

**THE FATE OF MYCOTOXINS IN NON-ALCOHOLIC
LACTIC ACID MAIZE MEAL FERMENTATION**

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I declare that the thesis herewith submitted for the MMedSc degree at the University of Natal, has not been previously submitted by me for a degree at any other University.

A handwritten signature in black ink, appearing to read 'S. M. M. M.', written over a horizontal line.

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ABSTRACT

This study was aimed at investigating the potential of lactic acid fermentation in reducing mycotoxin concentration in maize meal products. Maize meal was spiked separately with aflatoxin B₁, fumonisin B₁, and zearalenone, and fermented for four days. During this period the concentration of each toxin and the pH of the fermented maize meal were monitored. There was a significant ($p= 0.000$) decrease in the concentration of all the mycotoxins, with a percentage reduction of 55-69 by the third day and 68-75 by the fourth day, respectively. Commercial *amahewu* samples were also screened for the presence of these three mycotoxins, and the results indicated that the samples were not contaminated with detectable levels of these toxins.

An attempt was made to characterise the metabolic derivatives (by-products) of each mycotoxin following lactic acid maize meal fermentation. To achieve this maize meal samples were separately spiked with each of mycotoxin, fermented for four days and screened for specific mycotoxin derivatives (by-products) using GC/MS, HPLC and relevant standards (i.e. partially hydrolysed fumonisin B₁, aflatoxin B_{2a}, α - and β -zearalenol). None of the targeted derivatives could be detected in the fermented maize meal samples.

The potential cytotoxicity of the mycotoxin-spiked fermented samples was investigated using an SNO cell line. The fermented toxin-spiked maize meal samples with a starter culture were comparatively less toxic (29 - 36%) to SNO oesophageal cells than samples spiked with toxin without a starter culture (24 - 30%). However, this observed difference was not statistically significant ($p = 0.295 - 0.681$).

Furthermore, cells that were only inoculated with the cell culture medium had significantly ($p = 0.000$) high percentage cell viability.

This study indicates that it is possible to significantly reduce the concentration of mycotoxins using lactic acid maize fermentation to trace levels. However, such a reduction will not significantly alter the possible chronic toxic effects of such toxins in the diet, particularly a maize based diet containing poor quality protein. The trace amounts of these toxins in fermented and unfermented maize meal should continue to be a cause for concern.

LIST OF ABBREVIATIONS

AFB ₁	: aflatoxin B ₁
AFB ₂	: aflatoxin B ₂
AFB _{2a}	: aflatoxin B _{2a}
AFG ₁	: aflatoxin G ₁
AFG ₂	: aflatoxin G ₂
AFM ₁	: aflatoxin M ₁
API	: aminopentol of fumonisin
CFU	: colony forming units
DMSO	: dimethyl sulphoxide
DON	: deoxynivalenol
Dnase I	: deoxyribonuclease I
EMEM	: minimum essential medium with Earl's salt
FB ₁	: fumonisin B ₁
FB ₂	: fumonisin B ₂
FCS	: foetal calf serum
GC/MS	: gas chromatography/ mass spectrometry
HPLC	: high performance liquid chromatography
MeOH	: methanol
MS	: mass spectrometry
NaOH	: sodium hydroxide
NIV	: nivalenol
OPA	: ortho-phthaldialdehyde
SAX	: strong anion exchange
SPSS	: statistical programme for social scientists
TLC	: thin layer chromatography
ZEA	: zearalenone

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CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Maize and maize products are known to be susceptible to contamination by fungi that produce secondary metabolites called mycotoxins. Two of the well-studied groups of mycotoxins are aflatoxins and fumonisins that are produced, respectively, by species of *Aspergillus* and *Fusarium*. Groopman and Donahue (1988) described aflatoxins as extremely toxic and carcinogenic compounds, which appear to be ubiquitous in the environment. Aflatoxin B₁ is described as one of the most potent natural carcinogens known and is classified by the International Agency for Research in cancer as a human carcinogen (Massey *et al.*, 1995).

Fumonisin, on the other hand, have been described as hepatocarcinogenic in rats (Gelderblom *et al.*, 1988). Fumonisin-contaminated maize has been associated with the occurrence of human oesophageal cancer in Cixian and Linxian regions of the People's Republic of China and Eastern Cape Province of South Africa (Sydenham *et al.*, 1995) and implicated as a cause of pulmonary oedema in pigs (Colvin and Harrison, 1992). According to Sydenham *et al.* (1996), the International Agency for Research on Cancer has classified fumonisin B₁ as a Group 2B carcinogen (i.e., possibly carcinogenic to humans). In addition, de Nijs *et al.* (1998) confirmed that fumonisin B₁ can play a role in the aetiology of human oesophageal cancer.

Zearalenone (ZEA), another toxin produced by *Fusarium* spp., is receiving increasing attention because of its (and its metabolic derivatives) potential to cause hyperoestrogenism in humans (Schoental, 1983) and severe reproductive and infertility problems in animals, especially in swine

(Aucock *et al.*, 1980).

The rural populations in South Africa and other developing countries are exposed to food contaminated by fumonisins because they depend on maize as a staple diet. It is estimated that these communities can consume about 460 grams of maize per 70 kg person per day (Thiel, 1998). This could pose a potential problem since fumonisins can be found in the highest concentration under natural conditions in agricultural commodities such as maize. This problem is aggravated by the fact that these toxins are heat stable and cannot be easily degraded by physical methods without affecting food quality. There is, therefore, an urgent need to find affordable, readily available methods of mycotoxin decontamination to be used by rural communities.

One of the ways that rural communities consume maize meal is by non-alcoholic lactic acid fermentation in the form of *incwancwa* (a sour soft porridge) and *amahewu* (a sour beverage). The maize products obtained by this type of fermentation process are semi-solid with a fine texture and can easily be consumed by both children and adults. Fermentations have always offered a variation in the way rural communities have been consuming maize products over the years.

In addition, Motarjemi and Nout (1996) have highlighted the possibility that lactic acid fermentation of maize can be used as a method to render food safe for consumption by adults and as weaning food for babies. This is due to the fact that lactic acid fermentation of the maize meal reduces the pH of the final product, thereby, creating unfavourable conditions for growth of spoilage organisms. Lactic acid fermentation may have several other benefits; such as, increased shelf life of products, viricidal, antitumour, antileukemic, bacteriostatic, and bacteriocidal effects (Naidu *et al.*, 1999).

Gourama and Bullerman (1997) found that lactic acid bacteria involved in natural fermentation interfered with aflatoxin biosynthesis. Furthermore, lactic acid fermentation has proved to be commercially useful. For example, strains of *Lactobacillus* are used as probiotics, which are viable bacteria used as food additives producing beneficial effects by promoting the equilibrium of the intestinal flora (Zani *et. al.*, 1998). Because of the beneficial effects of lactic acid bacteria, there is a need to further explore and enhance the ability of the lactic acid fermentations to combat the mycotoxin problem.

1.2 Occurrence of mycotoxins in cereal grains

Mycotoxin-producing moulds are known to invade a whole range of agriculturally important crops with the result that none of the cereals (pre- and post-harvest) are immune to mycotoxin contamination. *Aspergillus flavus* is well known for infecting maize under poor storage conditions and the fungus may subsequently produce aflatoxins. *Fusarium verticillioides* infects mainly maize and fumonisins are formed in the field with often no mouldiness necessarily visible to the naked eye (Thiel, 1998).

1.2.1 Aflatoxins

Toxigenic strains of *Aspergillus flavus* and *A. parasiticus* are widespread in nature and produce aflatoxins (structures shown in Figure 1) whenever conditions are favourable. The occurrence of aflatoxin contamination is a global phenomenon with high levels of the toxin especially prevalent in developing countries. The incidence and level of aflatoxin contamination in various commodities has been monitored worldwide (Ce'spedes and Diaz, 1997). Humans are exposed to aflatoxins by

consuming foodstuffs that have been directly contaminated by fungal strains of *Aspergillus*. In the USA, maize is frequently contaminated with aflatoxins during growth, harvest, storage and transportation (Line and Brackett, 1995a).

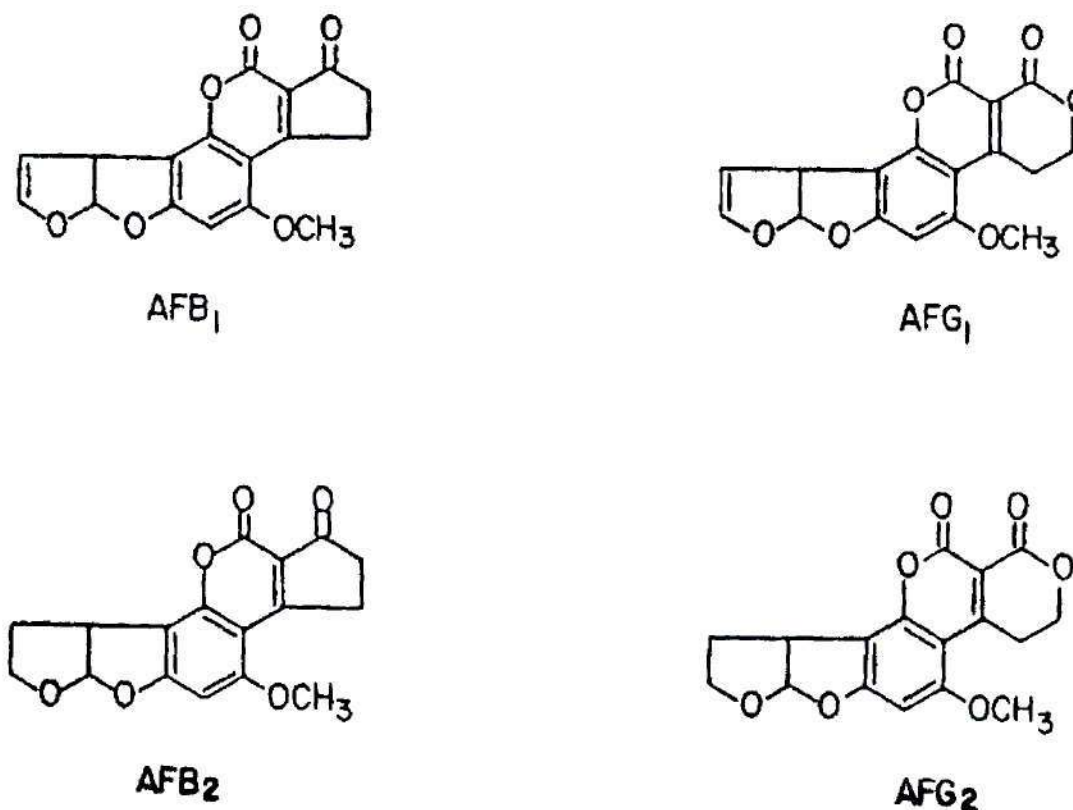


Figure 1. Structures of Aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), and G₂ (AFG₂) (Wilson 1982).

Many grains and foodstuffs, including maize, can be contaminated with aflatoxins. Contamination by moulds may be predominant within a geographic area and the levels of aflatoxins in the grain product can vary depending on the exact conditions. However the problem of assessing contamination is compounded by the unequal distribution of aflatoxin within a lot of grain (Groopman and Donahue, 1988). The present guideline for permissible aflatoxin contamination of

agricultural commodities is 15ppb. The presence of aflatoxin in feeds is a health hazard for animals and a potential public health hazard since animals can retain residues of aflatoxins or their metabolites (e.g aflatoxin M₁ found in milk of animals fed with aflatoxin-contaminated feed) in their tissues (Fernandez *et al.*, 1997).

Levels of aflatoxins and zearalenone were determined in 36 samples of stored maize in Brazil (Hennigen and Dick, 1995). Samples were obtained from May to September 1988, 23 samples were from silos and 13 samples were from farms. In 34.8% of samples from silos, aflatoxin B₁ +G₁ was detected at concentrations between 12 and 906 ng/kg. In 13% of samples from silos, aflatoxin B₂ was detected at concentration from 48 to 180 ng/kg. In 23% of samples collected directly from the farms, aflatoxin B₁ +G₁ was detected at concentrations between 6 and 11 ng/kg. Zearalenone was not detected in any of the samples.

Vasanthi *et al.* (1997) conducted a study where aflatoxin intake through the consumption of maize was assessed in 12 households in a rural population in southern India. This study was based on the measurement of aflatoxin in cooked maize and the quantity of consumption of maize. The average aflatoxin intake in nine out of 12 households ranged from 0.33 to 1.5 µg per day. Aflatoxin intakes calculated from aflatoxin levels in maize before cooking tended to be higher by 36% when compared with intakes calculated from cooked maize. This indicates that cooking can reduce aflatoxin levels to a certain extent.

In a study conducted by Taguchi *et al.* (1995), a total of 195 samples of imported foods were analyzed for aflatoxins. Samples, including cereals, were collected in Osaka prefecture (Japan) from 1988 to 1992. Eight samples were contaminated with aflatoxins, however, aflatoxins were not

detected in maize and other products like black pepper, garlic, rice, etc. It does appear from this study that these imported food samples would not pose a serious threat to the consumers as only 4% of the samples were contaminated. However, neither the levels of toxin/s nor the commodities were specified in contaminated samples for one to make an informed judgement.

Lotter and Krohm (1988) purchased maize meal 414 samples from supermarkets in Johannesburg and analyzed them for the presence of aflatoxins in the period from 1985 to 1986. In 1985, 33% of the samples were contaminated with aflatoxins, with no levels in excess of the legal limit (maximum of 10 µg/kg for total toxins and 5 µg/kg for aflatoxin B₁). In 1986 more samples were contaminated with aflatoxins, but the levels of contamination remained low, with only one sample exceeding the legal maximum limit.

Alozie *et al.* (1980) reported that sixteen samples of some Nigerian indigenous beverages and foodstuffs were analysed for their aflatoxin content. All eight samples of beverages that were tested were contaminated with aflatoxin. Of the eight samples of foodstuffs that were tested, all contained aflatoxin except *ewedu*, *dawadawa* and *shoko yokoto*. The names of the five contaminated samples/foodstuffs were not mentioned. The source of contamination in most of these products was ascribed to the use of contaminated grains and contamination by fungal propagules of the finished products. They suggest that products like *dawadawa* have a low pH due to lactic acid fermentation and thus inhibit microbial proliferation; and would be free of aflatoxins if clean maize or sorghum were used consistently.

1.2.2 Fumonisin

Fumonisin (structures shown in Figure 2) are produced by a limited number of morphologically related *Fusarium* species, which frequently infect maize crops around the world (Shepherd *et al.*, 1996). The following points are worth noting:

- Infection of crops with *Fusarium* spp. and its mycotoxins can be symptom-free.
- The mycotoxins that are present in the crop at harvest are not removed by processing of raw material for food production (de Nijs *et al.*, 1998).
- Fumonisin naturally occur in maize and maize-based feeds and foods.

The first conclusive report on the natural occurrence of FB₁ in maize was given by Sydenham *et al.* (1990a). In their study, mouldy home-grown maize collected from an area of the Eastern Cape (South Africa), was analysed for the presence of the fumonisin. Fumonisin B₁ (FB₁) was detected and quantified by HPLC in the sample extract, as were independently prepared derivatives of FB₁. Feeds associated with animal diseases and maize eaten by humans in areas of southern Africa and China with high oesophageal cancer rates may contain more than 100 µg FB₁/g (Scott and Lawrence, 1995).

Fumonisin are found to occur together with other *Fusarium* mycotoxins and less frequently with aflatoxins. In a study by Gonzalez *et al.* (1999), maize samples collected from the main production area in Argentina in 1995 were surveyed for the natural occurrence of *Fusarium* mycotoxins and aflatoxins. Fumonisin B₁, B₂ and B₃ were found in all samples. However, aflatoxins were not detected in the samples. In addition, the presence of mycotoxins in maize products available in Argentina was determined in order to assess dietary exposure of children (Solovey *et al.*, 1999).

