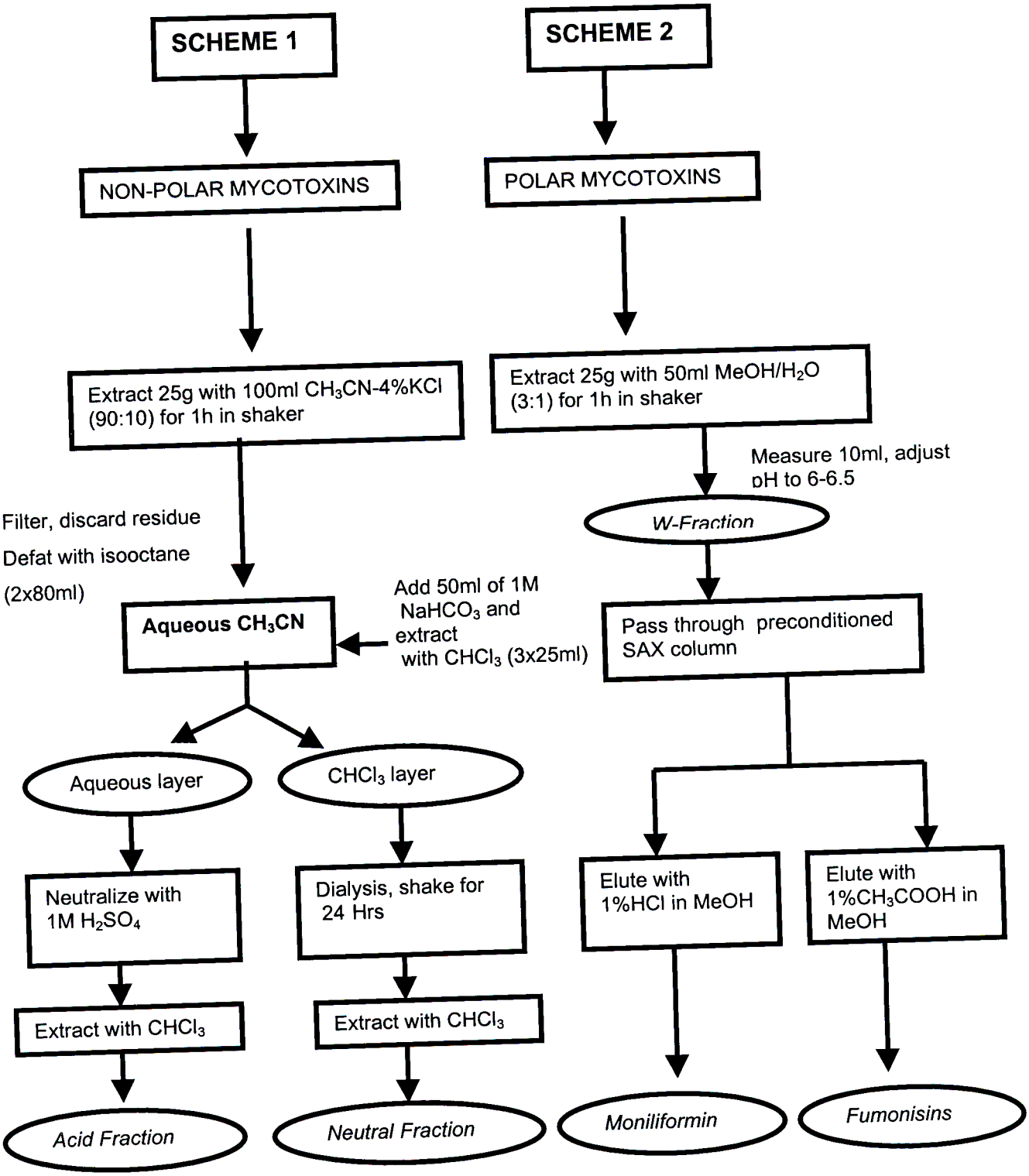


Figure 3.1: Current methods for mycotoxin analysis. Scheme 1 is the multi-mycotoxin method (adapted from Roberts and Patterson, 1979), while scheme 2 (Sydenham et al., 1992b) is a method for analysing polar mycotoxins. This study aims at incorporating scheme 2 into 1.

3.2 MATERIALS AND METHODS

All chemicals used in the present study were of Analar grade (supplied by BDH Chemicals, Poole, England) unless otherwise specified.

3.2.1 Solvents and Reagents

Iso-octane, acetonitrile, chloroform, acetone, methanol, concentrated hydrochloric acid, 1M sodium carbonate solution, sodium hydrogen carbonate, 1M sulphuric acid, acetic acid, anhydrous sodium sulphate, potassium chloride aqueous solution (4%v/w), cyclohexane, butanol, diethyl ether, carbon tetrachloride, formic acid, ethanol, o-phosphoric acid, o-pthaldialdehyde, 2-mercapthoethanol, sodium dihydrogen phosphate, disodium tetraborate, methyl eicosanoate, and distilled water.

Apparatus: Dialysis tubing (Sigma, St Louis, USA), Aluminium-backed fluorescent TLC plates (Merck, Darmstadt, Germany), solid phase extraction (SPE) Bond-Elut strong anion exchange (SAX) cartridges, 5ml capacity containing 500mg sorbent (Analytichem, Harbour City, CA 90710), rotary evaporator, freeze drier, pH meter.

3.2.2 Mycotoxin Recovery from Spiked Samples

Each sample (25g) of milled maize meal in 250ml Erlenmeyer flasks was spiked with various mycotoxin standards as indicated below (Table 3.1)

Table 3.1: Mycotoxin concentrations in μg/25g of spiked maize meal used to determine their recovery values on TLC plate and by HPLC.

Standard	Amount (μg)	Final Level(ppm)
Fumonisin B_1 (FB_1)	100	4
Aflatoxin B_1 (AFB_1)	50	2
Zearalenone (ZEA)	100	4
Sterigmatocystin (ST)	25	1
Cyclopiazonic acid (CPA)	100	4

The mycotoxin standards were dissolved in 10ml of chloroform except for FB_1, which was dissolved in 10 ml of methanol. Mycotoxin solutions were then added to milled maize, mixed well and left to dry (at room temperature) overnight in a fume cupboard.

3.2.3 Extraction and Clean-up (Adapted from Dutton, 1993)

Step 1

The spiked samples were then blended with 100ml acetonitrile/4% potassium chloride (KCl) (90:10, v/v) in 250 ml Erlenmeyer flasks. The flasks were placed in a shaking table (at 15 revolutions per minute) for one hour then filtered through a Whatman No.1 filter paper in a Buchner apparatus. An aliquot (10ml) of the filtered extract was set aside and processed as polar fraction (**W**-fraction) (Figure 3.1) by reducing the volume to 1-2 ml in a rotary evaporator at 60°C. The residue was then made to 10 ml with methanol/water (3:1, v/v). The pH of the extract was adjusted to 6.0 - 6.5 and passed through a solid phase strong anion exchange (SAX) cartridge previously conditioned by washing with 5 ml methanol and 5ml methanol/water (3:1 v/v). The flow rate was maintained at 2 ml/minute as described by Sydenham *et al.* (1992b). The column was then washed with 5ml of methanol/water 3:1 v/v, 3ml methanol and the FB_1 eluted at the rate of 1ml/min. with 1% acetic acid in methanol (10 ml). The eluate was dried under a stream of nitrogen at 60°C and stored at 4-8°C until further analysis.

Step 2

The rest of the filtrate (90ml) was transferred to a 250ml separating funnel and defatted twice with two equal volumes of iso-octane. Aqueous sodium hydrogen carbonate (1M, 50ml) was added and the mixture shaken gently, left to separate and the chloroform fraction collected at the bottom. The chloroform extraction step was repeated twice (25ml) and the extracts pooled. This was saved for further processing in step 4 below.

Step 3

The upper aqueous sodium hydrogen carbonate layer was neutralized with 30 ml of 1M sulphuric acid and extracted with 50ml of chloroform. The procedure was repeated with a further two 25 ml portions of chloroform. The extract was then passed through a bed of

anhydrous sodium sulphate (about 10g) placed in a filter paper supported by a funnel into an evaporatory flask (500ml). The combined chloroform extracts were dried by rotatory evaporation at 60°C. The residue was reconstituted in 2ml chloroform, transferred into a small vial and evaporated in a stream of nitrogen at 60°C. This was stored in a refrigerator as **ACID** fraction.

Step 4

The combined chloroform extracts obtained from step 2 above before neutralization with H_2SO_4 were further fractionated into phenolic and non-phenolic fractions by extracting twice (as in 2 above) with two 50ml volumes of 1M aqueous sodium carbonate solution. The lower chloroform fraction was collected and saved for step 5 below. The upper aqueous layer was carefully neutralized with 50 ml 2M sulphuric acid, extracted with chloroform as in step 2 above, dried and stored in the refrigerator as **P-** fraction.

Step 5

The lower chloroform extract (non-phenolic fraction) was dried by rotary evaporation at 60°C to give the **N**-fraction. This fraction was further cleaned up using a dialysis membrane. The dialysis tubing (25cm long, previously soaked in distilled water for at least 1 hour) was prepared by tying a firm knot at one end to form a closed sac. The N-fraction extract, reconstituted in 2ml acetonitrile, was transferred into the sac using a Pasteur pipette. A knot was tied at the top of the sac and placed in a boiling tube (15 x 25cm) containing 50 ml of aqueous acetone (30% v/v). The top knot was looped over the lip of the tube and a piece of aluminium foil was crimped over the top and secured with a rubber band. The tube was placed in a rack an a shaking table (150 RPM) overnight.

The dialysate was transferred into a 250ml separating funnel, washing out the boiling tube with a little acetone and adding the contents into the funnel. The dialysate was extracted with three 25 ml portions of chloroform, passing each extract through a bed of anhydrous sodium sulphate and into a clean rotatory evaporatory flask (500ml) as in previous steps above. The chloroform extracts were pooled and dried by rotary evaporation at 60°C to dryness, avoiding excessive use of heat. The residue was reconstituted with 2ml of chloroform and transferred

using a Pasteur pipette into a 1-ml vial. The residue was then dried in a stream of nitrogen at 60°C and kept in the refrigerator.

3.2.4 Thin Layer Chromatography

Aluminium-backed precoated with silica gel TLC plates (10 x 10 cm) were each marked at one corner with a pencil, 1 cm from the edges as the origin. The dry spiked extracts and a series of unspiked standards for FB_1 were reconstituted in 200µl of methanol and in chloroform for AFB_1, ZEA, ST and CPA (10, 20, 50 and 100µg/ml). Twenty microlitres of each mycotoxin extract were spotted on the marked origin of the TLC plate and dried in a warm stream of air. Extracts of CPA were spotted on the plates, which had been sprayed with 2% oxalic acid and dried in an oven. The spotted plates were then placed in the respective solvent mixture and allowed to develop until the solvent front just reached the top. They were then removed and dried. The plates were further developed at right angles to the 1st solvent system in the 2nd solvent mixture (Plate 3.1).

The following solvent systems were used to develop the TLC plates. Details of their composition and description are shown in Appendix 2.

(a). *W fraction toxins:* 1st dimension: CM2 (Chloroform/methanol, 3:2, v/v) and 2nd dimension, BWA, 12:5:3, v/v/v).

(b). *N and A fraction toxins*: 1st dimension, CEI (Chloroform/ethyl acetate/propan-2-ol, 90:5:5, v/v/v) and 2nd dimension, TEF (Toluene/ethyl acetate/formic acid, 6:3:1, v/v/v).

(a). *P fraction toxins:* 1st dimension, CtE (Carbon tetrachloride/ethanol, v/v) and 2nd dimension, ChE (Cyclohexane/ether, 3:1, v/v).

The plates were dried and viewed under ultraviolet light (UV) at 254 and 365 nm for any fluorescent or absorbing spots and marking them with a pencil then the plates were sprayed with their corresponding spray reagents.

a). *Fumonisins*: Plates were sprayed with p-anisaldehyde reagent (prepared by mixing 70 ml methanol, 10 ml acetic acid, 5ml concentrated. H_2SO_4 and 0.5ml p-anisaldehyde (p-methoxybenzaldehyde) and heated in the oven for 3 minutes. Fumonisin B_1 gave a purple colour.

b). *Sterigmatocystin*: When sprayed with 20% aluminium chloride in ethanol and heated in the oven at 120°C for 3 minutes, ST spot appeared as a bright yellow fluorescence under longwave UV.

c). *Afatoxin B_1:* When viewed under longwave UV light, AFB_1 appeared as blue fluorescing spots.

d). *Zearalenone:* The spray reagent (diazotized dianisidine) was made by dissolving 0.5g dianisidine in 20 ml of 1.5% HCl. Working spray reagent was made by mixing equal volumes of 10% sodium nitrite and dianisidine. When developed plates were sprayed with this reagent, zearalenone appeared as visible brick red spot at room temperature.

e). *Cyclopiazonic acid:* The spray reagent (Erhlich's) was prepared by dissolving 1g of p-dimethylaminobenzaldehyde in 10ml distilled water and 20 ml concentrated HCl added. When developed, plates were sprayed with Erhlich's reagent, cyclopiazonic acid appeared as a visible purple spot.

For identification, the retardation factors (R_f) values for the mycotoxins were determined for both dimensions from the resulting spots using the formula:

R_f = distance in travelled by mycotoxin/distance traveled by the solvent front (millimetres).

Concentrations were determined by comparing the colour intensity of the sample spots with those of standard spot series and deducing the concentration of sample per spot. The results were recorded and compared. Results are shown (Table 3.2).

3.2.5 Validation of the Multi-Mycotoxin Screen

3.2.5.1 Cyclopiazonic Acid

The acid fractions obtained from section 3.2.3 were further analysed by capillary electrophoresis (CE). The extracts were reconstituted in 200µl of methanol and filtered through and 0.22µm pore-size nylon filter. They were then injected into a capillary electrophoretic column (5µl). The CE system used was a P/ACE System 5510 (Beckman) fitted with a diode array detector set at 226 nm. Electrophoresis was carried out in uncoated capillary column, 75µm id x 50cm length and 100 x 800 aperture size with phosphate buffer

(10 mM) at pH 7.5 as a mobile phase. The buffer, was made by mixing equal volumes of 15mM di-sodium hydrogen phosphate and 10mM sodium di-hydrogen phosphate. The applied voltage was 15kV. A series of standards (100ng/ml, 1, 10, 50 and 200µg/ml, were run together with the samples. Concentrations of the CPA samples were read from the calibration curve or calculated from the equation of the curve (Appendix 3).

3.2.5.2 Fumonisin B_1

Quantitation of FB_1 was carried by out by HPLC using a Spectra Physics SCM400 SYSTEM, with a P2000 manual injector pump, Nova-Pak 4um C_{18} reversed phase analytical column (150 x 3.9mm i.d., from Waters, Milford, MA, USA) and a Spectra SYSTEM FL2000 fluorescent detector. Detector excitation and emission wavelengths were set at 335 and 440 nm respectively. The FB_1 extracts (W-fraction) were reconstituted in 200µl of acetonitrile/water (1:1 v/v) and filtered through nylon filter (0.22µm pore size). An aliquote (25µl) of this sample was mixed with 225µl of o-pthaldialdehyde (OPA). After one minute, a portion of the mixture (20µl), was injected into the column. The mobile phase [methanol/ 0.1M sodium dihydrogen phosphate buffer (80:20 v/v, pH 3.4)] was run isocratically at 1ml/minute. The peaks for FB_1 were detected in a fluorescent detector (Excitation 360nm and emmission, 440nm). The concentrations were determined by comparing the peak areas with those of the standards. Serial dilutions of FB_1 standard were used to construct a calibration curve from which the concentration of FB_1 was determined (Appendix 4). Alternatively, the calculation of FB_1 concentration in the sample was calculated as follows:

$$\frac{\text{Peak area of sample} \times [\text{standard } (\mu g/ml\ FB_1)]}{\text{Peak area of standard.}} = [\text{sample } FB_1\ (\mu g/ml)]$$

Where $[\text{standard } (\mu g/ml\ FB_1)]$ and [sample FB_1 (µg/ml)] are the concentrations of FB_1 standard and sample respectively.