None of the 38 samples contained any detectable amount of aflatoxins; however, fumonisin contamination was found in 95% of samples.

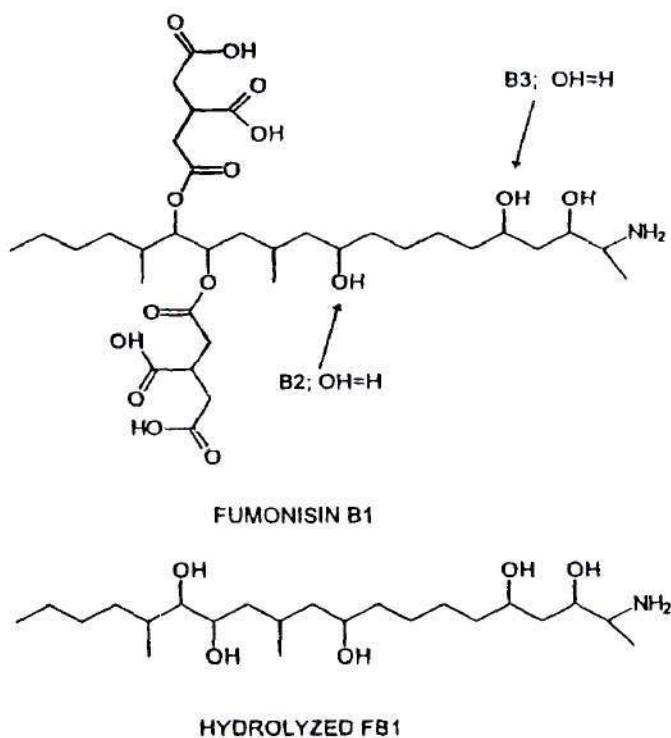


Fig 2. Chemical structures of fumonisin B₁ and fumonisin derivatives (Thakur and Smith, 1996).

Highest fumonisin levels were found in maize meal: FB₁ (range 60-2860 ng/kg), FB₂ (61-1090 ng/kg) and FB₃ (18-1015 ng/kg). Low levels of FB₁ were found in cornflakes samples (2-38 ng/kg). Fumonisin levels were greater than 1000 ng/kg in 24% (5/21) of maize meal samples. Although not the staple food in Argentina, maize consumption is very important, especially among children. Calculated at an average rate for all children (including non-consumers), daily intake was estimated at 0.9 ng/kg body weight.

Murphy *et al.* (1993) investigated fumonisin B₁, B₂ and B₃ content of maize from 1988-1991 crop

years and assayed maize screenings from 1989 in Iowa, Wisconsin and Illinois. They reported that FB₁ ranged from 14.9 µg/g to 15.8 µg/g in maize in crop years from 1988 to 1991. Maize screenings had about 10 times higher fumonisin content than intact maize. However, there was no size-related segregation of fumonisin content in maize screenings.

Hopmans and Murphy (1993) subsequently analysed selected maize containing foods from Iowa, USA, for the presence of fumonisins B₁, B₂, and B₃ and aminopentol (AP1). It was found that FB₁ and FB₂ contents ranged from 17-1410 µg/kg and from 0 to 414 µg/kg in these foods, respectively. Fumonisin B₃ was detected in 10 of 13 foods and AP1 was detected in tortilla, chips, and canned yellow maize. The FB₁ content (17µg/kg) is accounted by the fact that in the former investigation the toxin was analysed in maize only whereas in the latter, the samples contained other components in addition to maize. The amount of the toxin will invariably be lower in the final mixture as more of uncontaminated components are added to the maize.

The natural occurrence of fumonisins B₁ and B₂, the incidence of *Fusarium* spp. and the capacity of *Fusarium* isolates to produce fumonisins were investigated in maize-based samples from Spain destined for human and animal consumption by Sanchis *et al.* (1994, 1995). From 50 samples destined for human consumption, eight samples (16%) were contaminated with fumonisins. The levels of contamination were very low, with a mean of 80 ng/g. Of the 50 maize-based samples destined for animal feed, forty-four (88%) were found to be contaminated with fumonisin. However, levels of contamination were found to be very low, with a mean of 400ng/g in the samples. These findings suggest that the maize -base samples from Spain were safe for human and animal consumption. However, maize producers need to guard against fungal proliferation with a possible undesirable increase in fumonisin levels.

In another study, Katta *et al.* (1997) investigated the distribution of *Fusarium* moulds and fumonisins in commercial and experimental dry-milled maize fractions. *Fusarium* counts in the maize fractions were <100 colony-forming units (CFU)/g in flaking grits, <100- 6.4×10^4 CFU/g in bran, <100- 1.6×10^4 CFU/g in germ, and <100- 2.7×10^3 CFU/g in flour. Fumonisin concentrations were 30.1 µg/g in flaking grits, 0.02-1.1 µg/g in flour, 0.1-2.0 µg/g in germ and 1.5-3.2 µg/g in bran. It could be deduced from these results that there is no direct correlation of CFUs to toxin concentration, suggesting that toxin control should be attempted long before harvest time as the fungus may infect maize while it is still growing in the field.

Maize-based food and feed products purchased in Switzerland were analysed for fumonisins B₁ and B₂ by Pittet *et al.* (1992). Forty-four samples (36.7%) of 120 were found to contain FB₁ (55-790 ng/g), with only 15 containing detectable levels of FB₂ (50-160 ng/g). The highest frequency of positive samples and also the highest FB₁ concentrations were found in maize grits samples (61.8%, 790 ng/g), followed by maize-based poultry feed (27.3 %, 480 ng/g). Thirteen maize grits samples (23.6%) were positive for both FB₁ and FB₂, with mean concentrations of 460 and 100 ng/g, respectively. Of the 22 poultry feed samples examined, 6 were positive for FB₁ with an average content of 235 ng/g, while only two contained measurable levels of FB₂.

De Nijs *et al.* (1998) reported the occurrence of FB₁ in a total of 349 samples of maize from 18 countries worldwide and demonstrated the presence of FB₁ in 93% of the samples. The median FB₁ contamination of all the samples was 420 ng/g and the average contamination level was 1359 ng/g. Human intake of FB₁ was estimated based on the maize consumption of all people in the Netherlands in 1992. A probability distribution was derived to allow estimation of the exposure of the population to FB₁ intake in relation to maize intake. This showed that among those in the

group considered to be at risk, i.e., people with gluten intolerance such as people with celiac or Duhring's disease, 37% are estimated to be exposed to an intake of at least 10^5 ng of FB₁ per person per day. Ninety-seven percent of the risk group is exposed to an intake of at least 10^3 ng of FB₁ per person per day.

Ung-Soo *et al.* (1994) investigated the natural occurrence of fumonisin B₁ and B₂ in Korean maize kernels for feed. From the twelve maize kernel samples, FB₁ was detected in 5 samples at levels ranging from 53-1327 ng/g, while FB₂ was found in four samples. One sample showed the highest FB₁ and FB₂ contents as 1327 and 680 ng/g respectively. In the positive samples, the average concentrations of FB₁ and FB₂ were 506 and 288 ng/g, respectively.

Tseng and Liu (1997) conducted an investigation where maize-based human foodstuffs in Taiwan were analysed for fumonisin B₁ and fumonisin B₂ using HPLC. Fifty-two (33.9%) and 32 (20.9%) of 153 samples were found to contain FB₁ (73-2395 ng/g) and FB₂ (120-715 ng/g), respectively. The highest frequency of detection and also the highest FB₁ concentrations were found in sweet corn (50%, 1089 ng/g) and cornflower (50%, 608 ng/g), followed by cornflakes (23.5%, 1281 ng/g). During an analysis of the distribution pattern for the combined fumonisin levels of FB₁ and FB₂, it became apparent that more than 69% of test samples had fumonisin concentrations below 100 ng/g, while 11.1% (17 samples) contained in excess of 600 ng toxins per g of maize/product. This illustrated that commercially available maize-based foodstuffs for human consumption in Taiwan are frequently contaminated with FB₁ and FB₂.

Natural occurrence of FB₁ and FB₂ was investigated by Ueno *et al.* (1993) in maize and maize-based products sampled in Japan, Nepal, and China. As for the maize-based foodstuffs marketed in

Japan, no significant contamination with fumonisins was observed. Among 24 maize kernel samples in Nepal, 12 samples were positive for FB₁ and 7 for FB₂, and averaging 0.6 and 1.6 µg/g, respectively. One sample showed the highest fumonisin contents as 4.6 FB₁µg/g and 5.5 FB₂ µg/g, respectively. In maize samples harvested in Shanghai and Beijing (China), FB₁ and FB₂ were detected in various concentrations. These findings demonstrated that levels of contamination of fumonisins in maize and maize-based products in these Asian countries are generally lower than those found in areas with a high incidence of oesophageal cancer.

Maize and sorghum form the main dietary staple foods in Botswana (Siame *et al.*, 1998). These important food and feed commodities were analysed for the presence of aflatoxins, fumonisin B₁ and zearalenone. Aflatoxins were not detected in the maize samples analysed. Fumonisin B₁ was detected in 36% of the samples (all of which contained maize, as sorghum on its own is unlikely to be contaminated with FB₁ because the *Fusarium* strain occurring in sorghum is a mating type that does not produce FB₁). Maize samples were the most contaminated with FB₁ (85% of the samples) with concentrations ranging from 20 to 1270 ng/kg.

Doko *et al.* (1995), investigated the natural occurrence of FB₁ and FB₂ in 26 maize inbred lines grown in Italy and in two maize inbred lines grown in Croatia, Poland, Portugal, Romania, Benin, and Zambia. Countries could be placed into one of the two groups depending upon the level and incidence of FB₁ in their maize. The first with high contamination included Italy, Portugal, Zambia and Benin, with levels of 100, 100, 100, and 82% in the samples tested, and fumonisin (FB₁+ FB₂) levels up to 2850, 4450, 1710, 3310 ng/g, respectively. The second group, including Croatia, Poland, and Romania, showed very low (FB₁ + FB₂) levels of contamination (370 ng/g average) with 50 % incidence of positive samples.

In another study by Sydenham *et al.* (1990b), mouldy and healthy maize samples were collected from two human oesophageal cancer prevalence areas of the Eastern Cape, South Africa, during 1985, and screened mycologically. The mouldy maize was contaminated with *Fusarium* spp. Both mouldy and healthy maize samples from the two areas were screened for *Fusarium* mycotoxins. The mouldy maize samples were analyzed for the presence of several *Fusarium* mycotoxins, including deoxynivalenol (DON), Zearalenone (ZEA), FB₁, FB₂ and the tricarballic acid (TCA), a compound present in the structures of the fumonisins released when they are hydrolysed. The healthy maize samples were screened for the presence of FB₁ and FB₂. High concentrations of DON, ZEA, FB₁, and FB₂ were recorded in the mouldy samples. In addition, higher levels of both FB₁ and FB₂ were present in the healthy maize samples from the high oesophageal cancer area than in corresponding samples from the low oesophageal cancer area.

According to a study conducted by Shephard *et al.* (1996) commercially available refined maize products are generally contaminated by FB₁ at levels below 1µg/g, whilst individual products in certain countries can reach far higher levels. Homegrown maize intended for human consumption in certain rural areas, where it also constitutes the staple diet, can be contaminated at levels >100 µg/g (Thiel, 1998). Consumption of maize contaminated at these high levels has been associated with a high incidence of oesophageal cancer in these areas (Pineiro *et al.*, 1997).

A study was conducted by Thiel *et al.* (1992) in which the doses of fumonisins, to which humans and animals consuming maize products would be exposed, were compared with those doses known to produce leucoencephalomalacia in horses and hepatocarcinogenesis in rats. The findings of this study concluded that the known naturally occurring levels of fumonisin present a potential threat to human and animal health and realistic tolerance levels need to be set.

Desjardins *et al.* (1994) conducted a study in which strains of *F. moniliforme* from maize collected from four fields in north-east Mexico were tested for fumonisin production in culture. The results indicated that a diverse population of fumonisin-producing strains of *F. moniliforme* predominates and a potential exists for fumonisin contamination of Mexican maize. Meanwhile, fumonisin B₁ (FB₁) was present at levels of 300-366 µg/ml in *F. moniliforme*-infected Indian maize. Such high levels of toxin suggest that FB₁ ingestion via infected maize has become a health problem in India (Chatterjee and Mukerjee, 1994).

Pineiro *et al.* (1997) evaluated FB₁ and FB₂ in Uruguayan maize products and found that the levels for FB₁ varied from 50 ng/g (detection limit) to 6342 ng/g. Values were highest in feed samples (up to 6342 ng/g), unprocessed maize kernel (up to 3688 ng/g) and milled products (up to 427 ng/g). They were the lowest in processed maize kernel (up to 155 ng/g) and snacks (up to 314 ng/g). Fumonisin B₂ was determined in one-fourth of the total samples and detected at trace levels in only one feed sample. Polenta (Italian soft porridge) that contains fumonisins could be of concern because it is consumed in large amounts and are often the main nutrient source in Uruguay.

Patel *et al.* (1997) carried out a survey to determine the levels of fumonisins in a variety of maize-based foods and cereals available in the United Kingdom (UK). The fate of fumonisins during commercial processing of maize was also assessed at each stage of the process. Despite detectable fumonisin contamination in samples of the original maize, fumonisins were not detected in samples of foods containing maize-based thickeners, such as instant semolina pudding and instant custard powder. The highest levels of fumonisins were detected in samples of polenta (Italian stiff porridge) with concentrations ranging from 16 to 2124 ng/g. Overall, fumonisins were detected in

26% of the 291 retail samples analysed.

In Costa Rica, samples of maize grown in three different regions were collected and analysed for the presence of fumonisins (Viquez *et al.*, 1996). Fumonisin B₁ was present in 89% of the samples. Contamination was dependent on geographical region and period of collection. Samples of maize (kernels and /or powder) from north and south Vietnam were analysed for the concentrations of fumonisin B₁ and zearalenone. Zearalenone was not detected in any of the samples. Some of the samples were found contaminated with fumonisin B₁ (Nguyen, 1996).

Sydenham *et al.* (1995) reported that treatment of fumonisin-contaminated ground maize with 0.1 M calcium hydroxide [Ca(OH)₂] over a period of 24 h at room temperature resulted in the transfer of the majority of the FB₁ (mean =74.1%) to the easily separable aqueous fraction. In this fraction it was present predominantly as the aminopentol (AP1) moiety. Following similar treatment of intact maize kernels, only 5% of the original FB₁ concentration was retained in those kernels devoid of their outer pericarp. A possible application of these finding could be to add dilute Ca(OH)₂ to maize, wash with water and acidify it by lactic acid fermentation.

Chelule *et al.* (2001) analysed FB₁ in maize meal and *amahewu* and found that, of the 47 maize samples analysed, 15 were contaminated with FB₁ in the range 0.1-22.2 mg/kg. However, no FB₁ was detected in the 14 *amahewu* samples (non alcoholic lactic acid fermented maize meal). They attributed the absence of FB₁ in this product to lactic acid fermentation, although other factors were also taken into account.

1.2.3 Zearalenone

Zearalenone (structures shown in Figure 3) is an oestrogenic mycotoxin produced by *Fusarium graminearum* and several other species of *Fusarium*. Zearalenone is one of the most frequently reported mycotoxins associated with *Fusarium* contamination of agricultural products (Bennett and Shotwell, 1979). Numerous instances of its natural occurrence in a variety of agricultural products, principally maize and other grains have been reported. The hyperoestrogenic syndrome in pigs consuming ZEA-contaminated feed has been well-documented (Acock *et al.* 1980).

A survey was carried out from 1983 to 1994 to determine the natural occurrence of ZEA and aflatoxins in maize samples from Argentina (Resnik *et al.*, 1996). Among 2271 samples analysed, 1214 (53%) were contaminated with mycotoxins. Aflatoxin B₁ was identified in 445 samples (19.6%), AFB₂ in 92 samples (4.1%) and ZEA in 676 samples (29.8%).

A survey was carried out in the UK to determine the levels of mycotoxins in a range of ethnic foods (Patel *et al.*, 1996). It involved analysis of 121 samples of ethnic foods purchased from specialist shops, for aflatoxins, fumonisins, ZEA and other mycotoxins. Trace levels of aflatoxins, fumonisins and ZEA were detected in samples of cereal and cereal-containing products, such as maize flour, rice, noodles, etc. This suggests that the UK is not experiencing mycotoxin contamination of their cereal. However, study with a sample size needs to be conducted.

The toxigenicity of *Aspergillus flavus* (663 isolates) and *Fusarium* (459 isolates) from Russian crops (including cereals) were studied by L'vova *et al.*, (1993). Analysis of mycotoxins from 1038 grain samples and from 58 sunflower seed samples revealed that AFB₁ and AFG₁ and ZEA were the

main contaminants of the crops. Mycotoxin contamination was related to geographical source of the grain samples; the humid southern regions of eastern Russia were considered to be at particular risk.

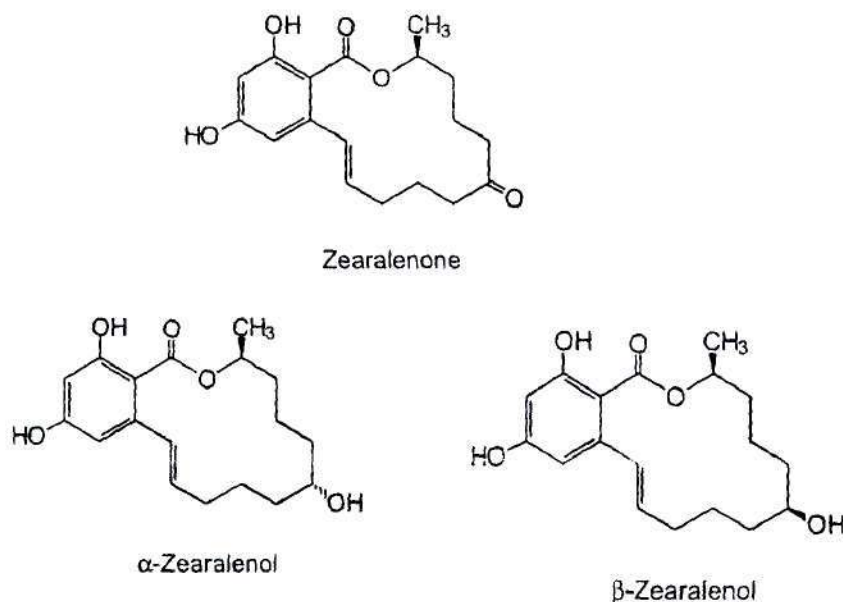


Figure 3. Molecular structures of zearalenone and its metabolites α -zearalenol and β -zearalenol (Krska, 1999).

Maize samples were collected from the Philippines, Thailand and Indonesia and surveyed for the natural occurrence of, among other mycotoxins, aflatoxins, fumonisins, and ZEA (Yamashita *et al.*, 1995). Zearalenone, ranged from 59-923 ng/g. This compares favourably with the study of Ali *et al.* (1998), who collected 16 samples from Indonesia and analysed them for aflatoxins, fumonisins and ZEA using HPLC and GC/MS (Ali *et al.*, 1998). Aflatoxins were detected in 11 (69%) samples at a mean level of 119 ng/g (max 487 ng/g) and fumonisins in all samples at a mean level of 895 ng/g (max 2970 ng/g). Zearalenone was detected in 2 (12%) samples at mean level 11 ng/g

(max 12 ng/g).

A survey was carried out on the occurrence of aflatoxigenic fungi and aflatoxins in staple Ugandan food crops (maize, groundnuts and soybeans) and in poultry feeds (Sebunya and Yourtee, 1990). 54 samples were collected during July and August 1987. Aflatoxigenic fungi were present in most of the samples analysed and in 77% of maize samples. Aflatoxin B₁ was detected at 20 ng/g in only 2 samples (1 of maize and 1 poultry feed). For the first time, ZEA (3 samples) was detected in Ugandan maize. Unfortunately, the authors did not indicate the concentrations of the ZEA detected.

The natural co-occurrence of fumonisins and zearalenone was investigated (Doko *et al.*, 1996) in forty randomly selected cereals and cereal-based commodities collected in 1994 from Botswana, Kenya, Malawi, Mozambique, South Africa, Tanzania, Uganda, Zambia and Zimbabwe. Fumonisin B₁ was detected in 37 samples (92.5%) at concentrations ranging from 20 to 1910 ng/g, while total FB₁, FB₂ and FB₃ concentrations in the same samples ranged from 290 to 2735 ng/g. The highest total fumonisin levels were detected in maize kernels from Zimbabwe (2735 ng/g). In contrast to the high incidence of fumonisins (92.5%), zearalenone was detected in only 5 samples (12.5%) at concentrations ranging from 40 to 400 ng/g.

The occurrence of zearalenone was investigated in raw and fermented cereal products from Swaziland and Lesotho (Martin and Keen, 1978). Zearalenone was detected in sorghum malt and mouldy maize samples from Swaziland and not in those from Lesotho. Twelve of 110 samples were found positive for ZEA, with concentrations ranging from 8 to 53 µg/g. However, no ZEA was found in the food-from-the plate samples. Seven-teen of 140 samples from Lesotho were

contaminated with ZEA concentrations ranging between 0.3 and 2 µg/g.

Lovelace and Nyathi (1977) conducted a study on the concentration of ZEA and aflatoxin in Zambian home-brewed and commercial opaque maize beverages. Zearalenone was present up to 4.6 mg/litre with a mean concentration of 0.92 mg/litre. ZEA was detected in the maize and maize malt used in beer preparation and it was found to dissolve preferentially in the liquid fraction of the beer. Just like in the case of Swaziland samples, the concentration was related to the district of maize growth.

Gimeno (1983) noted that ZEA has been found in maize, corn screenings, wheat, sorghum, barley, oats, sesame seed, hay silage and various mixed feeds at concentrations between 0.1 and 2909 µg/ml. Zearalenone was found at concentrations between 0.1 and 0.5 µg/ml in marketable maize. In addition it was pointed out that ZEA was found in maize from 2.4 to 12.8 µg/ml. These findings suggest that the concentrations and frequency of occurrence of ZEA in maize and other cereals could be fairly high.

1.3 Effect of fermentation on mycotoxins

1.3.1 Incidence of mycotoxins in fermented food or cereals

According to Boeira *et al.* (2000), six particularly well-known mycotoxins are important because of their incidence in agricultural commodities, viz. the aflatoxins, ochratoxin A, patulin, and the *Fusarium* mycotoxins, i.e. fumonisin, deoxynivalenol and zearalenone. Cereals such as barley, wheat, rice and maize are used as raw materials in beer production and other fermented products. When mycotoxin-contaminated grains are used for beer production, they may introduce mycotoxins

into the brewed products. For example, if mycotoxins are present in the barley used for malting or in a cereal adjunct (maize, wheat, rice), or if fumonisin-producing strain of *Fusarium* grows during the germination of barley in the malting process, then the final product is likely to be contaminated with mycotoxins (Scott and Lawrence, 1995).

1.3.2 Effect of fermentation and cooking on aflatoxin levels in maize meal.

In a study conducted by Kpodo *et al.* (1996), levels as high as 289 µg/kg for total aflatoxins was recorded during spontaneous fermentation (by both lactic acid bacteria and yeasts) of maize. In addition, they established that aflatoxins persisted throughout the traditional steeping and fermentation processes. However, cooking of fermented maize dough for three hours resulted in 80% reduction in aflatoxins B₁ and G₁ levels and 35 % reduction in aflatoxins B₂ and G₂. In a related study by Jespersen *et al.* (1994), when a mixed culture of yeast and fungi associated with maize fermentation was used as a starter culture, high levels of aflatoxins were observed in raw maize and these toxins were not affected during fermentation.

According to Moreau (1979), fungi and bacteria are well known for their activity as agents of bioconversions in which aflatoxin B₁ is transformed into a compound 18 times less toxic. The bioconversion products of AFB₁ are shown in Figure 4. Numerous organisms including *Aspergillus* spp, *Penicillium* spp and *Flavobacterium aurantiacum* (strain NRRL B 184) have been shown to be capable of metabolising aflatoxins. In acidified media, e.g., when citric acid has been added to aflatoxin B₁, hydroxy-dihydroaflatoxin B₁ (also known as aflatoxin B_{2a}) was formed.

Another derivative of AFB₁ is aflatoxin R₀ or AFL, which is also less toxic than AFB₁. Aflatoxin

R₀ is formed when the carbonyl group of the cyclopentenone ring in AFB₁ is reduced to a hydroxyl group. This derivative also known as aflatoxicol (Wilson, 1982) is only about 18 times less toxic than AFB₁ in day old ducklings. Since the reaction for the formation of aflatoxicol is reversible, it is possible that aflatoxicol could serve as a temporary *in vivo* reservoir of AFB₁.

Line and Brackett (1995a) investigated several factors affecting removal of AFB₁ by *F. aurantiacum* NRRL B-184. Using the spectrophotometric method, 72-h cultures of *F. aurantiacum* were observed to remove more toxins from solution than 24-h cultures. Likewise, populations of 10¹⁰ cells removed aflatoxin at a faster rate than did 10⁹ cells, although the total amount removed did not differ. It was concluded that high populations of viable cells (1 x 10⁹ CFU/ml) were necessary to effect toxin removal. Aflatoxin removal was not observed in cell populations 1000 times less concentrated (1 x 10⁸ CFU/ml). Heat inactivated cells were also unable to facilitate toxin removal. Since AFB₁ removal appeared to be a process mediated only by living cells, metabolism of the toxin was suspected.

Line and Brackett (1995b) conducted a study to investigate the effects of an added nutrient source and added aflatoxin on the ability of *F. aurantiacum* to degrade aflatoxin B₁. Radioactively labelled AFB₁ was added to test solutions containing cells in a phosphate buffer or tryptic soy broth. Nonlabelled AFB₁ was also added to similar flasks. Analysis of radioactive CO₂ and water- and chloroform-soluble portions of the cell supernatant fluids revealed that neither added nutrients nor added nonlabelled toxin had a significant influence on the microbial transformation of aflatoxin B₁. According to these authors, their results suggest that microbial degradation of AFB₁ by *F. aurantiacum* is probably a mineralization phenomenon (breaking down the substrate to CO₂ and water) and not a co-metabolism.

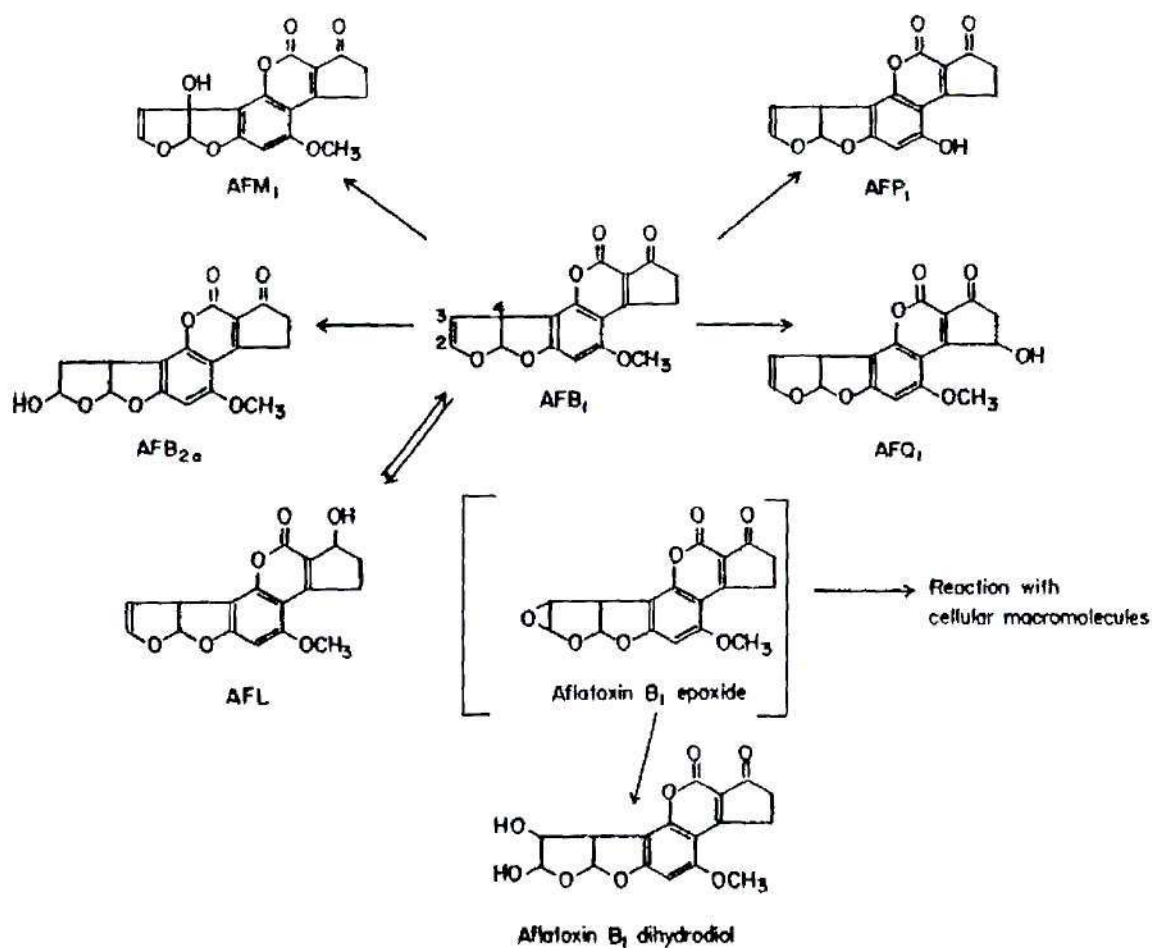


Figure 4. Aflatoxin B₁ metabolic conversion products (Wilson, 1982).

Smiley and Draughon (2000) evaluated the ability of crude protein extracts from *F. aurantiacum* to degrade aflatoxin B₁ (AFB₁) in aqueous solution. Crude protein extracts (800 µg of total protein/ml) reduced 74.5% of AFB₁ in solution. In a separate experiment, an average of 94.5% of AFB₁ was recovered after incubation with heat-treated crude protein extracts (800 µg of total protein/ml). DNase I-treated crude protein extracts reduced 80.5% of AFB₁ in solution, suggesting that removal of aflatoxin by *F. aurantiacum* was not due to non-specific binding with a bacterium's genomic DNA. Proteinase K-treated crude protein extracts degraded 34.5% of AFB₁, providing

evidence that degradation of aflatoxin is linked to a protein. Solution pH affected the amount of AFB₁ reduced by crude protein extracts after 24 h. Maximum degradation was observed at pH 7 (pH range tested: 5- 8), although AFB₁ reduction was observed at pH levels as low as 5 and as high as 8. Acidic pH levels were more detrimental to the ability of the crude protein extracts to degrade AFB₁ than was basic pH. These results indicated that the degradation of AFB₁ by *F. aurantiacum* might be enzymatic.