3.2.5.3 Zearalenone, Aflatoxin B_1 and Sterigmatocystin

Extracts from the neutral fraction in section 3.2.3 were further analysed using by HPLC system described in section 3.3.5.2. The extracts were reconstituted in 200µl of acetonitrile,

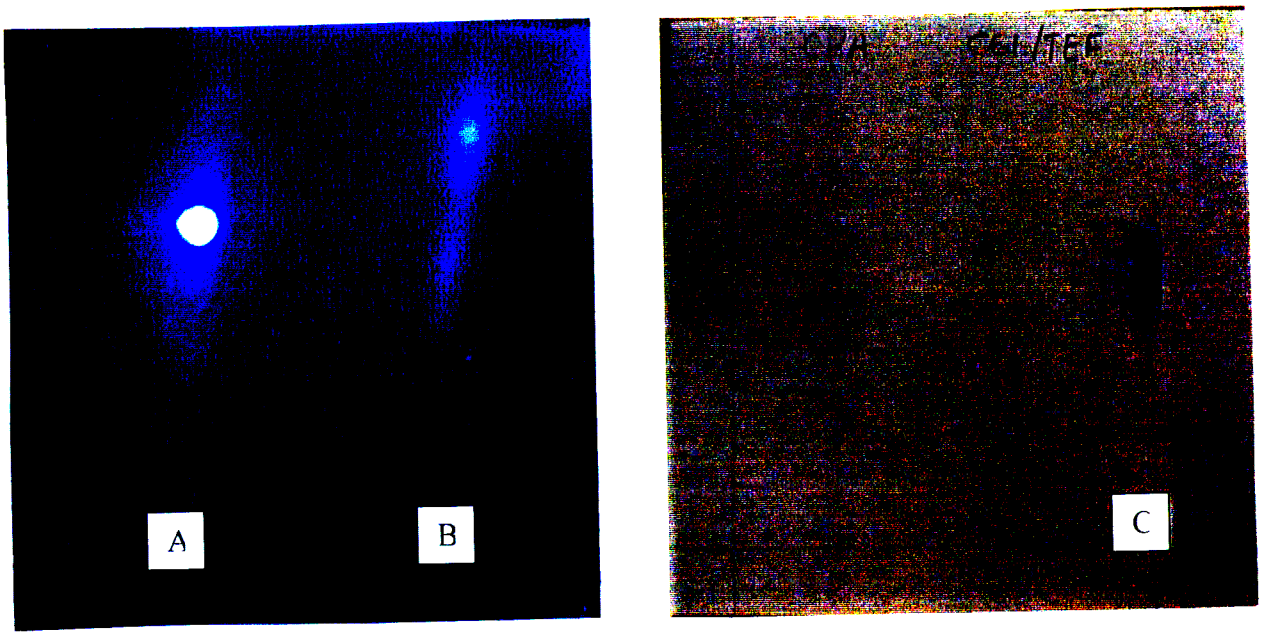
filtered through a 0.22μm pore-size nylon filter and an aliquot of 20μl injected into the HPLC without any derivatization. The mobile phase (acetonitrile/water (45:55 v/v)) was pumped isocratically at a rate of 1ml/minute. The peaks were detected in a UV detector set at 274, 325 and 360nm wavelength, the maximum peak absorbance wavelengths for ZEA, ST and AFB_1 respectively. Sample concentrations against those of standards were determined as described for FB_1 above (Section 3.3.2) or concentration read from the constructed calibration curves (Appendix 3-5). A chromatogram of recovered spiked standards is shown (Figure 3.3).

3.3 RESULTS

The TLC results show that co-extraction of FB_1 with other mycotoxins using in spiked samples is possible (up to a 100% recovery). Mycotoxins with a phenol functional group are thought form salts with aqueous sodium carbonate and therefore remain in the aqueous (P) phase while the neutral mycotoxins remain in the organic phase. Attempts to extract zearalenone and sterigmatocystin into P-fraction were very poor (only 5% of ZEA was recovered while no sterigmatocystin was recovered) (Table 3.2).

Table 3.2: TLC recovery results of spiked standards in 25g of maize meal.

Mycotoxin	Concencentration of mycotoxin (μg)	Solvent system	R_f1	R_f2	Recovered (μg)	%Recovery
FB_1	100	CM2/BWA	0.12	0.37	100	100
AFB_1	50	CEI/TEF	0.51	0.23	40	80
ZEA	100	CtE/ChE	0.23	0.62	100	100
ST	25	CEI/TEF	0.72	0.7	20	80
CPA	100	CEI/TEF	0.18	0.53	50	50
ZEA-N	100	CtE/ChE	0.23	0.62	90	90
ZEA-P	100	CtE/ChE	0.23	0.62	5	5



Pate 3.1 Appearance of aflatoxin B_1 (A), sterigmatocystin (B) and CPA (C) after two dimensional development on TLC. Plates were developed in CEI/TEF.

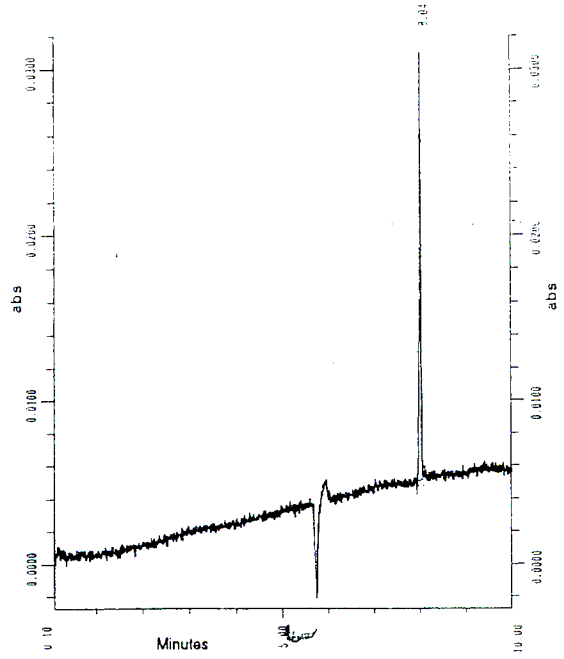


Figure 3.2: Appearance of CPA standard (A, 50ppm) electropherogram on a CE (P/ACE System 5510) under UV detection (226 nm).

When acid fraction extracts were analysed, CPA had a retention time of between 8-9 minutes. The detection limit of this method was 10 ng/ml. The average recovery value for two spiked samples (100µg/25g of maize meal) was 61.5µg (61.5%). An electropherogram of a standard is shown (Figure 3.2).

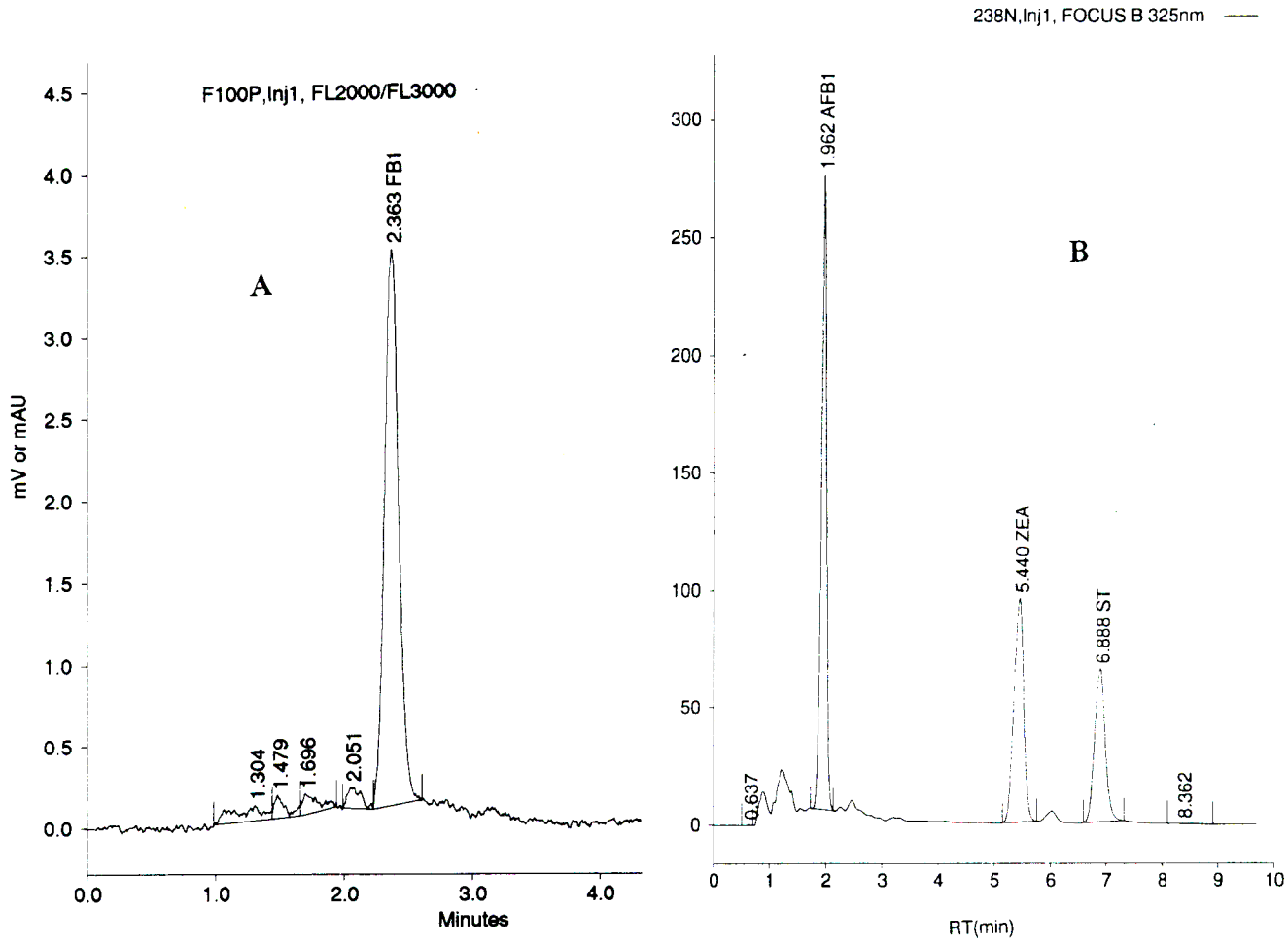


Figure 3.3 HPLC chromatograms of: FB_1: A, 100µg/ml standard derivatised with OPA and analysed by fluorescence detector (440 nm); B, appearance of AFB_1, ZEA and ST extracts from 25g spiked maize meal sample under UV detection (325 nm).

Table 3.3: Recovery of fumonisin B_1 (FB_1), aflatoxin B_1 (AFB_1), zearalenone (ZEA), sterigmatocystin (ST) and cyclopiazonic acid (CPA) spiked in 25 of maize meal. Recovery of ZEA from the neutral fraction (ZEA-N) and from the phenolic fraction (ZEA-P). The extracts were analysed by HPLC/fluorescence (FL) and ultra violet (UV) detection.

Mycotoxin	Original Conc. of toxin (µg/25g)	Conc. Recovered (µg)*	%Recovery	Method used
FB_1	100	97.40 (±2.4)	97.4	HPLC/FL
AFB_1	50	38.68 (±6.6)	77.4	HPLC/UV
ZEA	100	97.96 (±2.3)	98	HPLC/UV
ST	25	19.94 (±5.8)	79.8	HPLC/UV
CPA	100	61.5 (±5.7)	61.5	CE/UV
ZEA - N	100	92.79 (±3.5)	92.8	HPLC/UV
ZEA - P	100	6.15 (±0.8)	6.2	HPLC/UV

*Recoveries are a mean of two replicates

3.4 DISCUSSION

Results of recoveries on TLC are slightly higher than those of HPLC (and CE in case the of CPA) (Tables 3.2 and 3.3). However, the results from the three methods are consistent and comparable. Recovery of most mycotoxins (including FB_1) from the spiked maize meal, was remarkably good (over 75%) when acetonitrile/4% potassium chloride (9:1, v/v) was used as the extracting solvent. However, CPA, was the least recovered (61.5%)(Table 3.3). This recovery is comparable to that of Scudamore *et al.* (1998) where CPA recovery 65% in a spiked maize sample. The major improvement of multi-mycotoxin screen in this study was the co-extraction of other mycotoxins (AFB_1, ZEA, ST and CPA) efficiently with FB_1, which was not possible in the earlier method of Patterson and Roberts (1979). However, Patterson and Roberts (1979) did not give any reports on their recovery values. This makes it difficult to compare the efficiencies of the two methods. Their detection limits however, were similar to those reported in this study (10 ppb) for AFB_1, ZEA and ST.

Although the recovery of FB_1 using this method was high (over 97%), cleanup was rather lenghty when compared with that of Sydenham *et al.* (1992b) where maize extracts did not require any rotary evaporation before application into the SAX cartridges. In this study, direct application of the acetonitrile extracts into SAX cartridges during the clean-up process gave poor recoveries (up to 35.9%) (Table 3.3). It was assumed that acetonitrile lowered the polarity of the extracting solvent, thus preventing the retention of FB_1 in the cartridge. Previously, researchers who used aqueous acetonitrile, had to dilute it to insignificant levels with aqueous methanol before applying the extract to the SAX cartridge (Bennet and Richard 1994). In this study, acetonitrile was removed from the extract by rotary evaporation under reduced pressure and extracts reconstituted in methanol/water before clean-up by SAX cartridges. This procedure remarkably increased FB_1 recoveries.

Use of C_{18} cartridges to clean up acetonitrile/4% KCl (9:1 v/v) extracts is possible, although recoveries were increased when the proportion of acetonitrile in the extracting solvent mixture was reduced to 1:1 v/v, for the reason mentioned above. However, in general, extracts cleaned with C_{18} gave poorer recoveries, less cleanliness and more dilute solutions. In general, acetonitrile extraction was found to be more efficient when compared to methanol. Methanol extraction in other studies, gave 67-74% FB_1 recovery (Sydenham *et al.,* 1992b) which is lower than FB_1 recovery levels in this study.

Increase of extracting solvent to sample ratio was also found to increase FB_1 recoveries. In this study and that of Bennet and Richard (1994), the solvent/sample ratio was 4:1 v/w in comparison to 2:1v/w ratio of Sydenham *et al.* (1992b and 1996). This could also explain the variation in recovery values of FB_1 under the same extraction procedures. It is recommended, therefore, that a solvent/sample ratio of 4:1 be used in the extraction process for better recoveries.