Fernandez *et al.* (1997) investigated aflatoxin residues and their conversion products in lambs, and the clearance of these toxins after withdrawing the contaminated feed. Twenty-four lambs were intoxicated with aflatoxin at levels of 2.5 mg/kg in their feed for 21 days. Twelve lambs were slaughtered at day 21 and the remaining animals had an 8-day clearance period. Aflatoxins and their metabolites were detected in liver, kidneys, and faeces and urine using TLC and fluorescence densitometry. During the intoxication period, average value for AFB₁ (about 1.94 µg/kg) was higher than AFM₁ (0.35 µg/kg) in the liver. In the kidney AFM₁ (5.45 µg/kg) was higher than AFB₁ (1.29 µg/kg). There were higher concentrations of AFM₁ (27.2 µg/kg, 7.37 µg/kg, respectively) in faeces and urine than AFB₁ (17.25 µg/l, and 1.78 µg/l, respectively). Aflatoxin B_{2a} appeared in the kidney (0.05 µg/kg) and urine (0.35 µg/l). The clearance time of AFB₁ from faeces was 8 days and in all samples aflatoxin residues were still detected on the 8th day of the clearance period although in low quantities. These results suggest that aflatoxin transfer and elimination by the liver and the renal tissue is small and that the danger to humans consuming lamb meat is also small. Thus, examination of faeces and urine could be a useful marker to evaluate exposure of lambs consuming a contaminated diet.

According to Karlovsky (1999), enzymatic inactivation of fungal toxins has been useful for

decontamination of agricultural commodities. To that effect, some mycotoxins like aflatoxins are detoxified during ensiling and other fermentation processes, while others are transformed into toxic products or survive the fermentation unchanged. However, the detoxification of mycotoxins like aflatoxins, fumonisins and zearalenone by pure cultures is still a matter of continued investigations.

1.3.3 Effect of fermentation and processing on fumonisin levels

Fumonisin is stable on boiling in water and mostly survives yeast fermentations of maize and wort and its occurrence in commercial beer appears to be worth investigating (Scott and Lawrence 1995). Fumonisin could be introduced into beer by the use of contaminated maize-based brewing adjuncts. Dupuy *et al.* (1993) observed that FB₁ was not destroyed by the main drying processes of maize or thermal treatments used for producing maize products.

In a study to determine the effect of various added sugars on fumonisin B₁ (FB₁) levels in baked maize muffins and extruded maize grits (Castelo *et al.*, 2001), muffins containing added glucose had significantly lower FB₁ levels than muffins with sucrose, fructose, or no added sugar. Extrusion cooking of the grits resulted in significant ($p < 0.05$) reductions of FB₁ in all treatments relative to untreated controls. The addition of glucose to the maize grits resulted in greater reductions of FB₁ (45.3 to 71%) than did the use of fructose (29.5 to 53%) or sucrose (19.2 to 39%). The reduction in FB₁ levels during thermal treatment in the presence of sugars was suggested to be due to the non-enzymatic browning reaction of a reducing sugar with the amino group present in FB₁ (Murphy *et al.*, 1996; Hopmans *et al.*; 1997).

Lu *et al.* (1997) reported that modifying FB₁ with a reducing sugar such as fructose, eliminated FB₁

hepatocarcinogenicity in rats. N-(carboxymethyl) FB₁ was later characterized and identified as the principal reaction product following the heat treatment of FB₁ with reducing sugars in phosphate buffer at temperatures of 78 and 94 °C (Howard *et al.*, 1998). However, it is not known if sugars react with fumonisin in food, especially at temperatures and conditions used during processing (Castelo *et al.*, 2001).

Consumption of fermented maize pancakes has been associated with a high incidence of stomach cancer mortality rates in rural Linqu County in Shandong Province, China (Groves *et al.*, 1999). This group of workers point out that surveys of fungal contamination of maize in China have detected fumonisins as one of the prevalent mycotoxins. According to this study fumonisins were detected only at low levels (10 µg/g), and did not increase with fermentation. One can deduce from these findings that mycotoxin contamination may not be the sole cause of gastric cancer among those who consume fermented Chinese pancakes.

1.3.4 Effect of fermentation and processing on zearalenone levels

Fusarium mycotoxins deoxynivalenol (DON), nivalenol (NIV) and ZEA, which are present in barley or other grains used for malting, could be introduced into beer (Scott *et al.*, 1997). This is further exacerbated by the fact that cleaning of grain, removing *Fusarium* and mycotoxin infected grain proves ineffective (Patey and Gilbert, 1989).

In a survey by Shim *et al.* (1997) a total of 54 samples of Korean and imported beers was analysed for *Fusarium* mycotoxins. Deoxynivalenol was detected in 14 samples (26%) and NIV was detected in 43 samples (80%). Zearalenone was not detected in any of the samples tested. The

authors could not explain the reasons for the non-detection of ZEA. In another study, Groves *et al.* (1999) detected zearalenone (30.5 µg/g) ZEA in 15% of maize meal specimens. They further established that ZEA levels did not increase during fermentation. Kpodo *et al.* (1996) reported that neither ZEA nor α -zearalenol was found during spontaneous fermentation of maize. This may be due to use of uninfected maize and the fact that non-alcoholic lactic acid fermentation does not promote fungal proliferation and subsequent mycotoxin production.

Boeira *et al.* (2000) carried out an investigation to determine the interactive effect of combinations of the *Fusarium* mycotoxins DON, FB₁ and ZEA on the growth of brewing yeast. When a combination of mycotoxins at low concentration was added into the growth medium, no significant inhibitory effect on growth was observed compared to controls. However, when a combination of high concentrations of DON and ZEA, which individually inhibited yeast growth was examined, the interactive effect was shown to pass from antagonism to synergism depending on the ratio of the toxins used in the mixture.

1.4 Lactic acid fermentation

Lactic acid fermentation has several other beneficial actions other than food preservation. Lactic acid bacteria also produce antibacterial compounds called bacteriocins that inhibit gram-positive pathogens such as *Staphylococcus* that may be implicated in food spoilage (Nout *et al.*, 1996). The growth of lactic acid bacteria, which are facultative anaerobes, also alters the redox potential of food. The negative redox potential they generate slows down the rate of food spoilage by preventing the growth of aerobic spoilage microorganisms. In addition, as a result of fermentation, sugars and other nutrients are removed from food, thus denying them to spoilage organisms. As a

result, some lactic acid fermented foods have excellent shelf stability (Taylor, 1999).

In the production of Beninese *ogi* (a fermented, milled maize gruel containing 90% water and is similar to *amahewu*) (Coffi *et al.*, 1998), fermentation resulted in approximately 40% loss in total protein, but digestibility of the residual proteins increased by 20%. Although 50% of both macro- and micromineral elements were lost, there was an increase in the free ions of iron and sodium. The dominant microflora in Beninese *ogi* is a mixed population of lactic acid bacteria and yeasts. Fermentation rate is more intense at higher temperatures and viscosity increased during week 2 of fermentation (Nago *et al.*, 1998).

Uji is a thin, fermented cereal porridge from maize, sorghum or cassava flours, either singly or in mixtures. Spontaneous fermentation of *uji* resulted in the slowest decrease in pH, while the use of a starter culture led to the lowest final pH (3.5). The viscosity of the product was marginally affected by fermentation. Mainly organic acids were produced by fermentation with the starter culture (Masha *et al.*, 1998).

Sanni *et al.* (1998) explored the potential of sourdough starter organisms for the production of sour maize bread. They found that during fermentation, there was a decrease in the final pH from 4.9 to 3.8 and an increase in the acid equivalent and temperature of the spontaneously-generated (fermentation using indigenous maize microflora) sour maize meal at the end of the 24 h fermentation. There was also an improvement in the shelf life of the sour maize bread relative to the conventional wheat bread. Statistical analysis of sensory attributes revealed a consumer acceptance of sour maize breads, although ranking test showed preference for the baker's yeast leavened bread.

Lactic acid bacteria may exert an antibacterial effect, which is relevant in both product safety and inhibition of pathogens *in situ*. Lactic acid bacteria (isolated from Thai fermented fish products) were found unsuitable for use as live biopreservatives in chilled products but are likely to contribute to the safety and stability of fermented fish products (Ostergaard *et al.*, 1998). However, the pattern of antimicrobial factors was not species specific and the safety and storage stability of fermented maize was suggested to depend on a mixed population of lactic acid bacteria with different types of antimicrobial characteristics (Olsen *et al.*, 1995).

1.5 Inhibition of fungal growth and mycotoxin production

Thus, interaction between mould contaminants and lactic acid bacteria involved in the fermentation process may offer a possible biocontrol system. For instance, mould growth and aflatoxin production in foods and feeds is influenced by various factors, such as moulds species, substrates, temperature, pH, moisture content, atmosphere, and competing microflora. Aflatoxin-producing moulds are often present in an environment with other microorganisms, which can influence aflatoxin production. *Lactobacillus* spp have been reported to have antimycotic and antiaflatoxigenic effects (Gourama and Bullerman, 1995). In another study, Gourama and Bullerman (1997) also reported that a mixture of *Lactobacillus* species growing in a dialysis sack inhibited aflatoxin production by the *A. flavus* culture growing outside of the sack in broth without affecting mould growth.

1.6 Summary

Cereals are prone to fungal infection if stored under inadequate storage conditions. High temperature and humidity support fungal growth. The fungi can also produce mycotoxins as their secondary metabolites under these conditions. In the case of fumonisins, the fungus can start infection while the maize crops are still growing in the field and produce the toxin. If the infected maize kernels are not removed, fumonisin enter the final product. Contamination by aflatoxins is prevalent if maize is kept under poor storage conditions (post-harvest phenomenon).

Fungal infection of cereals is a worldwide phenomenon and can be exacerbated if appropriate agricultural practices are not in place. For instance, commercial farmers use fungicides to control mould infection of the maize crops. Many subsistence farmers do not have resources to procure the chemicals and relevant implements. Contaminated maize in this case is sorted out by hand; which can be problematic because symptoms of fungal infection of maize are not always obvious.

High levels of mycotoxins in cereals have been correlated to incidences of human (oesophageal cancer, liver cancer, etc) and animal mycosis in consumers of contaminated cereals. Therefore, measures must be put in place to control fungal growth in the field, followed by adhering to strict storage conditions that discourage fungal proliferation and mycotoxin production. Due to food shortages to communities remote from the urban centres, poor grade maize is sometimes used for human consumption. This is where strategies to reduce mycotoxin levels in cereal products are of crucial importance. Chemical and physical means of decontamination of maize may yield a product that has lost some of its nutritional and sensory properties. Biological methods (including fermentation) could be more suitable and have been used for centuries to preserve and process

cereal products.

1.7 Aims and objectives of the study

The aim of this study was to investigate the role of traditional non-alcoholic maize fermentations in reducing mycotoxin levels, with specific reference to AFB₁, FB₁ and ZEA, into less toxic products.

The central hypothesis is that natural lactic acid fermentation can detoxify mycotoxins and render maize food products safe for consumption. To test this hypothesis, experiments were carried out with the following objectives:

- Determination of recovery of aflatoxins, fumonisins and zearalenone from spiked maize meal.
- Optimisation of analytical techniques of the three toxins using advanced instrumental techniques.
- Analysis of commercial *amahewu* samples for aflatoxin, fumonisin and zearalenone using analytical techniques.
- Determination of the effect of lactic acid fermentation on toxin levels of spiked maize meal.
- Characterisation of the mycotoxin fermentation products using mass spectrometry.
- Assessment of the cytotoxicity of mycotoxin fermentation products using a cell culture.

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS AND STANDARDS

The *Inyala* maize meal and *amahewu* (Clover SA) samples were purchased from Checkers Supermarkets in Durban. All chemicals and solvents used were of Analar Grade and were purchased from Merck, South Africa. These chemicals were acetic acid, concentrated H₂SO₄, Sodium hydroxide (NaOH), acetonitrile, chloroform, benzene, methanol (MeOH), 1-butanol, p-anisaldehyde, toluene, ethylacetate, formic acid, potassium chloride, sodium tetraborate, o-phthaldialdehyde (OPA), mercaptoethanol, sodium dihydrogen phosphate hydrate (Na H₂PO₄·2H₂O), all from Merck (SA) and nitrogen gas (Afrox, SA).

Thin layer chromatographic plates (Merck aluminium backed silica gel G with fluorescence agent), Bond Elut- LRC Strong Anion-exchange columns (Varian Inc, USA), Sepak columns (Merck), Nova Pack C-18 column and Whatman filter No 1 (Anatech, South Africa), 0.45 µm Millipore filter (Microsep, South Africa), sample-injection vials, BioRad Protein Assay Kit I (SA Scientific). Mycotoxin standards (aflatoxin B₁, fumonisin B₁ and zearalenone) were purchased from Sigma (USA). SNO oesophageal cell culture, phosphate buffered saline (pH 7.2), Essential Medium with Earl's Salt (EMEM), 5% Foetal Calf Serum (FCS) and dimethyl sulphoxide (DMSO) salt solution all from Sterilab (SA).

Lactic acid bacterial mixed culture (LAB), obtained from Clover SA, was used as a starter culture.

Details of all equipment used are mentioned in the relevant sections.

2.2 DETECTION OF MYCOTOXINS BY THIN LAYER CHROMATOGRAPHY

2.2.1 Fumonisin B₁ extraction and thin layer chromatographic analysis

Maize meal (5g), spiked (see section 2.3) with 50 µg FB₁, was extracted with 5 ml of acetonitrile: water (1:1) by soaking for 18 h at room temperature. This was filtered through Whatman no. 1 paper. The filtrate was used to spot TLC plates but was cleaned up before HPLC analysis (section 2.3.2).

Thin layer chromatographic analysis of FB₁ was based on the method of Dupuy *et al.* (1993) except that different volumes of samples and standards were spotted on TLC plate and that the plate was developed in one solvent only. The extract (20 µl) and 20 µl FB₁ standard (50 µg) were spotted separately on the origin of a one-dimensional 20 X 10 cm TLC silica gel G plate (Merck.). Separation was carried out as follows: the plate was developed with 1-butanol: acetic acid: water (20:10:10). After drying, the plate was sprayed with 0.5% p-anisaldehyde (prepared by mixing 70 ml methanol, 10 ml glacial acetic acid, 5 ml of concentrated sulphuric acid and 0.5 ml of p-anisaldehyde), and heated at 110 °C for 10 min and fumonisin B₁ was observed as pink spots.

2.2.2 Aflatoxin B₁ extraction and thin layer chromatographic analysis

Maize meal (50g) spiked (see section 2.3) with 5 ml of 20 µg aflatoxin B₁ was added to 100ml of chloroform and placed on a shaker for 30 min. The suspension was then filtered through Whatman No.4 filter paper. The extract (10 µl) and 10 µl of 50µg AFB₁ standard were spotted on the origin

of pre-coated silica gel fluorescent plates 254 (Merck, SA) and developed with toluene: ethyl acetate: formic acid (6:3:1). The plates were air-dried and aflatoxin B₁ was viewed under UV light at (365 nm) as purple spots.

2.2.3 Zearalenone extraction and thin layer chromatographic analysis

Zearalenone was analysed using the method of Schaafsma (1998). Briefly, about 50 g of maize meal spiked (see section 2.3) with 5 ml of 20 µg zearalenone was added to 100ml of acetonitrile: 4% aqueous solution of potassium chloride (9:1) and placed on a shaker for 30 min. The suspension was then filtered through Whatman No.4 filter paper (Merck, SA). A sample of the extract (10 µl) and 10 µl of ZEA standard were spotted along on the origin of silica gel G with fluorescence indicator 254 TLC plates (Merck, SA). The plates were developed in chloroform: methanol (98:2), allowed to air-dry and sprayed with diazotized dianisidine to increase sensitivity. The plate was observed under short wave (254nm) UV light and ZEA was described as brick red spots

2.3 ANALYSIS OF THE EFFECT OF FERMENTATION ON MYCOTOXINS USING LIQUID CHROMATOGRAPHY

The HPLC system consisted of a spectraSYSTEM P2000 binary pump, a spectraSYSTEM AS3000 autosampler equipped with a 100 µl injection loop and SpectraSYSTEM FL 200 fluorescence detector and /or a SpectraFOCUS UV detector (ThermoSeparation Products, SMM Instruments, South Africa). The analytical column was a Waters NovaPak C18 cartridge (4 µm, 150 x 3.9 mm, Waters, Microsep, SA) preceded by an HIRPB-10c guard column (Hicrom Ltd, SMM Instruments,

SA). The three mycotoxins were all analyzed using the same instrument set at appropriate chromatographic conditions (Sections 2.3.2, 2.3.3, and 2.3.4).

2.3.1 Mycotoxin recovery experiments for fumonisin B₁, aflatoxin B₁, and zearalenone

The recovery of FB₁ was determined by spiking standards (0.5, 1.0, 3.0 µg/g FB₁) into a series of duplicate maize meal samples and analysed by HPLC as described in section 2.3.2. The recovery of AFB₁ was determined by spiking a series of duplicate maize meal with standards (0.25, 0.5, 1.0, 2.0 µg/g AFB₁) and analysed by HPLC as described in 2.3.3. The recovery of ZEA was determined by spiking (0.25, 0.5, 1.0, 2.0 µg/g ZEA) into a series of duplicate maize meal samples as described in section 2.3.4.

2.3.2 Effect of lactic fermentation on fumonisin B₁ on maize meal

Duplicate samples of maize meal (50g) in 50 ml centrifuge tubes were spiked with 20 µg FB₁ dissolved in 2 ml of methanol. After mixing to achieve homogeneity, the methanol was allowed to evaporate overnight in a chemical fume hood. Distilled water (6 ml) was added, followed by 1 ml of LAB culture mixture, to achieve a final concentration of 2000 ng FB₁/ml.

Duplicate controls were set as follows:

- (a) maize meal (3g) spiked with 20 µg FB₁, but no LAB culture was added. Water (7 ml) was added.
- (b) maize meal (3g) not spiked with FB₁ but inoculated with 1 ml of LAB culture and 6 ml water was added.

(c) maize meal (3g) not spiked with FB₁ and not inoculated with LAB culture. Distilled water (7 ml) was added.

Each tube was sealed aseptically with cotton wool and foil, vortexed and allowed to ferment in an incubator set at 25 °C. The pH, fumonisin B₁ concentration and total protein concentration were monitored daily for four days. Fumonisin B₁ was extracted from the spiked samples and controls by adding 25 ml of 75% (v/v) methanol-water to each test tube and shaken for 30 min at 200 rpm in a shaker. The extracts were filtered through Whatman No.1 filter paper. Part of the filtrate (2 ml) was retained for total protein assay. The rest of the filtrate was cleaned-up for FB₁ analysis by HPLC using a SAX cartridge (Thiel *et al.*, 1993).

Fumonisin B₁ was then analysed after the method of Thiel *et al.* (1993). A strong anion exchange (SAX) cartridge was fitted to a Vac-Elut. The cartridge was conditioned by washing with 5 ml methanol, followed by 5 ml methanol: water (3:1); maintaining a flow rate of 2 ml/min throughout. Without allowing the cartridge to run dry, 10 ml of the filtered extract was applied. The cartridge was washed with 8 ml methanol: water (3:1) followed by 3 ml methanol. FB₁ was eluted with 0.5 % (v/v) acetic acid in methanol into a collection vial. The eluate was evaporated to dryness under a stream of dry nitrogen at 60 °C. The dried residue was retained in a refrigerator (4 °C) before analysis.

The dried samples were dissolved in 1 ml acetonitrile water (1:1). A solution of o-phthalaldehyde (OPA) (80 µl) was added to 40 µl of FB₁ standard or sample. The OPA reagent was prepared by adding 600 µl methanol to 15 mg of OPA, followed by 20 µl of mercaptoethanol and 5 ml tetraborate buffer (obtained by dissolving 3.81 g of sodium tetraborate in 100 ml double distilled

water). After mixing for 15 sec the mixture was transferred into a vial insert and 50 μ l were injected into the system.

The mobile phase consisted of methanol: phosphate buffer (76:24). The buffer was obtained by dissolving 2.07 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 150 ml HPLC-grade water and the pH was adjusted to 3.30 using orthophosphoric acid. The buffer was filtered using 0.45 μ m membrane filter (Merck, SA). The flow rate was 1.5 ml/min. The column temperature was maintained at 23 $^\circ\text{C}$. Detector parameters were set as follows: fluorescent detector, excitation wavelength = 335 nm, emission wavelength = 440 nm. Identification and quantification was achieved by comparing retention times and peak areas of standards with those of the samples (analysed using HPLC under identical conditions).

2.3.3 Effect of lactic fermentation on aflatoxin B₁ in maize meal

Duplicate maize meal samples (3g) were spiked with 20 μ g AFB₁ dissolved in 5 ml chloroform and mixed using a sterile glass rod. The chloroform was evaporated in a fume cupboard for 6 hours after which 6 ml of water was added to the dry mixture, followed by 1ml of LAB culture inoculum, to obtain a final concentration of 20 μ g AFB₁/ml. The mixture was vortexed and allowed to ferment in an incubator set at 25 $^\circ\text{C}$.

The design of this experiment was similar to that described in section 2.3.2 with the following exceptions: AFB₁ was extracted after the method of Cepedes and Diaz (1997), leaving out the derivatization step (as a UV detector set at double wavelength obviates the need for derivatization). Maize meal (3g) spiked with AFB₁ was extracted with 10 ml acetonitrile–water (84:16) for 1 hour

by placing on a shaker at 200 rpm. The sample was filtered through Whatman No. 1 filter paper. The total resultant extract (9 ml) was purified with a Sepak (Merck, SA) cleanup column. A sample of the filtrate (2 ml) was kept for total protein assay as described below. Triplicate samples (200 μ l) of the purified extract were filtered and transferred into three vials. Samples were kept in a refrigerator for HPLC analysis after drying them under nitrogen gas.

The dried samples were dissolved in 1ml acetonitrile: water (1:1) and filtered using 0.2 μ m Whatman filter No 1. (Anatech, SA) into 2 ml vials. The sample (300 μ l) was injected into the HPLC system.

Chromatographic conditions were as follows: mobile phase, water-methanol (60:40 v/v). Flow rate was maintained at 1.0 ml/min; UV detector set at double wavelength; 350 nm (focus A) and 360 nm (focus B). Aflatoxin B₁ in each sample was identified and quantified by comparison with retention times and peak areas of standards, which were analysed under the same conditions as the samples.

2.3.4 Effect of lactic fermentation on zearalenone in maize meal

Duplicate maize meal samples (3g) were spiked with 20 μ g zearalenone dissolved in acetonitrile. The acetonitrile was evaporated in a fume cupboard for 6 hours. Water (6 ml) was added followed by 1.0 ml of LAB culture to obtain a final concentration of 200 μ g ZEA/ml.

The extraction procedure was based on that developed by Kpodo *et al.* (1996) except that a different analysis procedure was developed and optimized. Briefly, the sample was extracted with

20 ml chloroform from which the zearalenone was re-extracted into 5 ml of 0.1M sodium hydroxide (NaOH). The solution was acidified with 3-5 drops of 0.1 M solution of citric acid and the mycotoxin extracted into dichloromethane, which was evaporated to dryness. The residue quantitatively transferred into a vial with dichloromethane, which was evaporated off and the extract dissolved in 1.0 ml methanol by vortex mixing. The extract was then filtered through 0.45 µm Millipore filter and the filtrate was dried under nitrogen gas and was kept in a refrigerator. The dried sample was dissolved in 1ml acetonitrile: water (55:45). The solution (700 µl) was transferred into an injection vial and 50 µl of the sample was injected into the HPLC system.

The flow rate was maintained at 1.0 ml/min. The column temperature was maintained at 23 °C. The UV detector was set at 230 nm. For the fluorescent detector, the excitation and emission wavelengths were 236 nm and 418 nm, respectively. Identification and quantification of the zearalenone in each sample was achieved by comparison with retention times and peak areas of standards, which were analysed under conditions identical to the samples.

2.3.5 High performance liquid chromatographic analysis of commercial *amahewu* samples for fumonisin B₁, aflatoxin B₁ and zearalenone in maize meal

Samples (11) of commercial Mahewu No.1 (Clover SA) purchased from local supermarkets were fractionated into three sub samples of 25 ml. The first sub sample was analysed for fumonisins, the second one for aflatoxins and the third one for ZEA. Each sub sample was extracted with 50 ml of the appropriate extraction solvent mixture described in section 2.3.2-4 (i.e. FB₁ in section 2.3.2; AFB₁ in section 2.3.3; and ZEA in section 2.3.4). Sample extraction and analysis for the different mycotoxins was carried out following the methods used for the fermented maize meal samples

spiked with the mycotoxins.

2.4 CHARACTERIZATION OF MYCOTOXINS BIOCONVERSION PRODUCTS USING HPLC, LC-MS AND MYCOTOXIN STANDARDS

2.4.1 Characterization of partially hydrolysed FB₁

The preparation and isolation of partially hydrolysed FB₁ was based on the method described by Sydenham *et al.* (1995). Maize meal samples spiked with FB₁ and fermented over 4 days were screened for partially hydrolysed FB₁ (FB₁ with one or two of the carboxylate groups missing) using LC-MS (Finnigan Mat LCQ, USA), configured for electrospray mass spectrometry.

The dried samples in section 2.3 were dissolved in 1.0 ml of 40% aqueous methanol followed by direct infusion of the filtered solution (approximately 200 ng per component) into the MS at rate of 10 µl/min. The mobile phase was methanol: water (1:1). The mass spectra were obtained using a capillary voltage (34 V).

Fumonsin B₁ and its derivatives were identified by their relative abundance peaks with the following molecular weights:

FB₁ (MW=721.84), therefore the positive ion was set at -722.9;

PHFB₁ (MW =563.70), therefore the positive ion was set at -564.7;

API (MW= 405.61), therefore the positive ion was set at -406.7.

2.4.2 Analysis of aflatoxin B_{2a} in aflatoxin B₁-spiked fermented maize meal

Standard aflatoxin B_{2a} was prepared by transferring 20 µl aliquot of 0.5 µg/ml aflatoxin B₁ in benzene: acetonitrile (98:2) into a 10 ml volumetric flask and evaporating the contents to dryness under a gentle stream of nitrogen gas. Trifluoroacetic acid (100 µl) was added to the residue to completely convert aflatoxin B₁ to aflatoxin B_{2a}. The contents were then diluted in water: acetonitrile (9:1) to volume and mixed well.

Maize meal samples spiked with aflatoxin B₁ and fermented for 4 days were extracted (section 2.3.3) and screened for aflatoxin B_{2a} using an aflatoxin B_{2a} standard and HPLC. The mobile phase used was acetonitrile: water (105:195). The flow rate was set at 1 ml/min. Fluorescent detector was set at excitation wavelength of 330 nm and emission wavelength of 440nm. The UV detector was set at a wavelength of 360 nm. The dried samples were dissolved in 1 ml acetonitrile: water (1:9) filtered using a 0.2 µm Whatman No 1 and injected into the HPLC system.

2.4.3 Analysis of α-zearalenone and β-zearalenone

Samples spiked with maize meal and fermented for 4 days were extracted (section 2.3.4) screened for α-zearalenol and β-zearalenol (Sigma, USA), using standards and an optimized HPLC method. The mobile phase was acetonitrile: water (50:50). The flow rate was set at 1ml/min at ambient temperature. The fluorescence detector was set at excitation wavelength of 236 nm and emission wavelength of 418 nm. The UV detector was set at a wavelength of 230 nm. Identification and quantification of the α- and β-zearalenol in the samples was by comparison with retention times and peak areas of standards, which were analysed under conditions identical to the fermented maize

meal samples.

2.5 CYTOTOXICITY ASSAY USING A HUMAN CELL LINE

Flasks of confluent SNO human oesophageal cell line that were trypsinized and resuspended in a culture medium, i.e. Essential Medium with Earl's Salt (EMEM from Sterilab, Durban) supplemented with 5% Foetal Calf Serum (FCS, from Sterilab, Durban) were adjusted to give a cell number of 2×10^5 cells/ml using a Neubar counting chamber. An aliquot of 100 μ l of the cell suspension was dispensed into each well of a 96-well microtitre plate.

Mycotoxin standard solutions of 0.5 mg/ml in Phosphate Buffered Saline (PBS, Sterilab, Durban) with a pH of 7.2 were diluted serially so as to obtain a range of concentrations from 0.004 to 0.5 mg/ml. A volume of 100 μ l of each dilution was transferred to each well except eight wells, which served as a cell control (to which 100 μ l was added). For each mycotoxin concentration 4 replicates were performed.

The purified toxin extract samples containing the highest concentrations of fumonisin B₁, aflatoxin B₁ and zearalenone were reconstituted in 1 ml PBS (pH 7.2) and 100 μ l was transferred to each well of a 96-well microtitre plate. Four replicates were performed for each toxin tested. Plates were incubated in a moist chamber at 37 °C for 24 h.

Cytotoxicity was assessed using the MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, from Sigma, USA) assay (Hanelt *et al.*, 1994). A volume of 20 μ l of the MTT stock solution was added after 24 h to each well. The plates were incubated for another 24 h.

Supernatants were removed using a multi-channelled micropipette and 100 µl DMSO (Sigma, USA) was added to each well to dissolve the formazan crystals. The optical density of each well was measured spectrophotometrically with an ELISA-reader at an absorbance value of 570 nm and a reference filter of 650 nm.

The percentage cell survival was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of treated wells}}{\text{absorbance of untreated wells}} \times 100.$$

2.6 STATISTICAL ANALYSIS

The data was analysed by means of SPSS version 11 (SPSS Incorporated, USA, 2001).

CHAPTER 3 RESULTS AND DISCUSSION

3.1 QUALITATIVE ANALYSIS OF THE EFFECT OF FERMENTATION ON MYCOTOXINS

3.1.1 Thin layer chromatographic analyses of experimental samples

The TLC technique described (section 2.2.2) led to an identification of FB₁ in the spiked maize extracts. Fumonisin B₁ in the fermented maize meal extracts was identified by comparison of its R_f values with that of FB₁ standard on the same plate. Fumonisin B₁ was visualised as pink spots after spraying with p-anisaldehyde (Figure 5), with an R_f value for FB₁ of 0.79 and this value was higher than 0.65, obtained by Dupuy *et al.* (1993). The spots on the thin layer chromatogram corresponding to the FB₁ derived from the fermented maize samples were smaller in size but the R_f values were reproducible. Thus the TLC method served as a reliable preliminary screening technique for detection of FB₁ in the maize meal extracts prior to quantitation of the toxin by HPLC (Section 2.3.2).

Aflatoxin B₁ in the spiked maize meal sample extracts was identified as purple fluorescent spots (which had the same R_f value as the spot corresponding to the AFB₁ standard on the same plate) on the TLC plate viewed under UV light. The R_f value for AFB₁ was 0.38. Aflatoxin B₁ in the fermented maize meal sample extracts was then quantified using HPLC (Section 2.3.3).

The presence of ZEA in the spiked maize meal samples was detected as blue-grey spots (which had the same R_f as the ZEA standard spot/s on the same plate) when viewed under wavelength of 366

nm. The R_f value for ZEA was 0.60. Zearalenone was detected over the four days of fermentation using TLC as a qualitative technique and ZEA was then quantified in the fermented spiked maize meal samples using HPLC (section 3.2.4).