Further fractionation of N into P and non-phenolic fractions using aqueous sodium carbonate extraction was difficult. It was presumed that mycotoxins with phenolic structures form salts with sodium carbonate. In this study, two phenolic mycotoxins were studied. Whereas 6.2% zearalenone was retained in the sodium carbonate solution, sterigmatocystin was not (Table

3.1). This may be possible although neither of the toxins is particulraly polar, zearalenone is higher degree of polarity in comparison to sterigmatocystin. Aqueous sodium carbonate extraction of N fraction, possibly resulted in hydrolysis of zearalenone molecule, as brown unidentified pigments suspected to be degradation products of ZEA were observed on TLC. This step is, therefore, not very useful as a further fractionation of the N fraction. In addition, sodium carbonate, a component of the technique, is known to hydrolyze FB_1 molecule (Park *et al.,* 1996) and has been used in FB_1 decontamination. Thus FB_1 has to be determined in a separate portion of the extract. This is not a complete integration of FB_1 analysis into the multimycotoxin screen, but it is more efficient than carrying out a separate assay. It is also more appropriate for immunoaffinity cleanup as this requires low levels of FB_1.

CHAPTER 4

PRODUCTION AND PURIFICATION OF FUMONISIN B_1

4.1 INTRODUCTION

Fumonisin B_1 (FB_1), a mycotoxin which commonly contaminates maize has been associated with negative health impact in animals and humans (Marasas *et al.*, 1988a). Low levels have been known to alter sphingolipid biosynthesis in rodents fed with FB_1 contaminated feed (Howard *et al.*, 1996) resulting in altered biochemical processes which have been linked to cytotoxicity and cancer. Fumonisin B_1 occurrence in human foods has been linked to cancer of oesophagus (Rheeder *et al.*, 1992, Myburg, 1998). There is a worldwide interest in this mycotoxin and therefore, a need to provide pure FB_1 for use as an analytical standard and for toxicity studies to establish safe regulatory levels in food and feeds.

The current methods of obtaining FB_1 include solid and liquid culture methods using the *F. moniliforme* or *F. proliferatum* (belonging to the section Liseola), which have been shown to be the best producers (Thiel *et al.*, 1991). The highest producer of FB_1 (7,100µg/g) was *F. moniliforme* MRC 826 seconded by *F. proliferatum* (870µg/g). Greatest production of FB_1 has been achieved using solid media, namely, autoclaved maize (Leslie *et al.*, 1992a, b; Nelson *et al.*, 1993, Thiel *et al.*, 1991) and rice (Meredith *et al.*, 1996) (Table 4.1). Although substantial quantities of FB_1 can be obtained by this method, it is lengthy and involves use of considerable quantities of solvents. Liquid media have been preferred since they are less complex than the solid media. In addition, liquid media require no extraction with solvents. The only major disadvantage of liquid media lies mainly on poor FB_1 yields, in comparison to those of the solid media (Alberts *et al.*, 1994, Miller, 1994).

Growth of *Fusaria* and production of FB_1 indicates that FB_1 is a secondary metabolite, which appears at the secondary phase (idiophase) following the period of primary growth (trophophase). In trophophase, the metabolic reactions are finely balanced and secondary metabolites rarely accumulate. Most cellular growth occurs at this stage. Production of secondary metabolites (idiolites) takes place in idiophase. This phase begins when the

culture becomes nutrient limited and primary growth stops (Keller and Sullivan, 1996). Greatest production of FB_1 is, therefore, achieved by limiting some factors necessary for normal (primary) growth such as oxygen, pH, and temperature. This is only possible in an environment, which can be controlled, such as in a stirred jar fermenter. Production of FB_1 in liquid culture begins 3 days after inoculation and increases rapidly between days 4 and 14 when a plateau is reached. However, in solid cultures where growth can be much slower, the two phases often overlap, hence the stationery phase exceeds 14 days.

Table 4.1. Types of media used to produce FB_1 by *F. moniliforme* and *F. proliferatum

Medium	Conditions	%purity	FB_1 Conc.	Reference
Autoclaved maize	6 wks, 25°C	90%	2.64g/100g	Cawood *et al.*, 1991
Rice Culture	28-35 days	96%	770mg/kg dry wt.	Meredith *et al.*, 1996
Patty Culture (maize)			6-10mg/g dry wt.	Nelson *et al.*, 1994
Liquid media	21 days, 28°C		384mg/l	Blackwell *et al.*, 1994
Liquid Media	-	-	74mg/l	Jackson & Bennet,1990
Liquid Media	14 days, 28°C	97%	265.5mg/l	Miller *et al.*, 1994
Liquid Culture*		95%	750mg/l	Dantzer *et al.*, 1996

Isolation and purification of FB_1 from the culture material is difficult mainly due to its hydrophilicity and insolubility in organic solvents. Polar impurities like salts, sugars and peptides are co-extracted with it. A combination of purification procedures including hydrophobic microreticular polystyrene resins (e.g. XAD-2), silica gel, ion exchange and C_{18} column chromatography, have been used to obtain a reasonable degree of purity as shown by NMR in combination to mass spectrometry (MS) (Cawood *et al.*, 1991, Blackwell *et al.*, 1994, Miller *et al.*, 1994, Meredith *et al.*, 1996).

In order to achieve a higher degree of purity, other methods of purification need to be investigated. Several attempts have been made to derivatize fumonisin with other "bulky"

functional groups in order to obtain a crystalline pure compound for stereochemistry analysis by NMR (Bezuidenhout *et al.*, 1988, ApSimson *et al.*, 1994, Blackwell *et al.*, 1995). Methylation of tricarballylic acid residues of FB_1 using excess diazomethane yielded tetramethyl fumonisin A_1 ($FA_1(CH_3)_4$) derivatives which are soluble in organic solvents (Bezuidenhout *et al.*, 1988). However, no mention of de-methylating the derivatives, back to free FB_1, was made. From this previous work, it is possible that methylation of the polar side chains of FB_1 molecule renders it less polar, increasing the chances of isolating it from polar impurities which are usually co-extracted with FB_1 during purification steps. After methylation and substantial clean-up of FB_1 with the aforementioned techniques, methods of de-methylating the resultant tetramethyl FB_1 were investigated.

4.2 MATERIALS AND METHODS

4.2.1 Production of Fumonisin B_1 by Stirred Jar Fermenter

Production of FB_1 using the Brunswick Scientific Fermenter (Plate 4.2) was carried out using the method of Miller *et al.*, (1994), with minor modifications. A seed culture of *F. moniliforme* 826 (previously prepared by suspending conidia in 80ml of distilled water), was inoculated into ten Erlenmeyer flasks, (250 ml) each containing 100ml sterilized primary medium (containing 20g glucose, 2g malt extract, 2g yeast extract, 2g peptone, 2g potassium hydrogen phosphate, 2g magnesium sulphate heptahydrate, 0.2g ferrous sulphate heptahydrate and 3g ammonium chloride, per litre of distilled water). The flasks were incubated in a rotary shaker (28°C, 48 h). The contents were then transferred into a 9.3 litre-production medium (containing 40g sucrose, 10g glycerol, 1g ammonium hydrogen phosphate, 3g di-potassium hydrogen phosphate, 0.3g magnesium sulphate heptahydrate and 5g sodium chloride per litre) in a stirred jar fermenter. The incubation was carried out at 28°C, stirred at 380 RPM for 14 days.

Extraction and Clean-up

The mycelium was removed by centrifugation (5000 RPM at 4°C). The supernatant (pH adjusted to 6 with 2M ammonia solution) was cleaned up by passing it through diethylaminoethyl (DEAE) polystyrene resin (20g) in a glass column, previously regenerated using 2% formic acid (4 glass columns were used concurrently). The column was washed with 300ml of distilled water. Fumonisin B_1 was eluted with 200 ml of 2%

formic acid. The eluent was freeze-dried, reconstituted in 50% aqueous acetonitrile (5ml), loaded onto a C_{18} preparative column and washed with acetonitrile/0.1% aqueous formic acid (200 ml). Fumonisin B_1 was eluted with 0.1%TFA in acetonitrile. Fractions of 10ml were collected and FB_1 containing fractions were pooled and acetonitrile removed by rotary evaporatoion at 60°C. The procedure was repeated until there were no visible impurities on TLC. The dried extracts were then derivatized with diazomethane (prepared as described in 4.2.3.1).

4.2.2 Production of Fumonisin B_1 by Patty Cultures

Yellow maize kernels, ground to a fine meal (30g) were used to prepare patty media in Pyrex petri dishes (100 x 18mm) by the addition of distilled water (30ml) and mixed evenly (Alberts *et al.*, 1993). The dishes were then autoclaved for 1 hour at 121°C (15psi.) on each of two consecutive days. The patty culture plates were cooled and each inoculated with 1ml conidial suspension of *F. moniliforme* (MRC 826) previously prepared by re-suspending lyophilized conidia in 20ml of sterile distilled water. The plates were incubated in the dark at 28°C for 3 weeks.

Extraction

The patty cultures were first lyophilized, weighed and blended with acetone (1 litre/kg). Filtration of the extract was carried out using the Buchner apparatus. Acetone was removed by rotary evaporation and the residue saved. This first step was aimed at killing *Fusarium* spores. After acetone extraction, the patty culture material was extracted with aqueous acetonitrile (1:1, v/v) in the ratio of 100g culture material, to 500 ml of solvent. The extracts were stirred using a glass rod and vacuum filtered through a Whatman No. 1 filter paper and the residue saved. The extraction step was repeated, extracts pooled and the volume reduced to about half, by vacuum rotary evaporation at 60°C. The extracts were further cleaned up by extracting them with three equal volumes of chloroform in a separating flask, each time discarding the chloroform layer.

XAD-2 Clean-up

XAD-2 is a non-ionic macroreticular resin, which works under the principle of adsorption. Commonly, adsorption relies on non-specific dipole-dipole interactions between the solid support and the components of the analyte mixture. Separation depends on different components of the mixture having different relative adsorption effects on the solid support.

(mobile phase). The eluent (mobile phase) carries those components of the mixture, which are less tightly bound to the solid support much faster than those, which are bound tightly.

A gravity-fed column was set up with hydrophobic polymeric adsorbent XAD-2 (BDH Chemicals Co., Poole, England) by transferring 30g of dry XAD-2 material into a 30 x 2cm glass column plugged at one end with glass wool. The XAD-2 was washed with 200ml of 50% acetonitrile, until the eluent was clear, followed by 500ml distilled water. The extract was loaded into the column, washed with 1 litre of distilled water and drained completely. The washing was repeated with 200ml of methanol:water (1:3, v/v followed by 200ml of methanol/water (1:1 v/v). Fumonisin B_1 was eluted with 250 ml of methanol. Methanol was removed by rotary evaporation and the residue saved for further cleanup on preparative C_{18}.

4.2.3 Derivatization and Clean-up of Semi-purified Fumonisin B_1 Extracts

4.2.3.1 Preparation of Diazomethane

Safety Precautions

Diazomethane is a highly explosive and toxic compound, which needs to be handled with care. Glassware used for its preparation should have smooth (non-ground) fittings to avoid explosion in case of excessive gas build-up. Face masks, goggles and gloves must be worn during preparation of diazomethane. The procedure must be carried out in a fume chamber. Once prepared, it should be stored in a glass container with a loose fitting and stored at 4°C in the dark.

Procedure of Preparation

Ethanol (95%, 25ml) was added to a solution of potassium hydroxide (5g in 8ml of water) in a 100ml distillation flask fitted with a dropping funnel and an efficient condenser set for distillation. The condensate was run into 20 ml of ice cold ether in a flask and the whole apparatus was kept in a fume cupboard. The reaction flask was heated to 65°C in a water bath. A solution of 21.5g diazogen (99%, Acros Organics, Geel, Belgium) in 200ml ether was added via a dropping funnel over a period of about 25 mins. A further 40 ml of ether was added and slowly distilled until the distillate was colourless.

4.2.3.2 Derivatization of Fumonisin B_1 to Tetramethyl Ester with Diazomethane

Dry extracts of FB_1 from XAD-2 column, were methylated by adding excess diazomethane (CH_2N_2) at room temperature. The solution was stirred with a glass rod until effervescence stopped. Diazomethane was then left to evaporate at room temperature overnight in a safety hood. The extracts were reconstituted in 0.5 ml of methanol and spotted on TLC plates. The plates were developed in the first dimension using butanol/water/acetic acid (12:5:3, v/v/v) as solvent, to check the extent of methylation. The plates were sprayed with anisaldehyde and heated at 100°C to visualize FB_1.

4.2.3.3 Cleanup by C_{18} Column Chromatography

Diazomethane-methylated extracts of FB_1 were loaded into a C_{18} preparative chromatography column (25 x 250mm, 40-63µm diameter particle size). The column was washed with 250ml solution of acetonitrile/0.1% trichloracetic acid (TCA) 25:75 v/v, and the fraction containing fumonisin and its derivatives was eluted with acetonitrile/0.1%TCA (250 ml). Fractions of 10ml each were collected and analyzed for FB_1 by TLC. Fractions containing fumonisin and methylated derivatives were combined. The acetonitrile was removed under vacuum. Fractionation of the individual derivatives in the dried extracts was achieved using preparative TLC.

4.2.3.4 Preparative TLC plates

Glass plates (20 x 20 cm) were washed thoroughly in clean hot water, rinsed and dried with acetone. They were then placed on a spreading base (the spreading base can accommodate up to 6 glass plates). Slurry was prepared by mixing 30g of silica gel 60 (5-20µm particle size, Merck, Dermstad, Germany) with 70 ml of distilled water. It was mixed evenly to make a smooth paste. The paste was then poured into a spreader, which had been adjusted to 0.3mm. The spreader was held on both sides using both hands and pulled forward with a steady speed on top of the glass plates. The plates were coated evenly with slurry, air-dried, placed in a rack and activated at 110°C overnight.

4.2.3.5 Streaking the plates

Dried FB_1 extracts from the C_{18} cleanup (section 4.2.3.3) were each reconstituted in 500ul of butanol/water/acetic acid (12:5:3, v/v/v). The extracts were then streaked onto the preparative TLC plates (20 x 20 cm). Streaking was done on a line parallel to and 1 cm above the bottom edge using a Pasteur pipette. The procedure was repeated thrice to

concentrate the sample. The plates were then developed in butanol/water/acetic acid (12:5:3, v/v/v) and dried. Using another uncoated TLC plate, the developed plate was covered in such a way that it exposed 1.5 cm of the right edge. The exposed edge was sprayed with anisaldehyde (in a fume chamber) and heated at 110°C for 3 minutes. The same procedure was repeated for the left edge. The position of FB_1 derivatives on the developed plate were deduced by drawing parallel lines across the plate to join the coloured spots on both left and right edges. Each of the areas, (1-4, Plate 4.3) was scored off separately using a sharp needle and the silica scrapping from each the bands suspended separately in methanol. The extracts were filtered through a sintered glass funnel and dried by rotary evaporation at 60°C. Preparative TLC clean-up was repeated, until the spots were chromatographically pure. The spots were then scored off the plates separately and analysed in the next step (Section 4.2.4 below).

4.2.4 Conversion of FB_1-Tetramethyl Ester to Fumonisin B_1 using Esterase

4.2.4.1 Testing of Esterase Potency

Reagents

A. 10 mM Borate Buffer, pH 8.0 at 25°C

B. 0.1% (v/v) Ethyl butyrate solution

C. Ethyl Butyrate (Butyric Acid Ethyl Ester, Sigma Prod. No. B-2391.)

D. 10 mM Sodium hydroxide solution-Standardized (NaOH)

E. Esterase enzyme solution. Esterase solution (100 units/ml) was prepared in cold Reagent A, immediately before use, from the stock containing 3360 units/0.3ml.