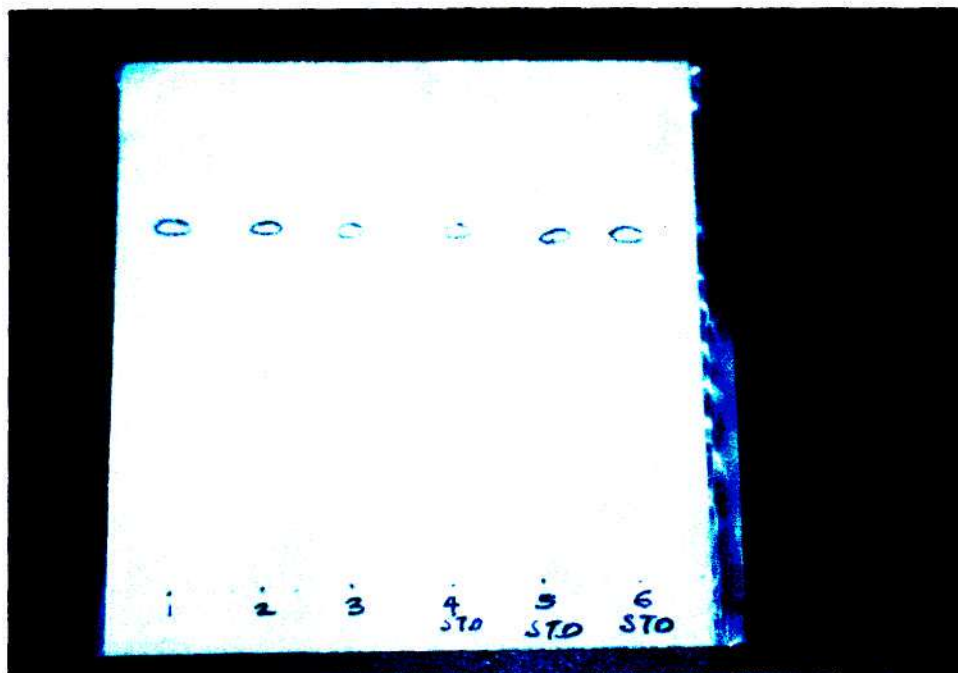


Figure 5. A thin layer chromatographic plate showing the effect of fermentation on fumonisin B_1 over 3 days of fermentation. Origin 5 and 6 ($10 \mu\text{l}$ of $50 \mu\text{g}$ standard fumonisin B_1), 1 (day 1), 2 (day 2), 3 (day 3), 4 (day 4) sample of fermented maize meal spiked with fumonisin B_1 .

3.2 QUANTITATIVE ANALYSIS OF MYCOTOXIN CONCENTRATIONS AFTER FERMENTATION IN MAIZE MEAL

3.2.1 Mycotoxin recoveries from spiked fermented maize meal

When 2.0 µg of each toxin was added to 5g maize meal samples, the recoveries were 80.6, 93 and 88.5% for FB₁, AFB₁ and ZEA, in order (Table 1). In the case of FB₁ the percent recovery of FB₁ is affected by neutral and alkaline pH and tends to improve as the pH is adjusted to 2.7-3.4 (Thiel *et al.*, 1993 and Chelule *et al.*, 2001). When the pH is not adjusted recoveries can be as low as 36% (Chelule *et al.*, 2001). Hence, the recovery of 80.6% obtained in this study was acceptable, considering the difficulty of extracting this toxin from a maize matrix. However, the maize meal was naturally contaminated with 0.018 µg/g FB₁, which is in keeping with reported levels of FB₁ contamination of commercial maize products for human consumption, which are less than 1 µg/g (Shephard *et al.*, 1996). The recoveries for AFB₁ and ZEA were satisfactory (93 and 88.5%, respectively) and the liquid chromatographic system had a high sensitivity for these two mycotoxins.

Table 1. Percentage recoveries of fumonisin B₁, aflatoxin B₁ and zearalenone added to maize meal at 2 µg/g of toxin

OFB ₁ (µg/g)				AFB ₁ (µg/g)				ZEA (µg/g)			
Added	Total	Net ^a	% Rec	Added	Total	Net ^a	% Rec	Added	Total	Net ^a	% Rec
0.0 (c)	0.018(0.00)	0.00	-	0.0 (c)	0.013	0.00	-	0.0 (c)	0.007	0.00	-
2.0	1.63(0.055)	1.61	80.6	2.0	1.87(0.01)	1.86	93	2.0	1.78(0.01)	1.77	88.5

^a = corrected for control (by subtracting toxin concentration found in unspiked samples from the toxin concentration in samples with added toxin)

(c) = controls (samples in which no toxin was added)

% Rec = % recovery.

x(sd) = mean and standard deviation; where x is a mean of two samples.

3.2.2 High performance chromatographic analyses of fermented meal samples spiked with fumonisin B₁, aflatoxin B₁ and zearalenone

Fumonisin B₁

The retention time for both the 1.0 µg FB₁ standard and FB₁ the spiked maize meal samples had a retention time of 8.1 min (Figures 6a and 6c). There was no peak at 8.1 min for the unspiked sample extract, implying absence of FB₁ in this sample extract (Figure 6b). The concentration of FB₁ in the fermented maize decreased from 1.97 to 0.5 µg/ml after 4 days (Table 2); with a %

reduction in FB₁ of 75. It was further observed that there was much higher FB₁ reduction in the presence of the microbial culture (75 %) than in the case of spontaneous fermentation absence (24 %) (Table 2). The results indicate that spontaneous fermentation was not as effective in lowering FB₁ levels than fermentation by added commercial cultures.

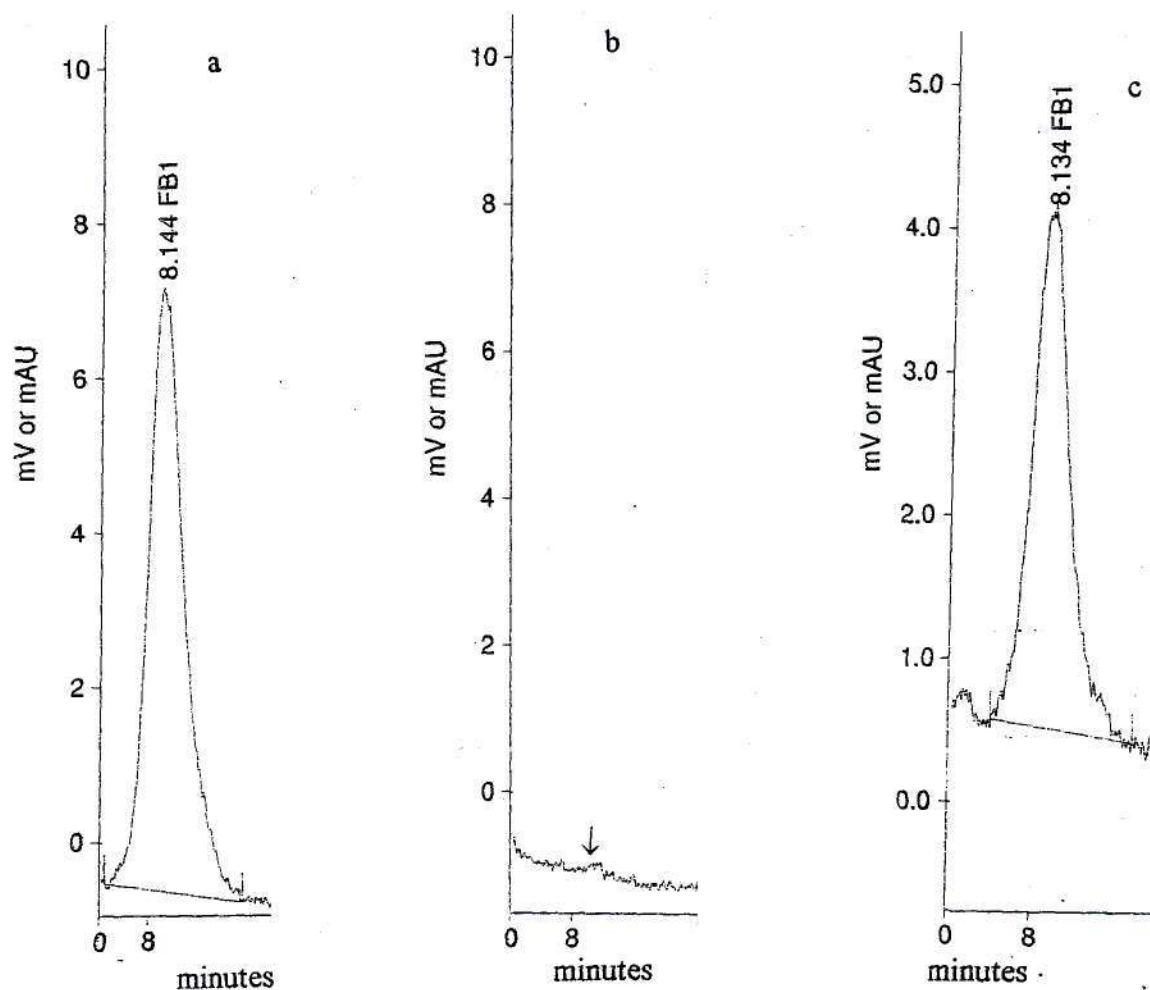


Figure 6. Typical high performance liquid chromatograms of (a) extracts of fumonisin B₁-spiked maize meal sample; (b) unspiked maize meal sample and (c) a 1.0 µg fumonisin B₁ standard. The mobile phase consisted of methanol: phosphate buffer (76:24). The flow rate was 1.5 ml/min. Detector parameters were set as follows: fluorescent detector, excitation wavelength = 335 nm, emission wavelength = 440 nm. The detection limit was 100 ng/ml. → = where FB₁ would have eluted.

It should be noted that the decrease in the amount of the toxin recovered does not necessarily mean it is metabolised by the lactic acid bacteria. It is possible that during lactic acid fermentation using the commercial cultures there is a reduction in available free FB₁. Therefore, it was crucial that the MS analysis of the fermented maize meal sample extracts be also carried out so as to verify the fate of FB₁ during lactic acid fermentation (Section 2.4.1). However, reductions in amount of FB₁ detected during lactic acid fermentation may not necessarily result in reduced toxicity, as it may not affect the bioavailability of the toxin. Therefore, cytotoxicity test of the sample extract had to complement the results of FB₁ analysis in order to confirm effective detoxification (section 3.6).

The analysis of variance for fumonisin B₁ (Table 3) indicated that treatment, duration, and treatment coupled to duration all had a significant contribution in reduction of FB₁ levels (p= 0.000). However, treatment type (fermentation) played a very significant role (F-factor = 3192.218) compared to duration and treatment coupled to duration (F = 133.328 and 67.914, respectively). Post Hoc Tests revealed that there was a significant difference among treatments 1, 2 and 3 or 4 (p=0.000). There was no difference between treatments 3 and 4 (p= 1.000). This was expected as both treatment 3 and 4 were controls.

Table 2. Effect of maize meal fermentation on fumonisin B₁ concentration

Treatment	Duration (Days)	pH	FB ₁ Average Concentration (µg/ml)	% FB ₁ Reduction
FB ₁ + LAB	0	6.6	1.97 (0.01)	0
	1	6.2	1.69 (0.16)	14.2
	2	5.4	1.61 (0.04)	18.3
	3	5.0	0.87 (0.03)	55.8
	4	5.0	0.50 (0.01)	74.6
FB ₁ + No LAB	0	6.6	1.97 (0.01)	0
	1	6.1	1.93 (0.04)	2.0
	2	6.0	1.71 (0.02)	13.2
	3	5.9	1.57 (0.08)	20.3
	4	5.4	1.49 (0.11)	24.4
No FB ₁ + LAB	0–4	6.6–3.6	ND	ND
No FB ₁ + No LAB	0–4	6.6–4.5	ND	ND

Key: LAB = microbial culture;
 No LAB = spontaneous fermentation;
 x(sd) = mean and standard deviation; and
 ND = not detectable.

Table 3. Analysis of variance for fumonisin B₁ in fermented maize meal

Source	Type III sum of squares	Degree of freedom	F- factor	Significance (p value)
Treatment	36.423	3	3192.218	0.000
Duration	2.028	4	133.328	0.000
Treatment* Duration	3.100	12	67.914	0.000

Aflatoxin B₁

The retention time for AFB₁ standard (Figure 7c) and the sample spiked to 0.1 µg levels (Figure 7a) in the HPLC chromatogram was 2.5 min. A relatively low amount of AFB₁ was detected in the unspiked sample (Figure 7b).

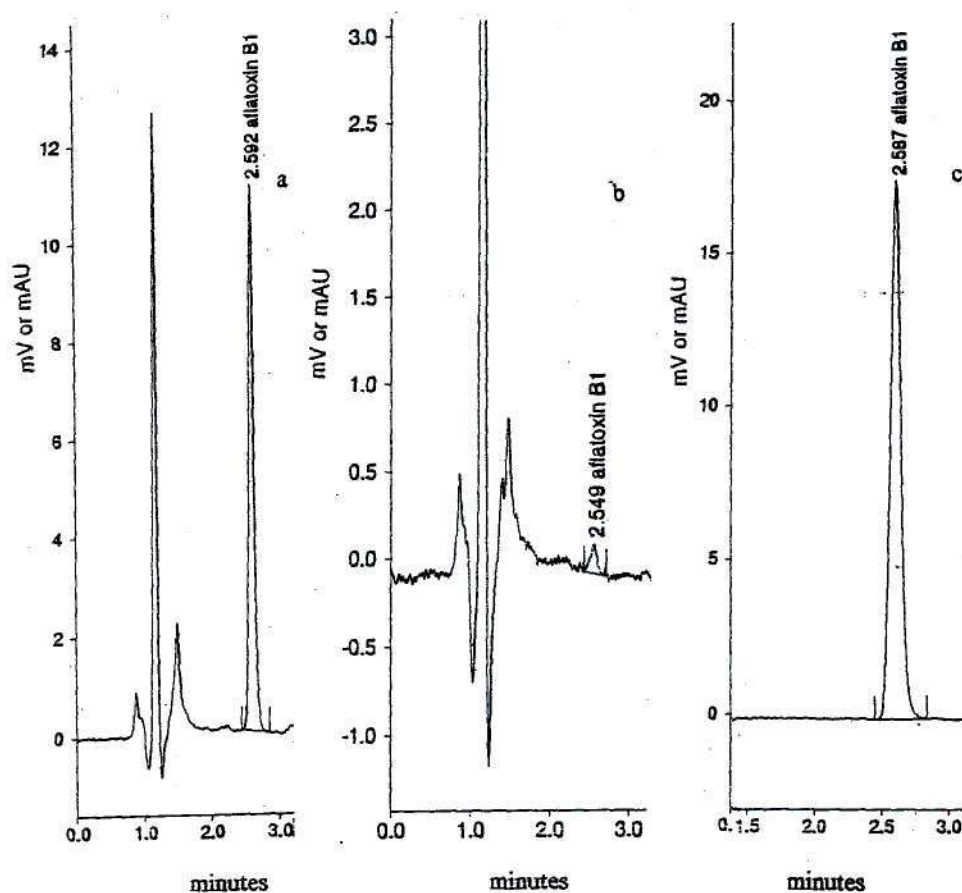


Figure 7. Typical high performance liquid chromatograms of (a) extracts of aflatoxin B₁-spiked maize meal sample, (b) an unspiked maize meal sample and (c) a 0.5 µg aflatoxin B₁/ml standard. The mobile phase was water-methanol (60:40 v/v). Flow rate was maintained at 1.0 ml/min; UV detector set at double wavelength; 350 nm (focus A) and 360 nm (focus B). The detection limit was 10 ng/ml.