F. Fumonisin B_1 tetramethyl ester. Prepared by methylating 0.5mg FB_1 standard with with diazomethane as in section 4.2.3.2. The dry derivative was reconstituted in 5 ml of borate buffer (Reagent A).

Principle of esterase activity

One unit of esterase (EC 3.1.1.1) hydrolyses 1.0 µmole of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25°C in a 10mM sodium borate buffer.

Procedure

Using a suitable pH meter in conjuction with a magnetic stirrer, 25 ml of Reagent B was pipetted into a 100ml conical flask. The solution was equilibrated at 25°C, then 0.025ml of Reagent C added. The pH was adjusted to 8.1 using Reagent D, then 0.1ml of Reagent E was added. Timing was started when the pH reached 8.0. The reaction was run for 5 minutes, recording the volume of sodium hydroxide used to maintain the pH at 8.0 every minute. The results are shown in Table 4.2.

4.2.4.2 De-methylation of Tetramethyl FB_1 Esters

A solution of methylated FB_1 standard (in 5ml methanol) was equilibrated to pH 8.1 and 25°C. The procedure was then repeated as described in Section 4.3.1.

4.2.4.3 Further Analysis of FB_1 Tetramethyl Ester Extracts

Samples of bands 1-4 (Plate 4.3) were further anlysed by liquid chromatoraphy/Electrospray –mass spectrometry (LC/MSD) at the Mass Spectrometry Unit, Cape Technikon in Cape Town. The Electrospray mass spectrometry was perfomed on a Hewlett Packard HP 1100 LC/MSD. Liquid chromatography was carried out on a C_{18} reversed phase column with methanol/ 0.1% HCOOH water (80/20, v/v) pumped isocratically at 1 ml per minute. The analyte was infused into electrospray mass spectrometer at 5µl/min by means of a sryringe. The spray needle was +4.5 kV. The mass spectra obtained from the different bands are shown (Figures 4.1 and 4.2).

4.3 RESULTS

In this study, production of FB_1 from the stirred jar fermenter by *F. moniliforme* yielded 160mg/l after 14 days of fermentation. There was no significant difference in the yields when fermentation time was increased to 21 days. Patty cultures yielded 6mg/g of dry weight (as crude FB_1). Although extraction and clean-up of stirred jar fermenter cultures were cheaper than those of patty cultures, the latter gave much higher yield of FB_1. Additional cultures from both methods using a subculture of *F. moniliforme* MRC 826 colonies yielded lower concentrations.

Plate 4.1 Appearance of *Fusarium moniliforme* on malt extract agar (MEA).

Plate 4:2 Three-day old culture of *Fusarium moniliforme* MRC 826 in a Brunswick stirred jar fermenter.

After methylation (4.2.3.2) and cleanup of the culture extracts, four spots with different R_f values were observed when the plates were developed and sprayed with anisaldehyde spray (Plate 4.3). It was deduced that methylation of FB_1 molecule did not occur only at the targeted functional group (TCA), but with other functional groups as well.

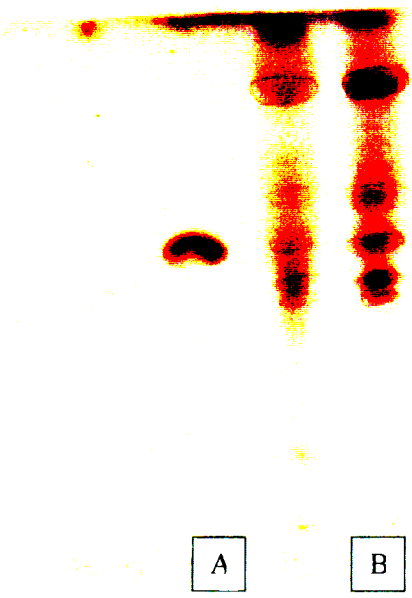


Plate 4.3 Thin layer chromatogram of methylated FB_1 from the culture material (B) and FB_1 standard (A) with butanol/water/acetic acid as a mobile phase The spots were visualized with anisaldehyde spray .

The potency of esterase was confirmed when the average amount of butyric acid liberated by esterase activity was 0.5ml/min (Table 4.2). Therefore, butyric acid liberated per min. = (0.5/1000ml) x10mM = 0.005mM or 5 µmole/min. This confirmed that the esterase was active. However, There was no change in pH after addition of enzyme solution to derivatized standards nor semi-purified FB_1 from the culture material, meaning that there was no reaction. It was concluded that esterase did not hydrolyze any ester bonds in FB_1 under the conditions used in this study.

Table 4.2: Esterase activity shown by the amount of 0.0M NaOH used to maintain the pH of 8.

Time (Min)	0	1	2	3	4	5	Mean Vol./min
0.01M NaOH (ml)	0	0.5	1.0	1.6	2.1	2.6	0.5
PH	8.0	8.0	8.0	8.0	8.0	8.0	

Further analysis of FB_1 tetramethyl ester extracts showed that Band 1 (Figure 4.1) had the highest peak of *m/z* 603, showing FB_1 dimethyl ester with a loss of one tricabarboxylic acid chain. However, a smaller peak at *m/z* 777.5 shows the presence of tetramethyl FB_1. Peak *m/z* 661.5 suggests dehydration of FB_1 with the removal of 3 molecules of water. Band 2 (Figure 4.1) has a highest peak at *m/z* 475 which suggests methyl esterification of 4 hydroxyl groups of hydrolysed FB_1 while the peak of m/z 817.5 is possibly a tetramethyl FB_1, with additional esterification of 3 hydroxyl side chains. Band 3 (Figure 4.2) appears to have a mixture of tetramethyl FB_1, *m/z* 790.5, 806.5 and 820.5 having additional methylation of 1-3 hydroxyl groups respectively. Band 4 has the highest peak of *m/z* 421 suggesting a structure of aminopentol (hydrolysed FB_1) with additional methylation of the side chains (hydroxyl or amino groups).

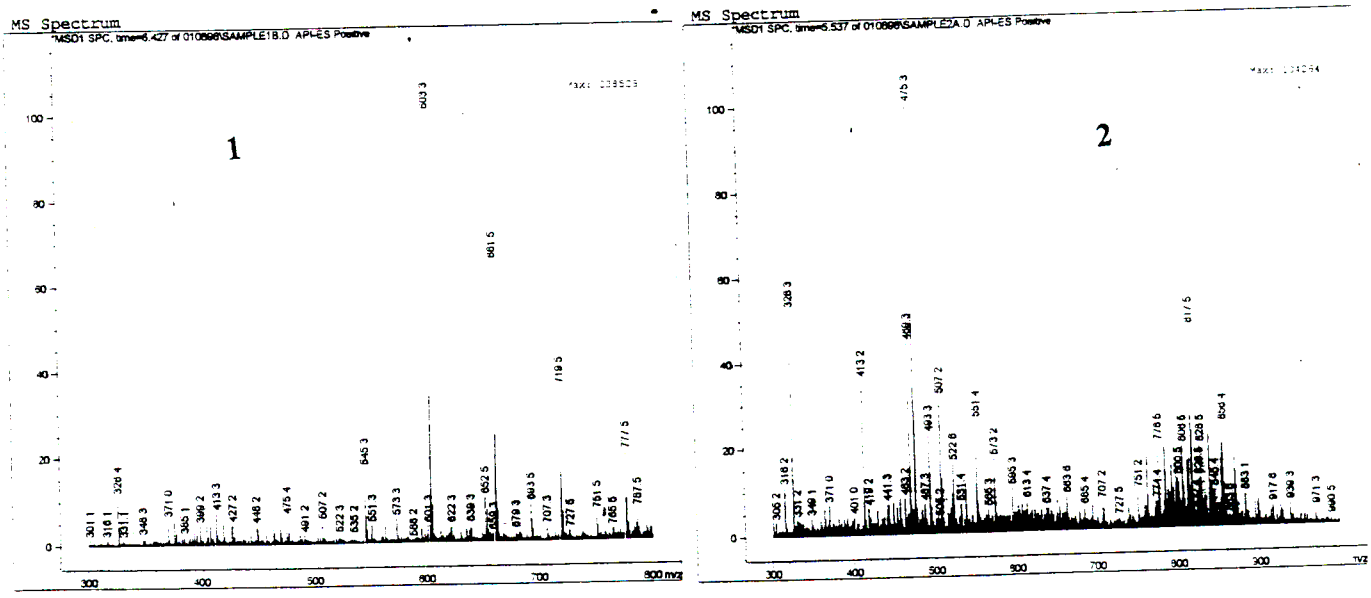


Figure 4.1 Mass Spectra of FB_1 methylated extracts, bands 1-2.

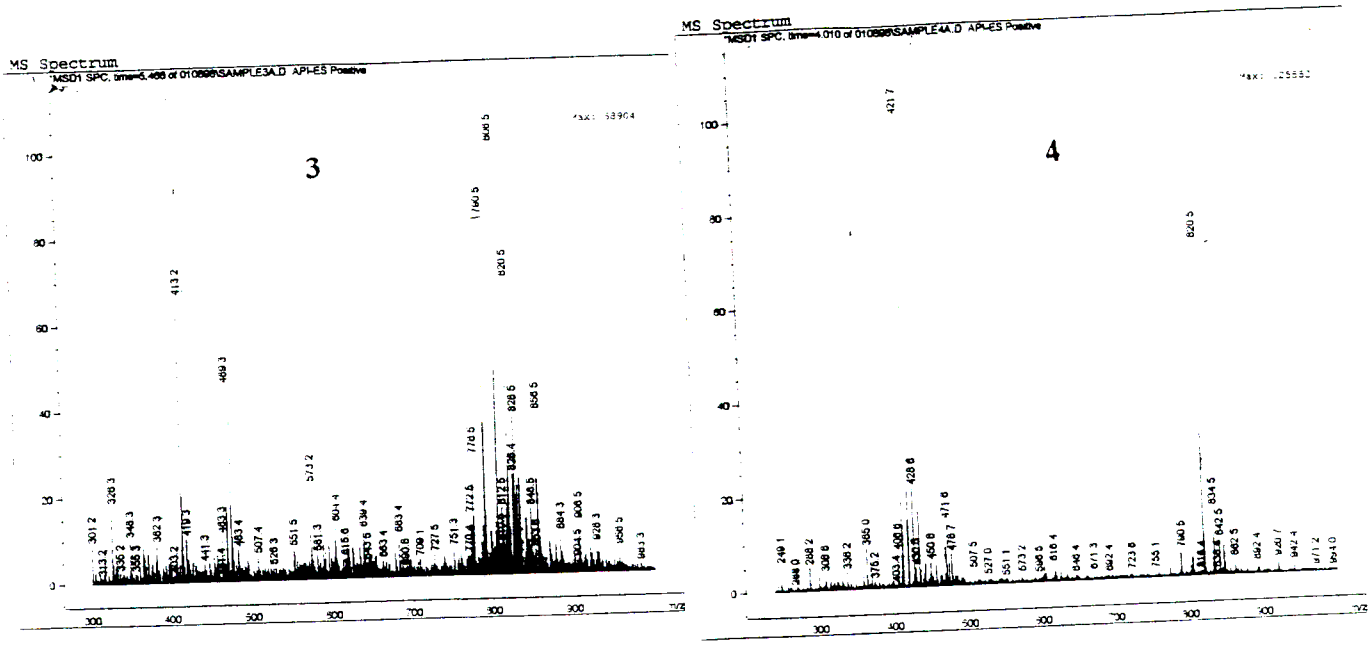


Figure 4.2 Mass Spectra of FB_1 methylated extracts, bands 3-4.

4.4 DISCUSSION

From the results of FB_1 production, it was difficult to immediately establish why additonal cultures from both methods (stirred jar fermenter and patty cultures) yielded lower concentrations with time. Attenuation of the organism from the original wild type by sub-culturing could have played a great role. Limiting culture medium nutrients (such as carbon source) so that the levels are never too high to inhibit production, is also a necessity for maximum production (Keller and Sullivan, 1996). Such conditions were not strictly monitored in the laboratory where this study was done and this could have adversely affected the production of FB_1. Although Miller *et al.*, (1994) reported much higher yields than those acquired in this study, the organisms used were different. Miller *et al.*, (1994) used *F. moniliforme* NRRL 13616 while *F. moniliforme* MRC 826 was used in this study.

It is observed from these results that although there was methylation of the targeted tricaballylic acid side chains of FB_1 (peak 777.5), extra functional groups like hydroxyl (OH^-) and amino (NH_2) groups were possibly methylated by CH_2N_2, which then made the interpretation of mass spectra difficult. Boyle and Kishi (1995), used carbobenzyloxy

chloride (CbzCl) to protect the amine and *tert*-Butyldimethylsilyl chloride (TBSCl) to protect the hydroxyl groups from methylation.

In this study, amine and hydroxyl groups were not protected and the reaction of the crude extract could have resulted in their methylation and hence made the purification and characterization of the extracts difficult. This is evidenced by the electrospray mass spectra of semi-purified methylated extracts (Figures 4.1 and 4.2). This technique could improve clean-up as it completely changes the polarity of FB_1, making its isolation from the polar impurities less laborious. However, it is recommended that the non-targeted functional groups (amine and hydroxyl) should be protected beforehand for better purification.

Although the stereochemistry of tricarballylic acid moieties has been elucidated, it is difficult to say why esterase could not hydrolyze the tricarballylic acid ester bonds to yield FB_1 from FB_1-tetramethyl ester. This is the area, which needs further investigation in order to achieve regeneration of the free FB_1.

4.5 CONCLUSION

The cost of production in the jar fermenter is much lower than the patty culture method. This is because no organic solvents e.g., methanol are used in the initial cleanup. Although methods of clean-up are tedious, it is much cheaper to produce FB_1 in the laboratory than to obtain it from commercial sources. Methylation of FB_1 is a potential technique for obtaining pure standards and needs further investigation.

CHAPTER 5

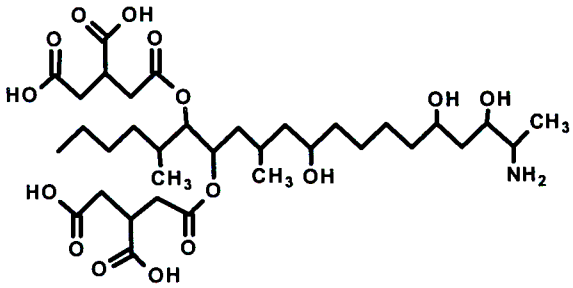
THE DETERMINATION OF FUMONISIN B_1 IN HUMAN FAECES: A SHORT TERM MARKER FOR ASSESSMENT OF EXPOSURE

5.1 INTRODUCTION

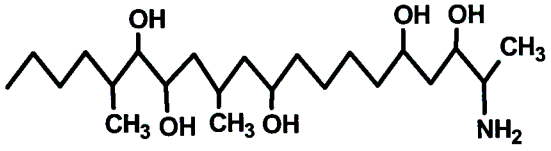
The ubiquitous occurrence of FB_1 in maize and its products, sometimes at elevated levels, presents a potential threat to human and animal health and realistic tolerance levels should be set. Hence, there is need to develop effective methods of analyzing food and to monitor human exposure using a suitable biomarker. The estimation of free FB_1 in blood is impractical, due to its poor absorption and rapid excretion from the gut leading to low plasma levels (Prelusky *et al.*, 1994). Furthermore, FB_1 does not seem to readily form conjugates like aflatoxin B_1 (AFB_1) does with either DNA or plasma proteins like albumin. Hence methods based on measurement of sphingoid bases, sphinganine (Sa) and sphingosine (So) which accummulate due to the action of FB_1, have been developed (Riley *et al.*, 1994). Sphingoid base measurement appears to give some hope of appraising human exposure to FB_1, over a time scale approaching to that given by AFB_1 adducts. The disadvantage of this method seems to be on poor and erratic recoveries of the sphingoid bases from physiological fluids and tissues.

As the fumonisins, including FB_1, mainly pass straight through the digestive tract and what is absorbed is mainly excreted in bile (Shephard *et al.*, 1994a), it seems reasonable to look for FB_1 and its degradation products in faeces rather than in blood and urine. The added advantage of this, is that faecal samples are taken as a matter of routine in hospitals and do not require sick patients to undergo further invasive techniques. Fumonisin B_1 analysis in the faeces of non-human primates and other animals has been carried out previously (Shephard *et al.*, 1994b; Prelusky *et al.*, 1994; Smith and Thakur, 1996). The results showed that when ingested, less than 1% of the administered dose was absorbed from the gastrointestinal tract. This may explain the high levels of contamination required (>5mg/kg) to produce symptoms of illness (Dutton, 1996).

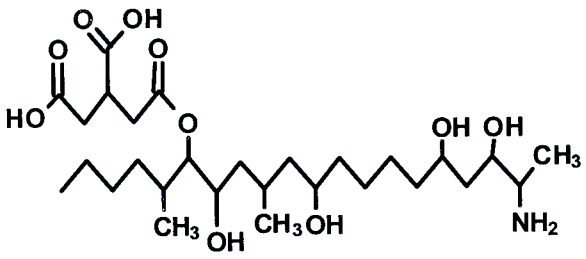
Fumonisin B_1 does not appear to undergo any significant biotransformation in the rat and is mostly excreted unmetabolized within 24 hours mainly in faeces (Shephard *et al.*, 1992) at the rate of 80% with trace amounts in urine. However there is evidence that at least three decomposition products exist: one fully hydrolyzed form (HFB_1), and two partially hydrolyzed forms of FB_1 ($PHFB_1$) (Figure 5.1) (Jackson *et al.*, 1996), which could be present in faeces. In this study, analysis of FB_1 in human faeces was carried out to monitor human dietary exposure to this toxin with a view of developing a routine short term marker.

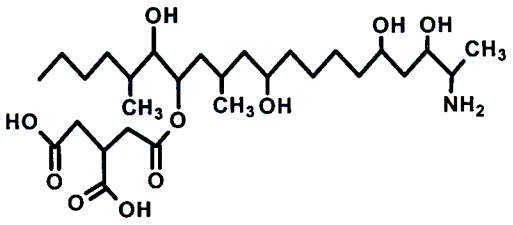


Fumonisin B1



Hydrolysed FB1





Partially hydrolysed fumonisin B_1

Figure 5.1: Structure of fumonisin B_1 and its derivatives.

5.2 MATERIALS AND METHODS

5.2.1 Study Area and Population

A total of 43 faecal samples were processed and analyzed for FB_1. Twenty of the samples were from Vulamehlo, a rural district school south of Durban and 23 others, were collected from the Durban metropolitan area. The samples were randomly selected from a set of 200, which were collected in the ongoing study on geohelminth infections in KwaZulu Natal. The geohelminth infection study was carried out at the MRC, Durban, South Africa. Samples for this study were selected after they were examined for helminthic infections. The selection of the samples did not take into consideration whether the included samples were positive or negative for the geohelminths. A laboratory list of numbered samples was used for selection.

5.2.2 Reagents and Materials

All reagents were of analytical grade unless otherwise specified.

a) *SPE C_{18} cartridges:*10 ml capacity; (Varian Bond-Elut from Analytichem, Harbour City, CA 90710) containing 500mg sorbent.

b) *Mobile phase:* Methanol: 0.1M sodium dihydrogen phosphate (80/20,v/v) adjusted to pH 3.4 with orthophosphoric acid and pumped at a flow rate of 1 ml/min.

c) *o-Phthaldialdehyde (OPA) reagent:* 40 mg of OPA dissolved in 1 ml of methanol and diluted with 5ml of 0.1M sodium tetraborate and mercapthoethanol (50 ul).

d) *Solvents:* Acetonitrile/water (1:1, v/v), butanol, acetic acid and methanol, both obtained from BDH Chemicals, Poole, England.

e) o-*Phosphoric acid* (concentration >85%, obtained from BDH Chemicals, Poole, England).

f) *Anisaldehyde spray:* This was prepared by mixing 70 ml of methanol and 10 ml of acetic acid. Concentrated H_2SO_4 (5 ml) was added followed by 0.5 ml anisaldehyde.

g) *Fumonisin B_1 standard.* (1mg/ml and 50µg/ml) dissolved in acetonitrile/water (1:1v/v). The standard was obtained from PROMEC, Cape Town, South Africa.

h) *Silica 60 aluminium backed TLC plates:* Obtained from Merck, Darmstad, Germany.

5.2.3 Extraction and Clean-up of Samples

Frozen faecal samples (from Section 5.2.1) were first lyophilized and then ground to a fine powder. A fraction (1.5g) of the sample was extracted thrice by vortexing for 1min in a

capped tube with 15ml of 0.1M ethylenediaminetetraacetic acid (EDTA, pH 5.2). The mixture was centrifuged at 2000g for 10min at 4°C, the supernatant removed and the extraction repeated a further 2 times. The supernatants were combined, acidified to pH 2.9-3.2 with 5M hydrochloric acid and centrifuged (4000g, 10min). A supernatant aliquot of 10ml was applied to a Bond-Elut C_{18} cartridge previously conditioned with 5ml methanol and 5ml of water. The sorbent was first washed with 5ml water, followed by 5ml methanol:water (1:3, v/v) and finally with 3ml of methanol/water (1:1, v/v). Fumonisin B_1 was eluted with 15ml of methanol and the solvent evaporated under a stream of nitrogen at 60°C.

5.2.4 Standard Recoveries on Spiked Samples

Three stool samples (1.5g) which showed no detectable FB_1 were each spiked with 50µg of FB_1 in 3ml of methanol. They were left to dry overnight at room temperature in a fume cupboard and were then extracted as described above (5.2.3).

5.2.5 Thin Layer Chromatography

The dried extracts were dissolved in 200µl of acetonitrile:water (1:1, v/v) and an aliquot of 20µl spotted on silica thin layer chromatography (TLC) plates (10x10 cm). Standards (10µl) of known concentration (5, 10 and 100µg/ml) were also spotted on the plate and developed uni-dimensionally in butanol/water/acetic acid (12:5:3, v/v); dried and sprayed with anisaldehyde. The plates were heated briefly for 3min at 110°C. Retardation factors (R_fs) were noted and the quantity of FB_1 in the extracts was deduced by comparing the intensity of the purple coloured spots of the samples to those of the known standards.

5.2.6 High Performance Liquid Chromatography

Quantitation of FB_1 in the extracts was carried by out by HPLC as described in section 3.2.5.2.

5.2.7 Statistical Analysis of Data

Data analysis was done using SPSS Version 6.13 (Microsoft Corporation NY, USA).

5.3 RESULTS

5.3.1 Thin Layer Chromatography

Fumonisin B_1 standards and spiked samples showed a purple visible colour with an R_f of 0.59. Some of the samples had purple spots at R_f 0.63. All the samples had yellow and brown pigments with R_f values of 0.75 and 0.78 respectively. Partial clean-up of FB_1 using C_{18} did not remove most of the impurities, making the TLC readings difficult. Some samples, which appeared positive for FB_1 on TLC, were negative on HPLC. Anisaldehyde spray, which reacts with fumonins to give a purple colour, is not specific. It could therefore, have reacted with other pigments in the faecal extracts to give a similar colour to that of fumonisins, leading to false positive results. Because of these problems, sample analysis by TLC was not regarded as being reliable and was discontinued.

5.3.2 High Performance Liquid Chromatography

Standard recovery results of FB_1 on spiked samples are displayed in Table 5.1. Using Tukey's one way analysis of variance (ANOVA) it was found that there was no significant difference between the means of each number of extractions (p=0.47). High performance liquid chromatography results in Table 5.2 show that 7 out of 20 (35%) rural samples had varying levels of FB_1 from 0.60 – 19.56mg/kg while the urban subjects had only 2 positive samples out of 23 (8.7%) having concentrations of 3.5 and 16.mg/kg. The HPLC chromatograms are shown (Figure 5.2).

The results (Table 5.2) whilst encouraging from the development of a biomarker point of view, were quite alarming. Thirty five percent of the rural samples were positive, although it might be argued that the highest concentration of about 20mg/kg of dry faeces is nothing to cause concern. What this represents in terms of dietary uptake, however, is unknown.

Table 5.1: Recoveries of FB_1 obtained from spiking faecal samples with 50μg FB_1. Analysis was carried out using HPLC.

	Amount recovered (μg)*	%Recovery
Extract 1 (3 times)	37.0 ± 2.3	74
Extract 2 (6 times)	42.5 ± 3.6	85
Extract 3 (9 times)	45.5 ± 6.2	91

* The mean value of recoveries was not significantly different (p=0.47)

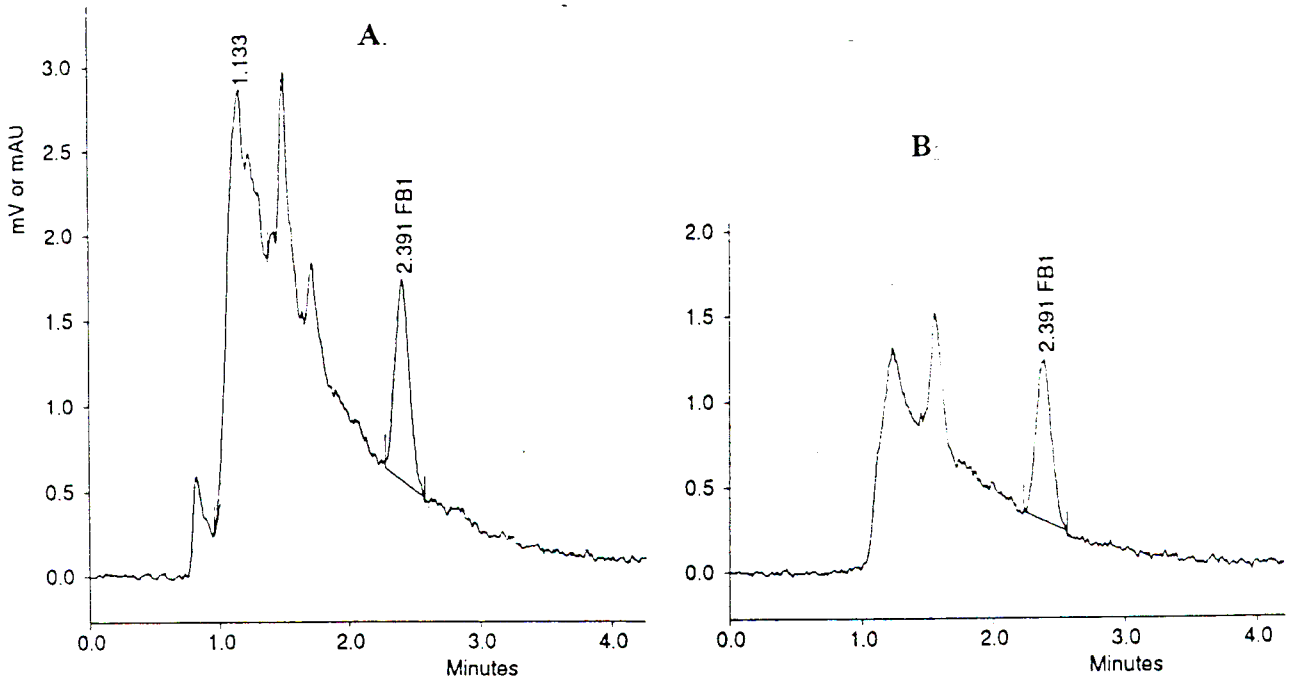


Figure 5.2 HPLC chromatograms of (A), FB_1 (50μg) recovered from 1.5g of freeze dried faeces, and (B) FB_1 from contaminated faeces.

Table 5.2 Concentration of fumonisin B_1 detected by HPLC in faecal samples of rural schoolchildren from Vulamehlo (V1-V20) and samples collected from the Durban Metropolitan area (T1-T23).

RURAL SAMPLES	[FB_1] μg /g*	URBAN SAMPLES	[FB_1] μg /g*
V1	ND	T1	ND
V2	ND	T2	16.2±4.6
V3	ND	T3	ND
V4	ND	T4	ND
V5	ND	T5	ND
V6	10.0±4.0	T6	ND
V7	11.0±4.7	T7	ND
V8	ND	T8	ND
V9	ND	T9	ND
V10	0.79±0.2	T10	ND
V11	ND	T11	ND
V12	18.4±5.7	T12	ND
V13	ND	T13	ND
V14	19.56±2.8	T14	ND
V15	ND	T15	ND
V16	14.7±4.4	T16	3.5±0.9
V17	0.6±0.1	T17	ND
V18	ND	T18	ND
V19	ND	T19	ND
V20	ND	T20	ND
		T21	ND
		T22	ND
		T23	ND

ND = Not Detected (below the detection limit of 50ng/g). *The mean concentrations of FB_1 between the two groups was significantly different (p=0.04, by Mann Whitney test).

Statistically, there was a significant difference between the rural and the urban groups (Fisher's exact t-test, $p = 0.059$ at 95% confidence interval). This showed that the rural group was at a higher risk of FB_1 exposure (6 times) than the urban population. However, no conclusion can be drawn from these results due to a small sample size and therefore, a wide confidence interval (1.016-31.478).

5.4 DISCUSSION

The present study revealed the presence of FB_1 in human faeces for the first time. More than 80% of FB_1 was excreted in faeces within 24 hrs in monkeys (Shephard *et al.*,1994b. The peak period of excretion is not known but it is logical to conclude that the amount of food taken and the frequency of bowel movements may play a role in transit time (clearance) of FB_1 from the body. It would not be possible therefore, to obtain the same concentration value of FB_1 from the samples collected at different times of the day. The HPLC method of analysis used in this investigation was developed by Shephard *et al.* (1994b) and modified in the present study. In their procedure, monkey faeces required at least six extractions to obtain approximately full recovery and nine extractions in case of rat and bovine faeces (Shephard *et al.*,1992 and 1995; Smith and Thakur, 1996). While high recovery is desirable, in this case it resulted in long extraction procedures and increased expense in both time and the amount of solvent used. In this study, extraction of spiked faecal samples showed that 74% of FB_1 could be recovered in three extractions and in addition, there was no significant difference between the mean number of extractions (Table 5.1). The number of extractions (three times) was adopted for all the samples. The latter had the positive effect of minimizing the amount of contaminating materials also being extracted. It was observed that addition of methanol into the extraction solvent (Shephard *et al.,* 1995), did not improve the recovery while it extracted more impurities, making the analytical procedure difficult. Thin layer chromatography was not useful as a tool for FB_1 analysis in faeces since the extracts had bile pigments which could have reacted with anisaldehyde spray and had the same R_f value as that of FB_1. This reaction made the results difficult to interpret.