By the fourth day of fermentation the AFB₁ levels in the spiked samples had decreased by 78.2%, with a corresponding pH of 3.2 (Table 3). There was a higher % reduction in toxin concentration than in the samples inoculated with the microbial culture (= LAB culture) than in the samples of spontaneously fermenting maize meal. However, about 50% toxin reduction was obtained in the spontaneously fermenting maize meal. In the control samples (natural fermentation where no AFB₁ was added) a pH of 3.1 was obtained. The reduction in the levels of AFB₁ during lactic acid fermentation might not have been due to metabolism of the toxin to CO₂ and H₂O, but AFB₁ may bind to cellular material. There are several possible metabolites of AFB₁, most of which could still be toxic to cells (Wilson 1982). In this study, the samples were screened for AFB_{2a} (Section 2.4.2), as it is one of the toxic products of AFB₁.

The analysis of variance for aflatoxin B₁ (Table 5) indicated that treatment, duration, and treatment coupled to duration all had a significant contribution in reduction of AFB₁ levels (p= 0.000). However, treatment type (fermentation) played a very significant role (F-factor = 904.614) compared to duration and treatment coupled to duration (F = 49.665 and 25.063, respectively). Post Hoc Tests revealed that there was a significant difference among treatments 1, 2 and 3 or 4 (p=0.000). Multiple comparisons of the treatments, there was no mean difference between treatments 3 and 4 (p= 1.000). This was expected as both treatment 3 and 4 were controls that did not have AFB₁.

Table 4. Effect of maize meal fermentation on aflatoxin B₁ concentration

Treatment	Duration (Days)	pH	AFB ₁ Average Concentration (µg/ml)	% AFB ₁ Reduction
AFB ₁ +LAB	0	6.5	59.4(3.3)	0
	1	4.9	36.8(15.8)	38.1
	2	3.7	19.3(9.3)	67.5
	3	3.2	18.4(3.6)	69.0
	4	3.2	15.1(7.0)	75
AFB ₁ + No LAB	0	6.6	57.0 (0.6)	0
	1	6.6	52.2(2.8)	8.4
	2	5.7	48.9(4.0)	14.2
	3	4.5	41.9(4.7)	26.5
	4	4.4	41.3(4.7)	27.5
No AFB ₁ +LAB	0 - 4	6.5 - 3.1	ND	ND
No AFB ₁ + No LAB	0 - 4	6.5 - 3.1	ND	ND

Key: LAB = microbial culture;
 No LAB = spontaneous fermentation;
 x(sd) = mean and standard deviation; and
 ND = not detectable.

Table 5. Analysis of variance for aflatoxin B₁ in fermented maize meal

Source	Type III sum of squares	Degree of freedom	F- factor	Significance (p value)
Aflatoxin B ₁ treatment	25710.592	3	904.614	0.000
Duration	1882.094	4	49.665	0.000
Treatment* Duration	2849.340	12	25.063	0.000

Zearalenone

The retention time for the ZEA standard (Figure 8c) and ZEA extracted from maize sample spiked to 0.2 µg levels (Figure 8a) was 3.02 min. The ZEA peak was not found in the unspiked samples (Figure 8b). The levels of ZEA in the test samples were reduced by 68.3%, with a pH of 4.2 by the fourth day of lactic acid fermentation using the LAB culture (Table 4). The natural fermentation (no LAB culture added) was not effective in lowering ZEA levels. As in the case of FB₁ and AFB₁, the reduction in ZEA levels may not be necessarily due to microbial metabolism. ZEA might bind to certain fermentation products of lactic acid fermentation and as such become more difficult to recover from the fermented maize meal matrix (Haskard *et al.* 2000).

The latter statement is in agreement with the work of Kazanas *et al.* (1984), who noted that aerobic fermentation of grains did not degrade zearalenone but contributed to the formation of a toxic substance of unknown identity. It is now known that α- and β-zearalenone are ZEA derivatives associated with fermentation (Scott *et al.*, 1992). Hence the samples were further screened for these two derivatives of ZEA (Section 2.4.3).

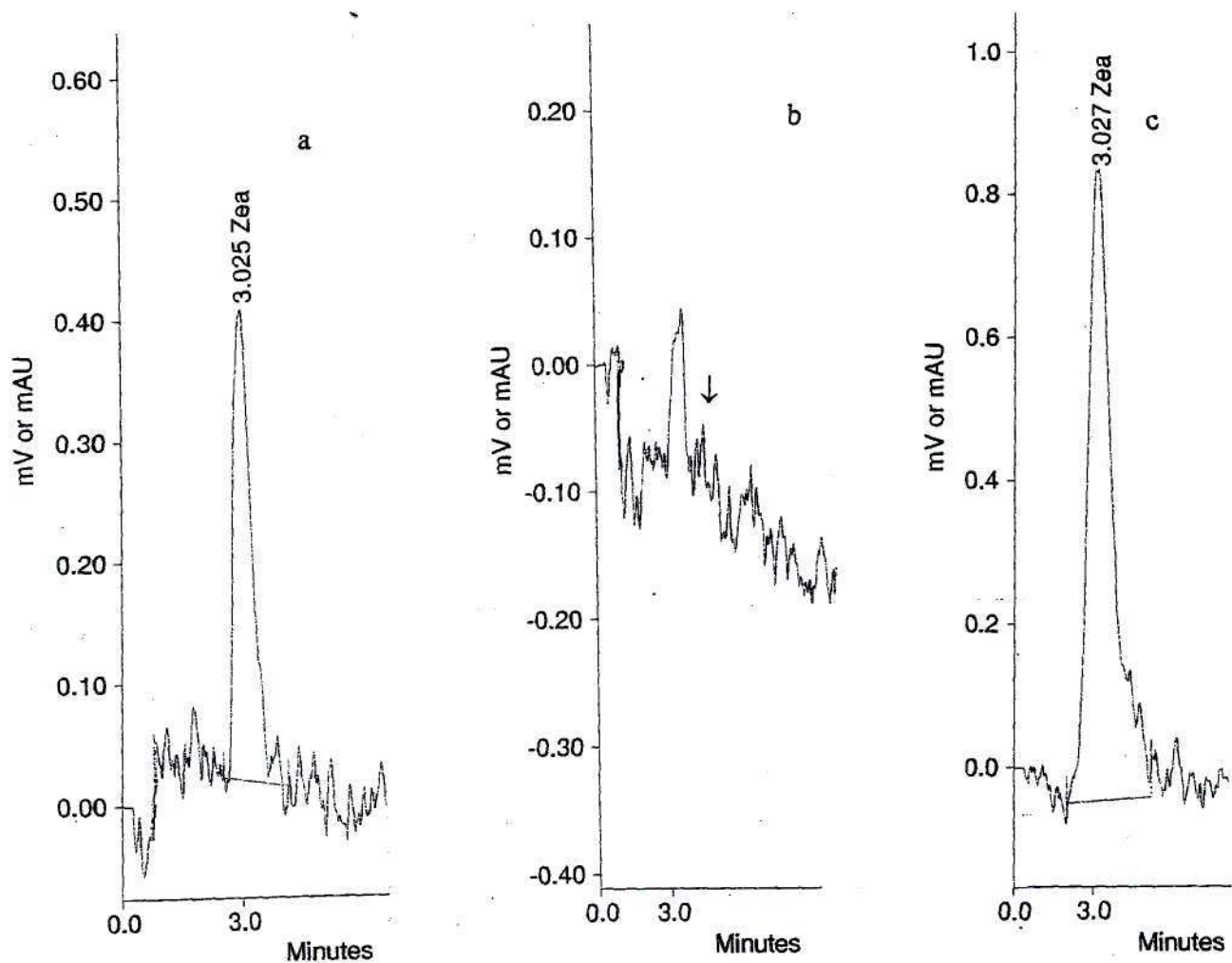


Figure 8. Typical high performance chromatograms of (a) extracts of a zearalenone-spiked maize meal sample, (b) extracts of an unspiked maize meal (\rightarrow = where ZEA would have occurred) and (c) a 1.0 μ g zearalenone standard. The mobile phase was acetonitrile: water (55:45). The flow rate was maintained at 1.0 ml/min. The UV detector was set at 230 nm. For the fluorescent detector, the excitation and emission wavelengths were 236 nm and 418 nm, respectively. The detection limit was 10 ng/ml. The detection limit was 10 ng/ml.

Table 6. Effect of maize meal fermentation on zearalenone concentration

Treatment	Duration (Days)	pH	ZEA Average Concentration (ng/ml)	% ZEA Reduction
ZEA + LAB	0	6.5	184.6(1.9)	0
	1	6.2	124.9(2.9)	32.3
	2	5.6	116.7(3.6)	37.0
	3	4.55	61.3(4.6)	67.0
	4	4.2	58.6(2.1)	68.2
ZEA + No LAB	0	6.5	184.7(1.7)	0
	1	6.5	181.7(2.8)	1.6
	2	5.8	157.0(7.9)	15.0
	3	5.5	134.6(4.1)	27.1
	4	4.3	121.4(3.4)	34.3
No ZEA + LAB	0 - 4	6.8 - 4.3	ND	ND
No ZEA + No LAB	0 - 4	6.8 - 4.8	ND	ND

Key: LAB = microbial culture;
 No LAB = spontaneous fermentation;
 x(sd) = mean and standard deviation; and
 ND = not detectable.

The analysis of variance for zearalenone (Table 7) indicated that treatment, duration, and treatment coupled to duration, all had a significant contribution in reduction of ZEA levels ($p=0.000$). However, treatment type (fermentation) played a very significant role (F-factor = 8236.163) compared to duration and treatment coupled to duration ($F=408.382$ and 172.413 , respectively). Post Hoc Tests revealed that there was a significance decrease in ZEA concentration among treatments 1, 2 and 3 or 4 ($p=0.000$). There was no mean difference between treatment 3 and 4,

and the p value was 1.000.

Table 7. Analysis of variance for zearalenone in fermented maize meal

Source	Type III sum of squares	Degree of freedom	F- factor	Significance (p value)
Treatment	279920.799	3	8236.163	0.000
Duration	18506.117	4	408.382	0.000
ZEA treatment* Duration	23439.069	12	172.413	0.000

The estimated marginal means of outcome indicated that fermentation was most efficient in reducing ZEA levels ($p = 0.000-0.006$) and least effective in reducing FB_1 levels ($p = 0.141-0.481$). It further indicated that the effect of fermentation was optimal on the second day of fermentation, whilst the fourth day did not make a difference in reduction of mycotoxin levels ($p = 0.269$ for FB_1 , 0.438 for AFB_1 and 0.006 for ZEA). This means that duration was not a significant factor for FB_1 and AFB_1 but had a significant role in reducing ZEA levels.

The decrease in pH in all the experiments conducted (with the three mycotoxins) is an indication that fermentation was taking place in samples inoculated with the microbial culture and also in the spontaneously fermented samples. On the basis of these results, the microbial culture was able to grow and ferment maize meal in the presence of mycotoxins; suggesting existence of a mechanism to reduce mycotoxin levels.

3.3 SCREENING OF COMMERCIAL AMAHEWU SAMPLES FOR MYCOTOXINS

The *amahewu* samples from different supermarkets in Durban were found to be free of fumonisin B₁ and zearalenone (Figure 9 and 11); in the case of AFB₁ one sample was contaminated at a level of 150 ng/kg (Figure 10). However the amount of toxin was too low (150 ng/kg) to have a toxic effect on humans consuming this beverage. However in the case of zearalenone there was a compound that could be mistaken for zearalenone around the retention time of 3 min. By using a dual wavelength, it was possible to conclude that the compound was not zearalenone because it had much more fluorescence at wavelength 236 nm (Figure 9) than would be possible with ZEA. These results are in keeping with the study conducted by Chelule *et al.* (2001), who reported that no FB₁ was found in *amahewu* and sorghum, but was present in maize and maize porridge. It is known that the decrease in pH inhibits growth of many microorganisms in products produced by lactic fermentation. Hence, there was a need to characterize mycotoxins bioconversion products in fermented mycotoxin-spiked maize meal (Section 2.4).

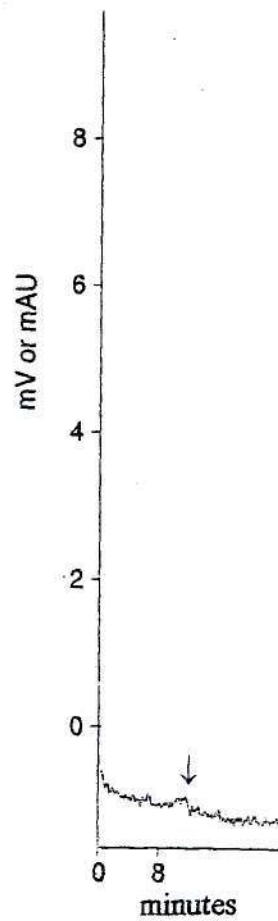


Figure 9. High performance liquid chromatogram of a commercial *amahewu* sample screened for fumonisin B₁. The mobile phase consisted of methanol: phosphate buffer (76:24). The flow rate was 1.5 ml/min. Detector parameters were set as follows: fluorescent detector, excitation wavelength = 335 nm, emission wavelength = 440 nm. → = where FB₁ would have eluted. However, no FB₁ was detected in any of the samples.

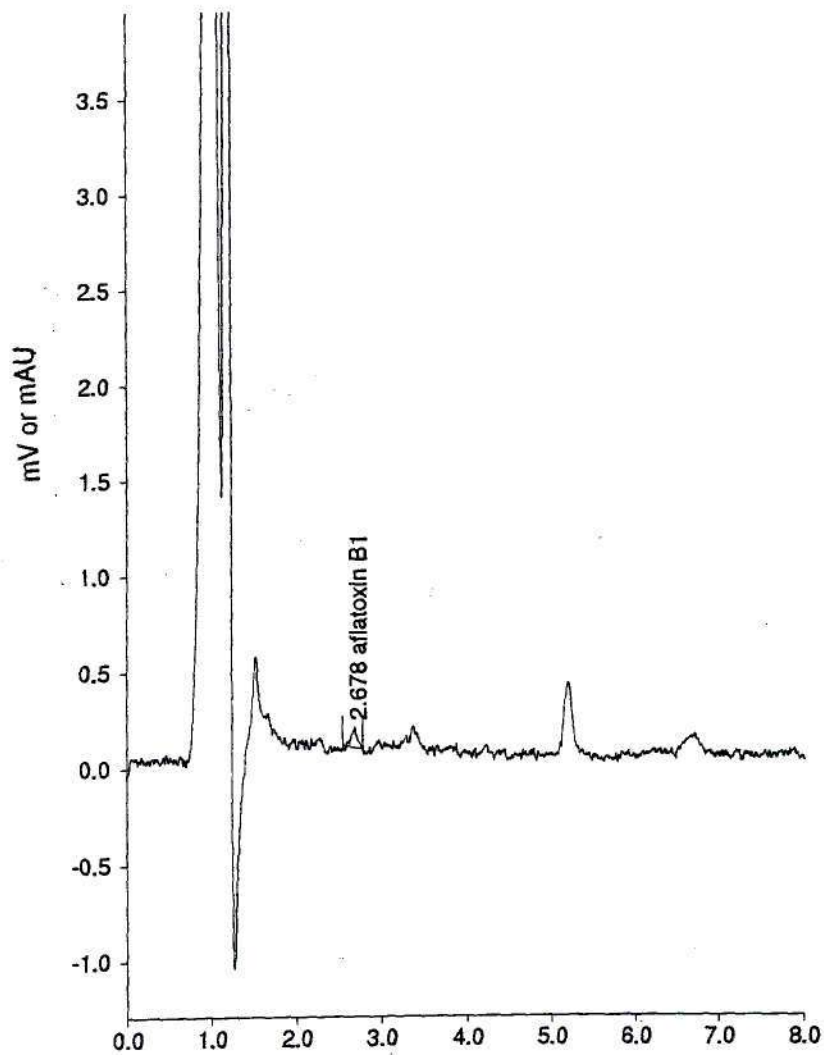


Figure 10. A high performance liquid chromatogram of a commercial *amahewu* sample screened for aflatoxin B₁. The mobile phase was water-methanol (60:40 v/v). Flow rate was maintained at 1.0 ml/min; UV detector set at double wavelength; 350 nm (focus A) and 360 nm (focus B). No AFB₁ was detected in any of these commercial *amahewu* samples.