Fumonisin B_1 hydrolyzed products, usually formed due to acidic nature of the stomach and the microbial activity of the lower gastrointestinal tract (GIT), have not been studied in detail. Little is known about the gastrointestinal absorption of FB_1. A limited amount of FB_1 is

absorbed into the bloodstream in its intact form. However when it loses the tricarballylic acid side chains by hydrolysis, absorption is enhanced (Hopmans *et al.,* 1997) and this may explain the greater toxicity of HFB_1 in comparison to FB_1. One school of thought is that FB_1 is polyanionic and may interfere with its own absorption by binding cations such as sodium, potassium, and other large molecules, which are required for active transport across the intestinal membrane. Polycations have been known to inhibit active transport of sugars and amino acids across the intestinal membrane in rats (Elsenhans *et al.,* 1983).

Although $PHFB_1$ and HFB_1 (FB_1 degradation products, Figure 5.1) have been analysed alongside FB_1, in monitoring FB_1 exposure in animals (Shephard *et al.,* 1994b, Smith and Thakur, 1996), their concentration in faeces is relatively small in comparison to that of FB_1 and their standards are not readily available. In addition, different chromatographic conditions from those used for FB_1 analysis in this study are necessary in order to separate $PHFB_1$ from FB_1 since they co-elute (Shephard *et al.,* 1994b). These, render the technique unnecessarily lengthy. In this case, analysis of FB_1 alone was found to be satisfactory.

Clearly, the rural population have greater exposure as they consume locally produced maize, which is often contaminated with FB_1, since maize and its products are not subject to any regulatory restrictions in South Africa at the moment. Fisher's exact test showed that the rural population is at a higher risk of exposure to FB_1 than the urban population. Considering the losses due to degradation during digestion, recovery, dilution effects and the time interval to when food was consumed, the quantities detected might be equivalent to considerable amounts of fumonisins in the food. Clearly the children from the rural area, who were not regarded as suffering from any disease, are exposed routinely to FB_1, which is resident in their GIT for considerable periods of time, depending upon personal habits. This situation cannot be conducive to good health in later life. An even more interesting result was found in the samples from the urban area in Durban. If positive results were to be found, it was predicted that these would be from the rural population since maize is the staple food. Surprisingly, 2 volunteer subjects (9%, Table 5.2) from the urban area were positive at levels in the higher range of contamination (3.5 and 16.2mg/kg respectively). Whether this represents a real situation remains to be seen from further studies. It is possible that the positive volunteers

could have traveled from the rural area to the city after ingesting contaminated food. Usually, urbanized African people eat maize products, presumably from urban stores, and, therefore, may be complacent about its quality.

Statistically, there was a wide confidence interval in the mean results as shown by Fisher's exact test, indicating that the sample size in this study was too small to make a general conclusion to the general population in the study area. This study therefore serves to point out the need to carry out a much bigger study with a large sample size in order to arrive at a more representative conclusion.

In conclusion, it would seem that the measurement of FB_1 in human faeces is the basis for a short term biomarker for exposure to FB_1. Presence of FB_1 in the human faeces as detected in the present study is of great concern since maize is a staple diet and FB_1 may pose a threat to the human health. Further studies should concentrate on the significance of FB_1 in infected maize and its impact on the human health.

CHAPTER 6

A STUDY OF THE OCCURRENCE OF FUMONISIN B_1 IN A KRANSKOP RURAL COMMUNITY, KWAZULU NATAL

6.1 INTRODUCTION

Several epidemiological studies to determine the levels of exposure and factors which contribute to fungal contamination of food have previously been carried out on aflatoxins and liver cancer (Ngindu *et al.*, 1982, Van Rensburg, 1985 and Peers *et al.*, 1987), fumonisins and oesophageal cancer (Rose, 1982, van Rensburg, 1985, Rheeder *et al.*, 1992; Dutton *et al.*, 1993, Chu and Li, 1994, Bhat *et al.*, 1997). In South Africa, *F. moniliforme* Sheldon has been identified as the major fungus that infects maize especially in the home grown crop intended for human consumption (Marasas *et al.*, 1988a). Maize and maize based products form the main portion of the rural daily diet, increasing this population's chances of exposure to mycotoxins (especially fumonisins), through contaminated food. Studies carried out in two districts of Transkei (Butterworth, Kentani), linked high oesophageal cancer (OC) rates to consumption of fumonisin contaminated maize as compared to low levels in control areas (Lusikisiki and Bizana) (Marasas *et al.*, 1988a, Sydenham *et al.*, 1990a). At the moment, there are no reliable tolerance levels for fumonisins in maize and maize products in South Africa. These levels can only be established following a comprehensive exposure and hazard assessment through epidemiological studies.

This study was carried out in rural locations of Mphise and Ngcolosi both in the magisterial district of Kranskop, KwaZulu Natal, to determine the level of FB_1 in uncooked and cooked food.

Geographical Information Systems (GIS) is a computer-assisted information management system of geographically referenced data. It contains two closely integrated databases: one spatial (locational) and the other, attribute (statistical). The spatial database contains information in the form of digital coordinates, usually from maps or from remote sensing, using geographical positioning system (GPS) by a sattelite tracking device. The attribute data contains information about the characteristics and qualities of the spatial features. The use of GIS in health has been applied to epidemiological investigations in S. Africa

(Abdool Karim *et al.*, 1992, Le Sueur *et al.*, 1997). This has played a great role in eradication and control of malaria. Geographical Information Systems technique is used in this study to map out the rural homes for easy follow-up.

Plate 6.1 A typical rural home in Tugela valley

6.2 MATERIALS AND METHODS

6.2.1 Area of Study

Kranskop area (Figure 6.1) is one of the magisterial districts of KwaZulu Natal with the small town of Kranskop, about 200 km north of Durban, as the headquarters. The area is under Umvoti-Kranskop Health District with Ntunjambili hospital as the headquarters serving a catchment area of up to 300,000 people. The upper plateau, which encompasses the town has abundant rainfall and is mainly occupied by sugarcane farms and wattle tree plantations. It is scarcely populated because of sugarcane farming. The majority of the population in this area, who are rural African, occupy the lower Tugela valley. The rainfall, which is usually abundant in the months of November to April, is lower in the valley than in the upper region. Tugela river is the main source of water. The crops grown are mainly maize, sorghum and beans and are for local consumption being occasionally supplemented with commercial supermarket products, especially in times of drought. The main cereal is maize, which is the major portion of the staple diet of the Tugela valley community. Cattle, goats, sheep and chicken are reared and are the major source of meat. Many of the people are peasants and pensioners, while some are employed in the urban areas. Magisterial

districts are subdivided into locations governed by chiefs with the assistance of *indunas* (headmen). The area of study has two local clinics; Mandlalathi (at Ngcolosi location) and Mphise health clinics. This area was mapped out using GIS (Figure 6.1).

Kranskop was chosen for the study because the residents were approachable and willing to assist in the present investigation. Moreover, the climate of the area was comparable to that of OC endemic areas (semi-arid with unreliable rainfall and altitude below 1000m above sea level) where the conditions are likely to cause plant stress leading to fungal infection. Since maize production is done at low scale in this area, very little or no consideration is given to optimize storage conditions. Poor storage facilities coupled with variations in environmental conditions promote growth of storage fungi and post-harvest spoilage.

6.2.2 Collection of Samples

Two locations in Tugela valley (Mphise and Ngcolosi villages) were identified for the study. After obtaining permission to conduct the study from the two chiefs (N.C. Cele (Mphise) and N.V. Bhengu (Ngcolosi) the two communities were addressed at an *imbizo* (gatherings) at their respective tribal courts, explaining the objective of the study and seeking wider approval. An *imbizo* is the highest decision making meeting in these communities. On obtaining permission, recruitment of homes started by systematic random sampling (choosing every fifth of those who volunteered to take part in the study). The recruited volunteers were visited in their households and further consent for sampling was obtained. In order to assess the home situation, family size, health history, food source and storage, a questionnaire was then formulated in Zulu and distributed to the occupants of the homes under study. Trained Zulu interpreters were asked for assistance. Processed foods (cooked mealimeal, *phutu*, sour porridge, *amahewu* and local brew, *isizulu*) and unprocessed stored cereals, were also collected. Faeces were collected the day following that of cooked food collection, from the same households and stored in a refrigerator at Ntunjambili hospital. Analysis of samples was done at the University of Natal.

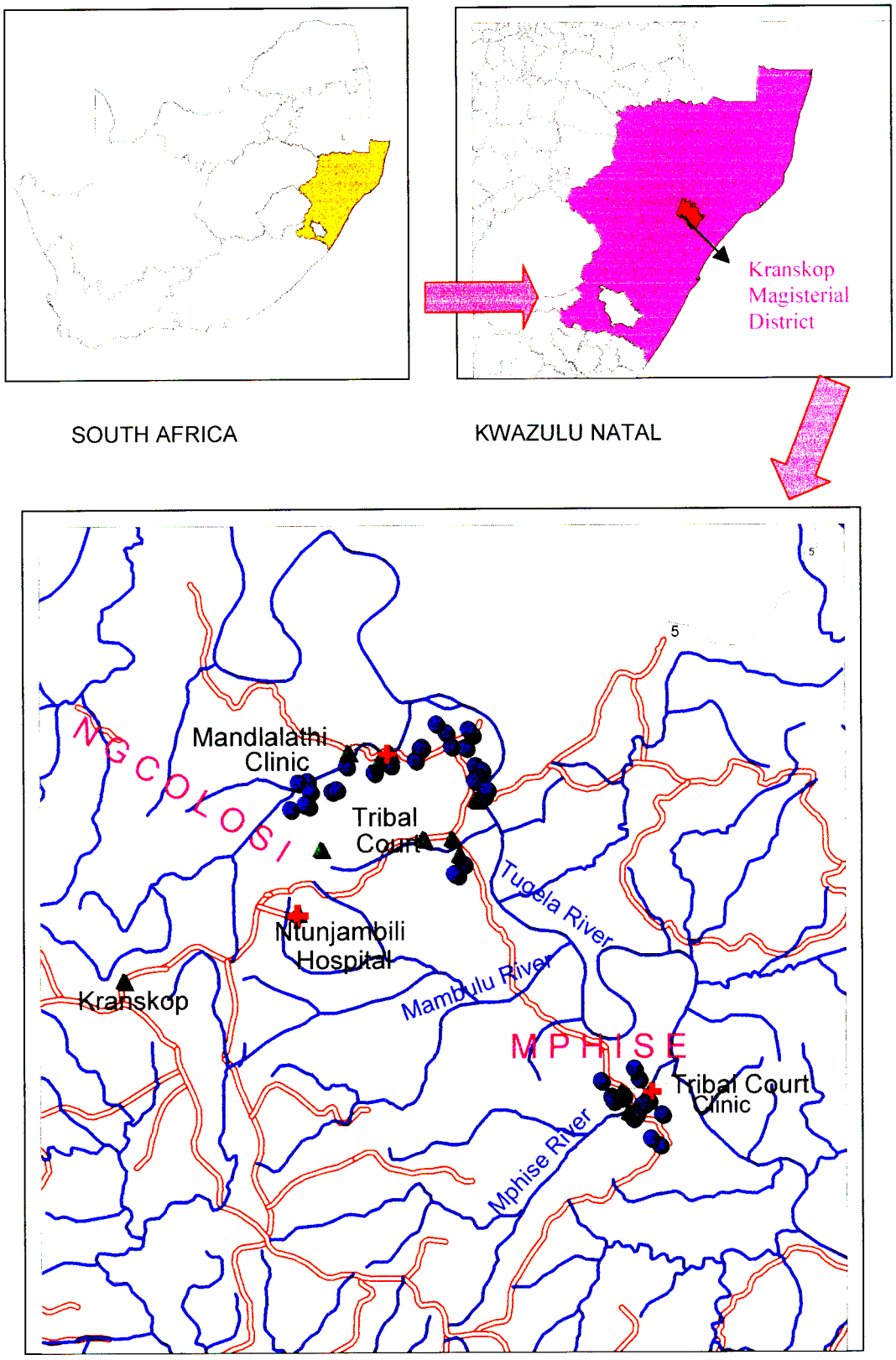


Figure 6.1 Plot of dwellings in the study area derived from GIS

A similar study was carried out within Durban Metro as a control group. Households were visited and the questionnaire survey carried out. The households varied from formal to informal settlements. Food and faecal samples were also collected and analyzed as in the rural study. It was important to include this control group because of its urban character and therefore, people from this region consume a more mixed diet. Where maize is consumed, it is of commercial grade for human consumption and is more likely to be mycotoxin free.

6.2.3 Data Analysis

The questionnaire survey data were analysed using Excel (Microsoft Corporation, NY, USA) and SPSS statistical package (Microsoft Corporation, NY, USA). Geographical data were processed and household locations displayed on a map using Mapinfo professional software package (Mapinfo Corporation, Troy, NY, USA) (Figure 6.3)

6.2.4 Reagents and Equipment

All chemicals unless otherwise specified were of Analar Grade.

a) *SPE C_{18} cartridges:* 10 ml capacity; (Varian Bond-Elut from Analytichem, Harbour City, CA 90710) containing 500mg sorbent.

b) *Mobile phase:* Methanol/0.1M sodium dihydrogen phosphate (80:20,v/v) adjusted to pH 3.4 with orthophosphoric acid.

c) *o-Phthaldialdehyde (OPA) reagent:* 40 mg of OPA dissolved in 1 ml of methanol and diluted with 5ml of 0.1M sodium tetraborate and mercapthoethanol (50 ul).

d) *Solvents:* Acetonitrile/water (1:1, v/v), butanol, acetic acid and methanol, both obtained from BDH Chemicals, Poole, England.

e) o-*Phosphoric acid* (concentration >85%, obtained from BDH Chemicals, Poole, England).

f) *Fumonisin B_1 standard.* (1mg/ml and 50µg/ml) dissolved in acetonitrile/water (1:1v/v). The standard was obtained from PROMEC, Cape Town, South Africa.

6.2.5 Analysis of Maize Samples

6.2.5.1 Extraction and Clean-up

Ground maize samples were mixed thoroughly, a 25g sample was extracted with 100ml methanol/water, 3:1 v/v for 1 hour in a homogenizer as described in the method of

Sydenham *et al.* (1992b). Samples (50ml) of homogenized liquid foods, *isizulu* and *amahewu*, were extracted with equal volumes of methanol/water, 3:1 v/v. Samples (25g) of cooked food, *phutu*, were extracted using 100ml of 50% aqueous acetonitrile at pH 2.7 as described by Murphy *et al.*, (1996). The extracts were filtered and 10 ml aliquots of each sample extract dried by rotary evaporation under low heat (60°C). The dry extracts were then reconstituted in 10 ml of methanol water 3:1 v/v, pH adjusted to 6-6.5 and applied into strong anion exchange (SAX) solid phase cartridges previously conditioned by washing with 5 ml methanol and 5ml methanol/water (3:1 v/v). The columns was then washed with 5ml of methanol/water 3:1 v/v, 3ml methanol and the FB_1 eluted at the rate of 1ml/min. with 1% acetic acid in methanol (10ml). The eluates were dried under a stream of nitrogen at 60°C and stored at 4-8°C until further analysis.

6.2.5.2 Analysis and Recovery of Fumonisin B_1 in Maize

Four samples of maize meal (25g) containing no detectable FB_1 in 250ml conical flasks, were thoroughly mixed with 10 ml of methanol containing 100µg of FB_1 and were left to dry overnight in a fume cupboard at room temperature. Two of the spiked samples were used to prepare *phutu,* a common traditional Zulu dish. Boiling water (20ml) and a pinch of sodium chloride were added to each of the two samples in 250 ml conical flasks on a hot plate. Glass rods were used to stir the mixture to an even paste. Temperature of the cooking mixture was noted (93°C). The temperature of the hot plate was then reduced to about 70°C and the samples left to simmer for a further 15 minutes. After cooling, the cooked samples were each extracted with 100ml of 50% aqueous acetonitrile at pH 2.7. The same solvent was used to extract the uncooked samples but with no pH adjustment. The samples were continuously homogenized for 1 hour in a shaking table (150 revolutions per minute), filtered and cleaned up using SAX cartridges as described in section 3.2.5.2. Extracts were analyzed for FB_1 by HPLC with OPA derivatization and fluorescent detection as described in section 3.2.5.2.

6.2.6 Analysis of Faecal Samples

Faecal samples were processed and analyzed as previously described in section 5.2.3.

6.2.7 Quantitation by High Performance Liquid Chromatography

Quantitation of FB_1 in maize and faecal extracts was carried out using HPLC with OPA derivatization and fluorescent detection as described in section 3.2.5.2. The detection limit of the method was 50 ng/g FB_1 .

6.3 RESULTS AND DISCUSSION

6.3.1 Questionnaires

In the rural area, a total number of 47 households were visited and consent obtained. However, 7 later withdrew for other reasons from the study. This resulted in 40 houses being sampled with a total number of 50 personal contacts (in some households, more than one person was interviewed). In the urban control area, 46 households were visited and 50 household members interviewed. The findings of the questionnaires are summarized in Table 6.1. A copy of the original questionnaire is enclosed (Appendix 6).

Although the sample size is relatively small, there are trends, which indicate that the consumption of home grown maize in the rural population group (RG) is low. This is the case even when considering households that use both home grown and commercial maize. As for the response of the questionnaire, the incidence of smoking in the RG is much lower than that of the urban population group (UG), which can be explained on the greater affluence of the latter and access to tobacco products. It is highly likely, however, that there are smokers of non-tobacco products (i.e., *Cannabis*) in the RG, which would not be admitted to because of legal implication of such practices. Smoking has been suspected as one of the factors causing OC (section 2.4.2).

Percentage of alcohol consumption and source of water were different. The RG drink a higher percentage of home brewed beer, which is the potential source of mycotoxins, in particular from maize. The source of water for daily use, including drinking and cooking, since it is rain water from rivers, lakes and dams, the health of RG is of great concern. On the other hand, investigation revealed that 80% of UG use tap water, which is controlled. Not only is the possibility of the RG being exposed to microbial pathogens greater, but also dangerous ions, such as nitrite which has been implicated in OC induction (Rose, 1982, Li and Cheng, 1984) (section 2.4.2).

Table 6.1: Demography, smoking status, and sources of food and water (all the subjects in the study were African in rural and urban areas).

Subject/Activity	Incidence (%)	
	Study Area (%) Rural	Reference Area (%) Urban
Homes	40	46
Participants	50	50
Smokers/Non-smokers	18 (36)/ 32 (64)	43 (86)/ 7 (14)
Source of Food (Maize)		
Home grown	6 (12)	1 (2)
Shops	35 (70)	40 (80)
Farm and shops	9 (18)	5 (10)
Drinks Taken		
Local brewed beer	39 (78)	6 (12)
Beer from shops	4 (8)	16 (32)
Soft Drinks	7 (14)	28 (56)
Source of Water		
River	47 (94)	5 (10)
Rain	1 (2)	1 (2)
Tap	0	40 (80)
Well	2 (4)	0

Maize samples spiked with FB_1, on extraction and analysis gave over 90% recovery (Table 6.2). However when spiked samples were cooked, extraction with unmodified solvent (50% aqueous acetonitrile), gave 35.9% while the modified extraction solvent (with pH of 2.7) gave a recovery of over 85% FB_1.

Table 6.2 Results of FB_1 recoveries from 25 g of cooked and uncooked maize based food on extraction with 50% aqueous acetonitrile

	Conc. of FB_1 spiked (μg)	Conc. Recovered*** FB_1(μg)	%Recovery
FB_1 in phutu (M)*	100	89μg (±3.4)	89.1
FB_1 in phutu (U)**	100	36μg(±2.9)	35.9
FB_1 maize meal	100	97μg(±1.3)	97.4

* modified solvent, pH 2.7

** unmodified solvent pH

*** Recovery is a mean of two results

6.3.2 Food analysis

A summary of results on food analysis for FB_1 (Table 6.3) shows that maize from the rural area had the highest number of positives 15 from 47 samples (31.9%) while the UG had only three positives from 49 samples (6.1%). The mean concentration of FB_1 in the rural maize was significantly different from that of the urban area (p = 0.014, and corrected χ^2 test of 6.03). This difference in FB_1 distribution in maize is noteworthy as it shows that the rural populace is at a higher risk of fumonisin-associated disorders (Fisher's exact test showed that the rural group is 6 times more at risk to FB_1 exposure than is the urban group).

Cooked food- *phutu,* showed that only 29% of rural samples were positive while there were none from the UG, therefore, no statistical comparison was done. This clearly reflects the incidence of FB_1 in maize. As was shown in the recovery experiments, the preparation of *phutu* does not appreciably degrade the toxin. The high end of the range of over 22mg/kg, is of some concern but is not as high as that found in the Transkei high OC regions of Kentani and Butterworth, where infected maize contained 117mg/kg (Rheeder *et al.,* 1992). This may explain the lack of elevated OC levels in Tugela Valley.

Fermented foods and sorghum were all negative for FB_1 from both population groups. While the *F. moniliforme* mating population "A" is the main contaminant of maize, sorghum often gets infected by type "F" mating population which is the lower producer of fumonisins (Klittich and Leslie, 1992). Although a limited amount of FB_1 is degraded

during fermentation of contaminated maize (Bothast *et al.,* 1992, Scott *et al.,* 1995) in these fermentations, i.e., *amahewu* and *isizulu*, fermented maize is used as a starter, with sorghum, the main component. This explains the absence of FB_1 in the fermented products. From these results, it seems that the rural population should be encouraged to consume traditional fermented products in preference to straight maize derived dishes.

6.3.3 Faecal analysis

Of the forty samples of rural faecas analyzed, 13 (32.5%) were positive for FB_1 (Table 6.3) while for the urban samples, only three samples were positive out of 44 (7%). The mean concentration of FB_1 in the samples of rural faeces was significantly different to that from the urban area ($p = 0.014$, and corrected Chi Square (χ^2) test of 6.03). These results reflect the incidence of FB_1 in the maize consumed and is important in showing that analysis of faeces is a useful short term biomarker for FB_1 exposure, as the faecal samples were taken 24 hours after maize consumption. Fisher's exact test also showed that the rural group is 6 times more at risk to FB_1 exposure than the urban group is. Other risk factors are also of importance here. These include personal habits, amount of food consumed, body weight, gut flora and body metabolism. However, it does not seem unreasonable to assume a working rule that, after 24 hours of ingestion, a similar magnitude of FB_1 will be found in the dried faeces, although this conclusion is not as clear cut in the urban group. A time course study after the ingestion of contaminated maize is required to evaluate the analysis of faeces as a practical marker of FB_1 exposure. In general, however, it is obvious that the rural group is routinely exposed to FB_1 from the diet, a conclusion arrived in Chapter 5 of this thesis.

Table 6.3. Fumonisin B_1 content in cereals/stool from Tugela valley and Urban control groups

Sample	Region	Samples analysed	No. of Positives For FB_1	Range (mg/kg)	%positives
Maize	Rural*	47	15	0.092-22.225*	31.9
	Urban*	49	3	0.205*	6.1
Sorghum	Rural	13	ND	NA	NA
	Urban	NS	NA	NA	NA
Amahewu	Rural	14	ND	NA	NA
	Urban	NS	NA	NA	NA
Isizulu	Rural	11	ND	NA	NA
	Urban	NA	NA	NA	NA
Phutu	Rural	28	8	0.104-0.364	28.6
	Urban	39	ND	NA	NA
Faeces	Rural	40	13	0.513-39.0	32.5
	Urban	44	3	0.607-16.2	6

NA = Not analysed NS = Not sampled ND = Not detected (below the detection limit of 50ng/g.

*The mean concentration of FB_1 was significantly different ($p = 0.014$).

6.3.4 Storage

From the questionnaire survey, the common storage containers in rural homes included: 10sacks (35%), 10 metal drums (20%) and 25 plastic drums (50%). According to the questionnaire survey, majority of the households (65%) stored their food for 7-12 months (Table 6.4). Long storage periods may increase the chances of food contamination by fungi if the storage environment is not kept dry or insect free. However, there was no correlation between the occurrence of FB_1 in stored cereals and type of storage. This may be explained by the fact that *F. moniliforme* which produces fumonisin, is mainly a field fungus. Other fungi could have been present but they were not investigated, as analysis of fumonisin B_1 was the main objective of this study.

Plate 6.2. A metal drum is a popular form of storage container in Tugela valley homes

Table 6.4: Duration of food in storage in the rural and urban areas

Storage Period	Region	No. of households*	Percentage No. of households
< 1month	Urban	43	93
	Rural	11	27.5
1-6months	Urban	3	6.5
	Rural	11	27.5
7-12 months	Urban	0	0
	Rural	26	65
> 1 year	Urban	0	0
	Rural	2	5

*n = Total number of households (46 for urban and 40 for rural)

6.3.5 Clinical Conditions

Chronic respiratory symptoms (tuberculosis, and asthma) were the major complaints in the RG (24%) followed by heart related disorders, mainly hypertension (12%) (Figure 6.2). The main causes of these non-infectious disorders were not known but considering the type of dwelling and surrounding, it was likely that airborne fungi were involved (Bardana,

from the urban group showed similar trend of respiratory complaints as the rural group, for non-infectious conditions which were mostly asthmatic (9 cases out of 50 (18%). This may be possible because most of the volunteers were from informal and poorer housing sector where exposure to indoor fungal spores is high (Gqaleni, *et al.,* 1998b). The questionnaire survey did not record any cases of OC. However, the recorded incidence of OC in the local hospital serving the communities (300 000 people) under the study is given in Table 6.5. These records are comparable national average records of 4.8/100 000 and 10.8/100 000 in females and males respectively (Sitas *et al.,* 1997) and does not indicate the undue high incidence observed in the Transkei.

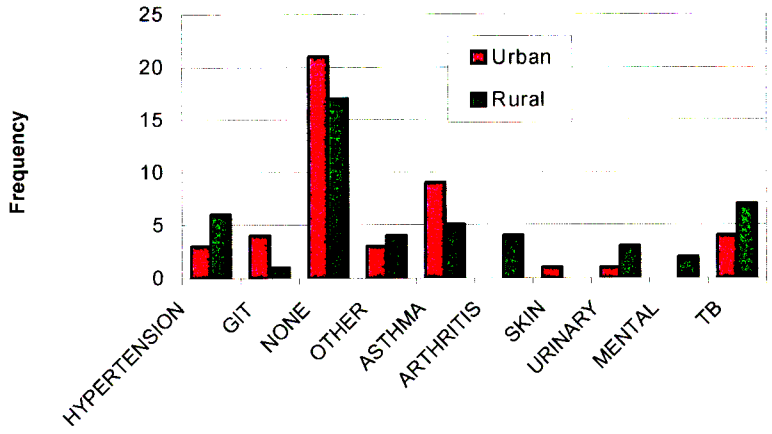


Figure 6.2 Urban control and Rural study groups showing the distribution of the common medical complaints

Table 6.5 Oesophageal Cancer (OC) cases recorded at Ntunjambili hospital (January 1995 – September 1998)

Year	Male	Female
1995	9	5
1996	5	4
1997	4	4
1998	3	2

6.3.6 Climatic Conditions

The rainfall and temperature patterns for the known OC districts in Eastern Cape (Butterworth and Lusikisiki with altitudes above sea level as 534 and 594m respectively) were compared to those of Kranskop, Mandini and Durban (Altitudes 1148 and 109m and 15m respectively, above sea level) (Figure 6.3 and 6.4). Climatic data from Mandini were used to represent Tugela Valley where the study was carried out for two reasons: there was no weather station within the study area and secondly, Mandini is the nearest station with the same altitude as Tugela Valley villages. Kranskop and Durban had the highest average annual rainfall, than the rest of the districts. There was no observed drastic change in average annual temperature pattern in the five regions (mostly over 20°C). Rainfall patterns were similar except for Durban and Lusikisiki which had unusually heavy precipitation in the dry months of between May and September. Although Durban, the home of the urban group, had high annual rainfall in comparison with other areas, much of the food consumed comes from the farms further inland, hence the observed weather data may not have any association with the type of food taken by the urban group. Environmental factors like rainfall and soil types are among the factors implicated in the aetiology OC in endemic areas (van Rensburg, 1985). The weather data gathered in this study show that the areas have similar climatic conditions. However, they may not be used alone to predict the existence of OC in this area. Other environmental factors like soil type and minerals in drinking water, should be analyzed as well.

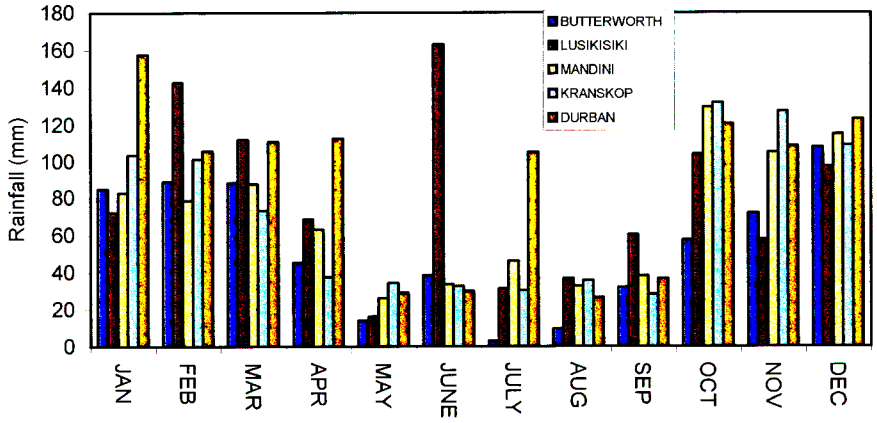


Figure 6.3 Average annual rainfall (1993-97), for Butterworth, Durban, Lusikisiki, Kranskop, Mandini and Durban (Data was obtained from South African Weather Bureau, Johannesburg).

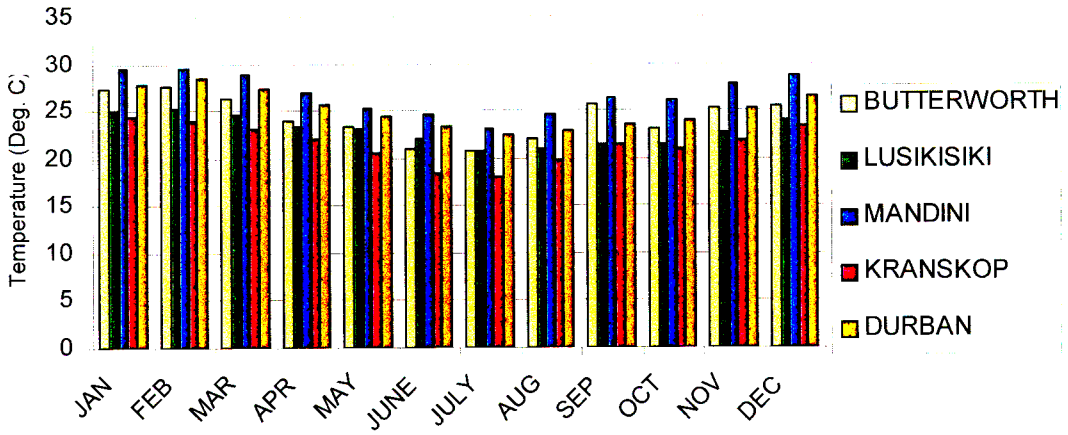


Figure 6.4 Annual Average Temperature °C (Maximum) (1993-97) for the districts of Butterworth, Lusikisiki, Kranskop and Mandini (Data from South African Weather Bureau, Johannesburg

6.4 CONCLUSION

It would be of interest to know whether other rural populations of South Africa, are exposed to FB_1 at the same level as in Transkei. Analysis of faecal samples was also carried out as a short term marker for FB_1 exposure. It can be deduced from this study that faecal analysis for FB1 is a useful tool in assessing individuals for early exposure to fumonisins. The aim of this study was also to find out if FB_1 levels in food commodities correlated with the incidences of OC in this community. The level of FB_1 and OC incidence was minimal in comparison to high OC areas of Transkei. An essential requirement to carrying out this kind of study was the use of Geographical Information Systems (GIS). This proved to be a useful tool in locating the study homes.

CHAPTER 7

SUMMARY

This study amongst other things showed that:

1. Fumonisin B_1 can be co-extracted with other mycotoxins in maize samples using one solvent. However, FB_1 could not be completely be integrated into the multi-mycotoxin screen. The modifications introduced in the multi-mycotoxin screen used in this study, makes the method more efficient, economical and time saving, since one solvent is used to extract most mycotoxins including FB_1. With the change of solvent pH to 2.7 the recovery of mycotoxins, especially FB_1 in cooked food, was improved.

2. Production of FB_1 is best done using stirred jar fermenter, as clean-up of standard produced from this method, is much cheaper compared to that from patty cultures. Methylation of FB_1 extracts for better cleanup may be possible but is not effective if the untargeted functional groups are not protected.

   Analysis of faeces for FB_1 shows that the rural communities are at a higher risk of exposure to FB_1, than their urban counterparts.

3. Having determined FB_1 in faeces and food, the Fisher's exact test shows that the rural group from Tugela valley in Kranskop are 6 times more at risk of FB_1 exposure when compared with the urban group. It may be concluded, therefore, that measurement of FB_1 in faeces or food can be used to monitor the risk of exposure to FB_1. However the method is preferable since it is specific for each individual, whereas the latter method is a more general method for screening FB_1.

RECOMMENDATIONS

1. For full integration of FB_1 into the multi-mycotoxin screen, there is need to modify the method such that the sodium hydrogen carbonate used in the procedure can be avoided. This is because sodium hydrogen carbonate has a hydrolytic effect on FB_1 molecule. Improvement of recovery values of mycotoxins can be achieved by increasing the extracting solvent/sample ratio from the usual 2:1 v/w to 4:1 v/w.

2. For better methylation of FB_1 molecule, protection of the untargeted functional groups need protection, using appropriate reagents, in order to avoid diazomethane esterification.

3. Fumonisin B_1 analysis in faeces can further be optimized such that it can be used a biomarker of exposure to FB_1. Knowledge of excretion rate, effect of bowel movement, the role that different diets play in faecal transit time needs to be studied.

4. A follow up of the rural study needs to be carried out with a large sample size in order to arrive at a better exposure risk assessment to FB_1. This will provide valuable information towards prevention and control of OC and FB_1 related disorders in southern Africa and worldwide.

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APPENDICES

APPENDIX 1

PREPARATION OF C_{18} PACKING MATERIAL FOR REVERSED PHASE LIQUID CHROMATOGRAPHY

Chemicals: Octadecyltrichlorosilane and trimethylchlorosilane (Sigma Chemical Co., St. Louis, USA), Silica gel 60 (40-63μm diameter particle size) was purchased from Merk, Dermstard, Germany. Analytical grade (AR) reagents: Toluene, methanol, concentrated sulphuric and nitric acids.

Method: The packing material was prepared according to the method of Kingston and Gerhart (1976), with slight modifications. Silica gel 60 (300g) was heated (100°C, 12 hours,) in a mixture of 500ml concentrated nitric acid and 500ml concentrated sulphuric acid. After cooling and decantation of excess acid, the residue was washed with distilled water by decantation. The washing procedure was repeated until the pH of the distilled water was neutral. The residue was then dried (110°C, 12 hours). The dry silica gel was suspended in 800ml of dry toluene, 90 ml octadecyltrichlorosilane added, and the mixture heated under reflux for 14 hours. Excess toluene and chlorosilane were decanted, and the residue washed with toluene to remove any remaining chlorosilane. The bonded chlorosilane was then hydrolyzed by stirring the silica gel with 50% aqueous acetonitrile for 2 hours at room temperature. The aqueous mixture was then decanted, the residue washed in distilled water and dried. Final protection of the free hydroxyl groups was achieved by reaction of the dry powder with 10% trimethylchlorosilane in dry toluene (500ml) for 2 hours under reflux, followed by final washes with toluene and methanol and drying (110°C, 3 hours).

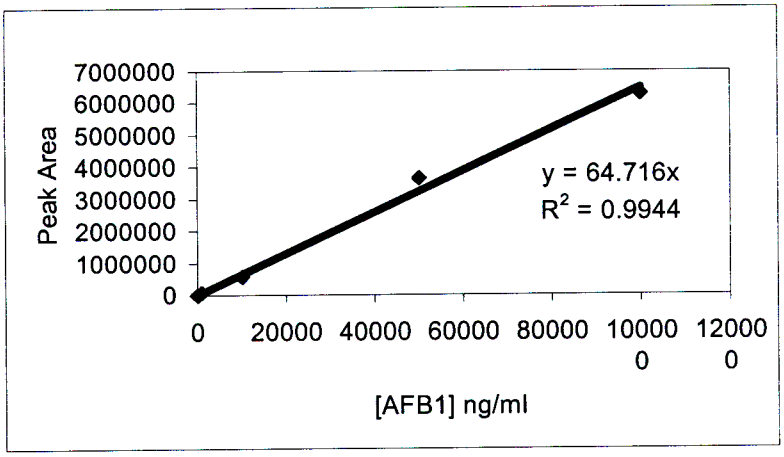
The performance of the C_{18}, obtained by the above procedure, was compared to that of commercially available C_{18} solid phase cartridges (Bond Elute).This was done by using both of them to clean up FB_1-spiked sample of maize meal (using the procedure described in section 3.2.1) and estimating the percentage recoveries of FB_1. The recovery value of FB_1 from prepared C_{18} was 10% less than that from commercial C_{18} cartridges.

APPENDIX 2

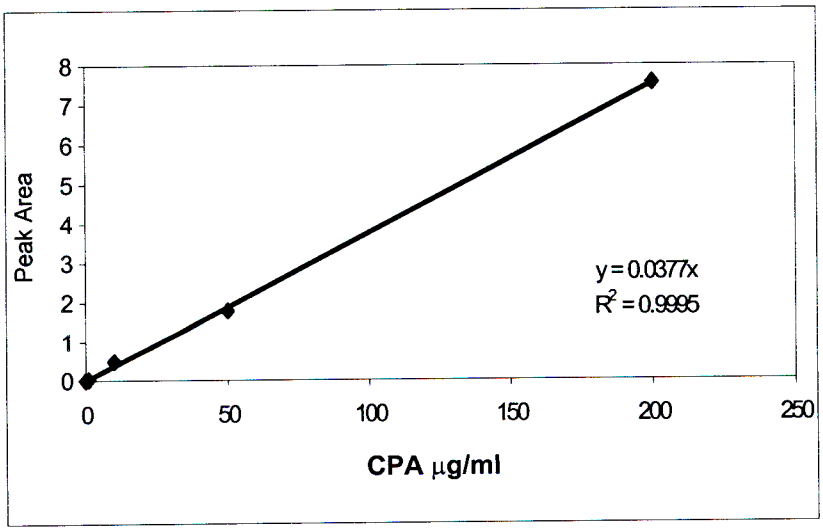
SOLVENT SYSTEMS USED FOR TLC PLATE DEVELOPMENT

1) CEI = Chloform/Ethyl Acetate/Propan-2-ol (90:5:5, v/v/v)
2) TEF = Toluene/Ethyl Acetate/ Formic Acid (6:3:1, v/v/v)
3) CtE = Carbon Tetrachloride/Ethanol (98:2 v/v)
4) CM2 = Chloroform/Methanol (95:5, v/v)
5) ChE = Cyclohexane/Ether (3:1, v/v)
6) BWA = Butanol/water/Acetic Acid (12:5:3, v/v/v)

APPENDIX 3

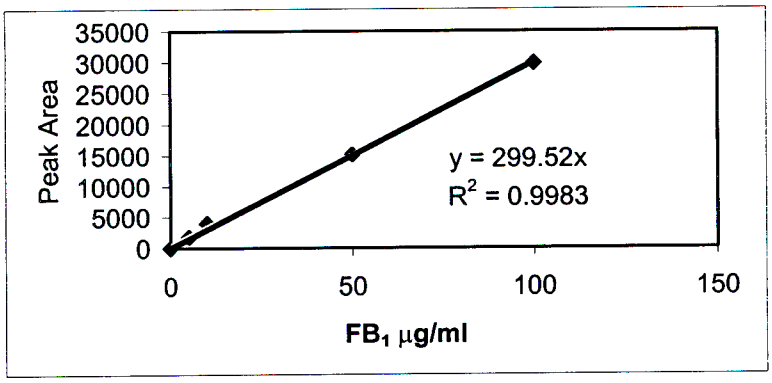


Standard curve for the determination of aflatoxin B_1 concentration using high performance liquid chromatography with UV detection

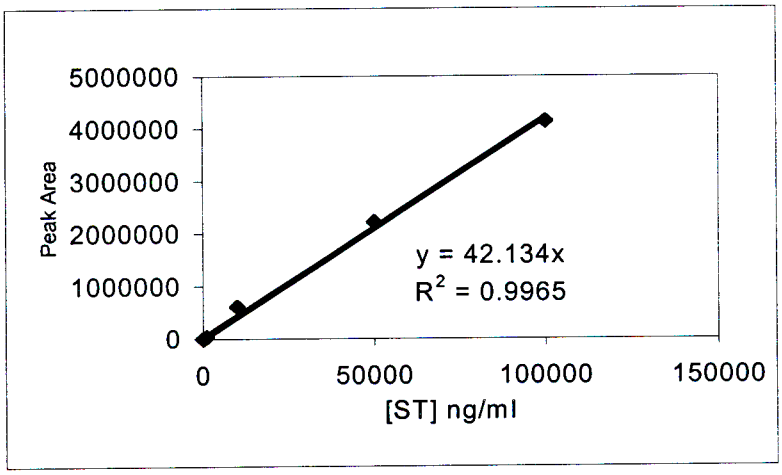


Standard curve for the determination of cyclopiazonic acid concentration using capillary electrophoresis (CE).

APPENDIX 4

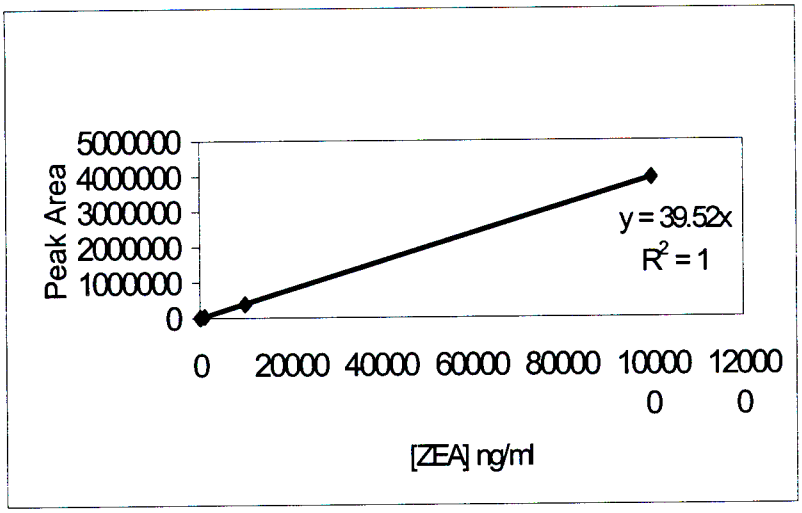


Standard curve for detemining fumonisin B_1 (FB_1) using high performance liquid chromatography



Standard curve for determining sterigmatocystin (ST) by high performance liquid chromatography

APPENDIX 5



Standard curve for determining zearalenone (ZEA) concentration using high performance liquid chromatography

APPENDIX 6

A COPY OF THE QUESTIONNAIRE USED IN THE RURAL SURVEY

MYCOTOXIN UNIT
DEPARTMENT O F PHYSIOLOGY: QUESTIONNAIRE

Where appropriate, mark selection with a cross (X).

A. Home Situation

Head of Family (Family contact person)

1. Surname:_____ Forename_____
2. Home location _____
3. Number in family_____
4. Number between ages 1-6 years_____
5. What is the health state of the family? Mark with an "X" where applicable

1	Not good	
2	Fair	
3	Good	

6. If not healthy, what is the nature of sickness?

1	Respiratory		7	Skin	
2	Gastrointestinal		8	Liver	
3	Urinary tract		9	Blood related	
4	Mental		10	Skeletal	
5	Cardiac		11	Cancer	
6	Reproductive		12	Other	

7. How long has the sickness lasted?
   (a) Less than a week___ (b) Longer than a month___ (c) longer than a year___

8. How many members of your family smoke?
   (a) One _____ (b) More than one _____ (c) None_____

B. Food and drinks taken

9. What type of food do you eat and how frequently? Mark 'D' if daily or 'W' if once in a week: (a) Cooked mealimeal_____ (b) Samp _____ (c) Chicken _____
   (d) Red meat_____ (e) Wild vegetables _____ (f) Peanuts _____
   (g) Other_____

10. Where do you obtain your food?

(a) Locally in the farm____ (b) From the shops_____ (c) Farm and shops_____

11. How do you prepare your food before eating?
    (a) Cook _____ (b) Ferment_____ (c) Other (specify) _____

12. What type of drinks do you take?

    (a) Milk____
    (b) Soft drinks____
    (c) Locally brewed beer _____
    (d) Beer from shops____
    (e) Other _____

13. Where do you obtain your water?
    (a) River _____ (b) Well_____ (c) Rain_____ (d) Tap_____

14. How is the water treated before drinking?
    (a) Not treated _____ (b) Boiled_____ (c) Other_____

C. Food Storage

15. Where do you store your food?
    (a) In metal drums _____ (b) In plastic drums_____ (c) Other _____

16. Is the food stored inside or outside the house? (a) Outside____ (b) Inside____

17. How long does the stored food last?
    (a) Less than one month_____
    (b) 1 - 6 months_____
    (c) 7-12 months_____
    (d) more than 1 year____

18. Do you clean the storage containers before putting in new grain?
    No _____ Yes_____

Details supplied by: _____

Interviewer _____