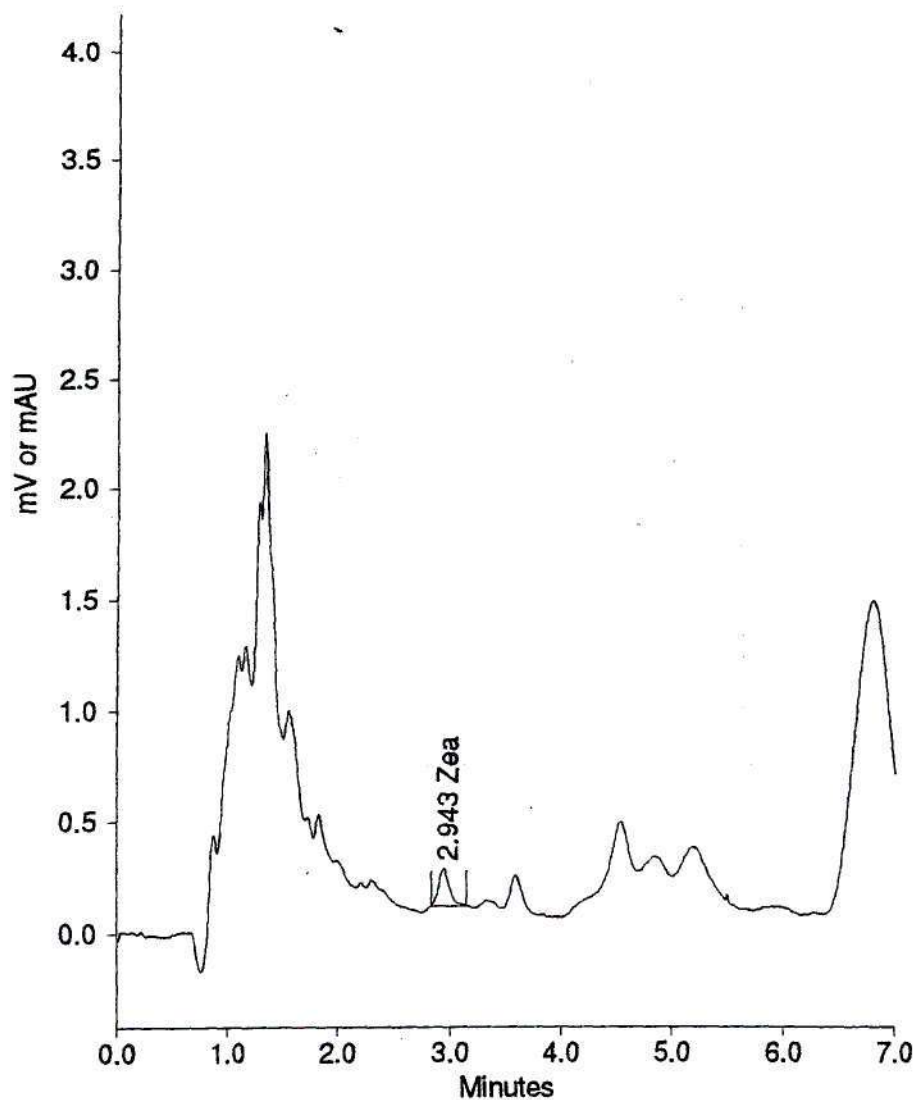


Figure 11. A high performance liquid chromatogram of a commercial *amahewu* sample extract screened for zearalenone. The mobile phase was acetonitrile: water (55:45). The flow rate was maintained at 1.0 ml/min. The UV detector was set at 230 nm. For the fluorescent detector, the excitation and emission wavelengths were 236 nm and 418 nm, respectively. The detection limit was 10 ng/ml. None of the samples had a detectable amount of ZEA.

3.4 ANALYSIS OF MYCOTOXIN PRODUCTS IN FERMENTED MAIZE MEAL

Mass Spectrometric analysis of the FB₁-spiked maize meal indicated FB₁ as the main compound present up to the fourth day of fermentation (Figures 12 and 13). High performance liquid chromatographic results (Table 2) showed that the amount of FB₁ recovered in the fourth day was lower compared with that from the other days. The MS results indicated that although the carboxyl groups (MW = 45) and partially hydrolyzed FB₁ (MW = 577) could be formed to a lesser extent, FB₁ (MW = 723) was found to be still stable in the culture medium, as shown by a high relative abundance in Figures 12 and 13, which are the day 0 and day 4 sample chromatograms, respectively. The aminopentol of FB₁ (MW = 406) was not found in the samples analysed.

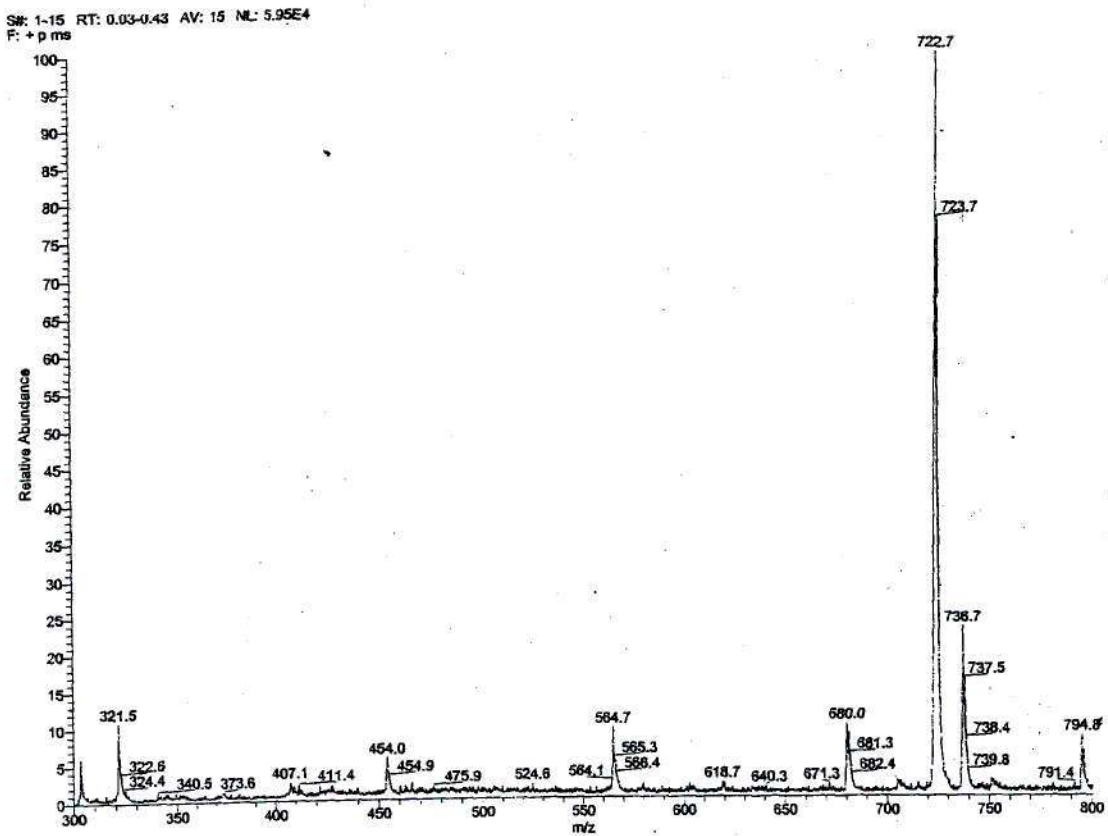


Figure 12. A mass spectrometry of a fumonisin B₁-spiked maize meal sample fermented over 4 days.

S#: 1-15 RT: 0.01-0.47 AV: 15 NL: 2.37E4
F: + p ms

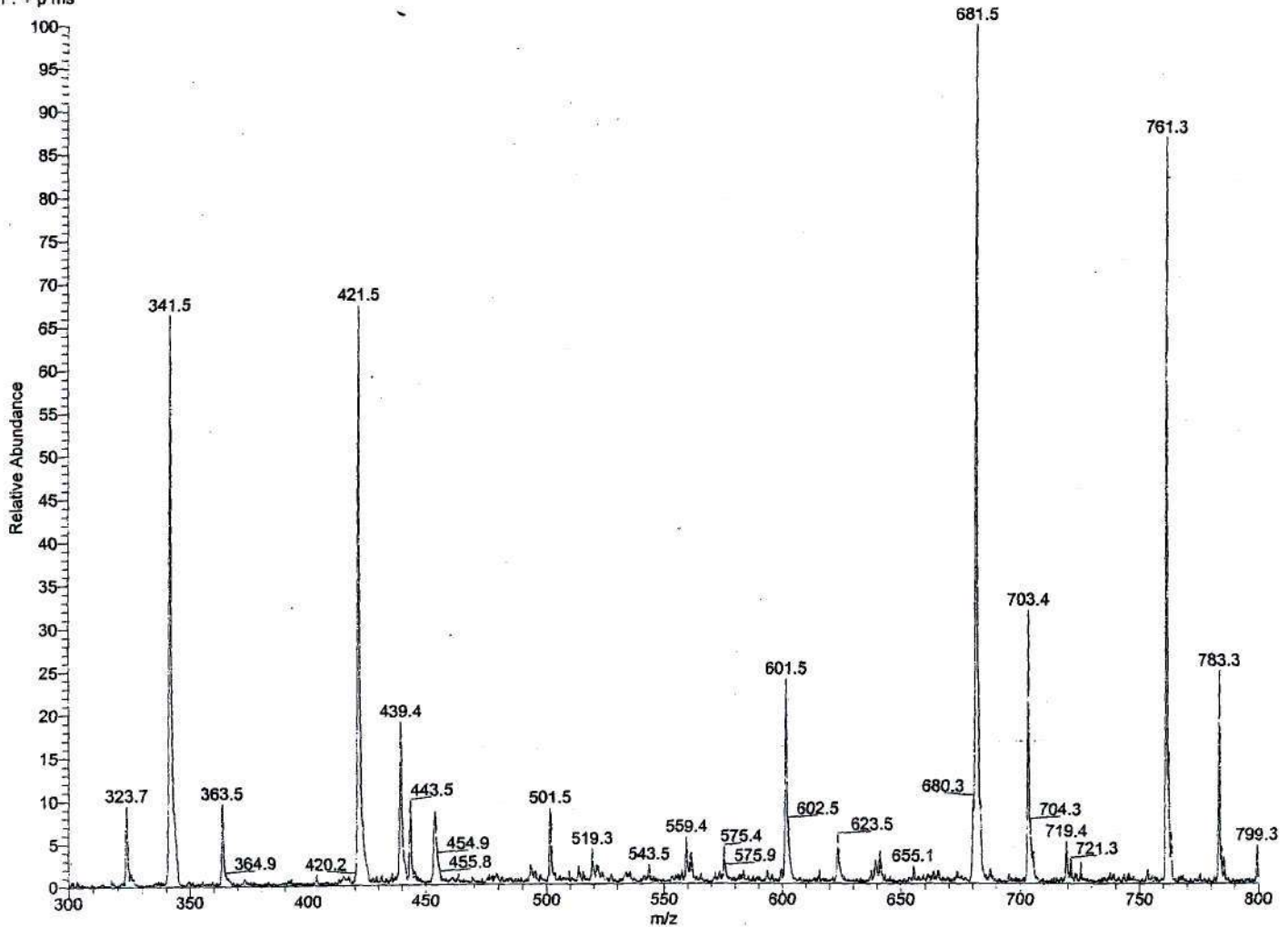


Figure 13. A mass spectrogram of an unspiked maize meal sample fermented over 4 days.

Aflatoxin B_{2a} standard eluted at 2.8 min and the detection limit was 200ng/ml (Figure 14). None of the samples spiked with AFB₁ had a peak at 2.8 min (Figure 15). Aflatoxin B_{2a} was expected to be the main possible product of AFB₁ subjected to lactic acid fermentation, however this does not preclude the presence of other AFB₁ derivatives in the culture medium. The absence of AFB_{2a} in the AFB₁-spiked samples indicates means that AFB₁ was not converted to AFB_{2a} but to other metabolites which can be lesser toxic than AFB₁ and AFB_{2a}. The cytotoxicity test was performed to assess the toxicity of these unidentified AFB₁ metabolites (Section 3.6).

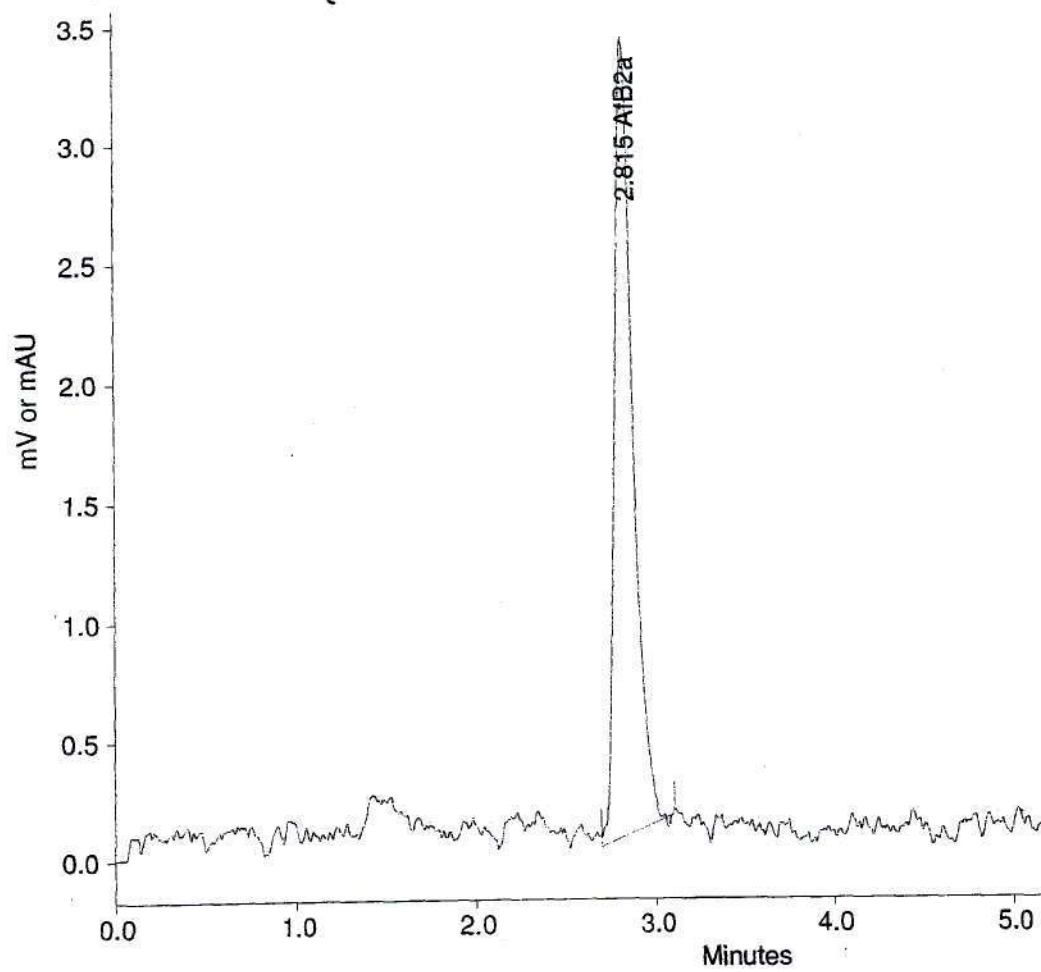


Figure 14. A high performance liquid chromatogram of a 1.0 μg AFB_{2a} standard. The detection limit was 200ng/ml.

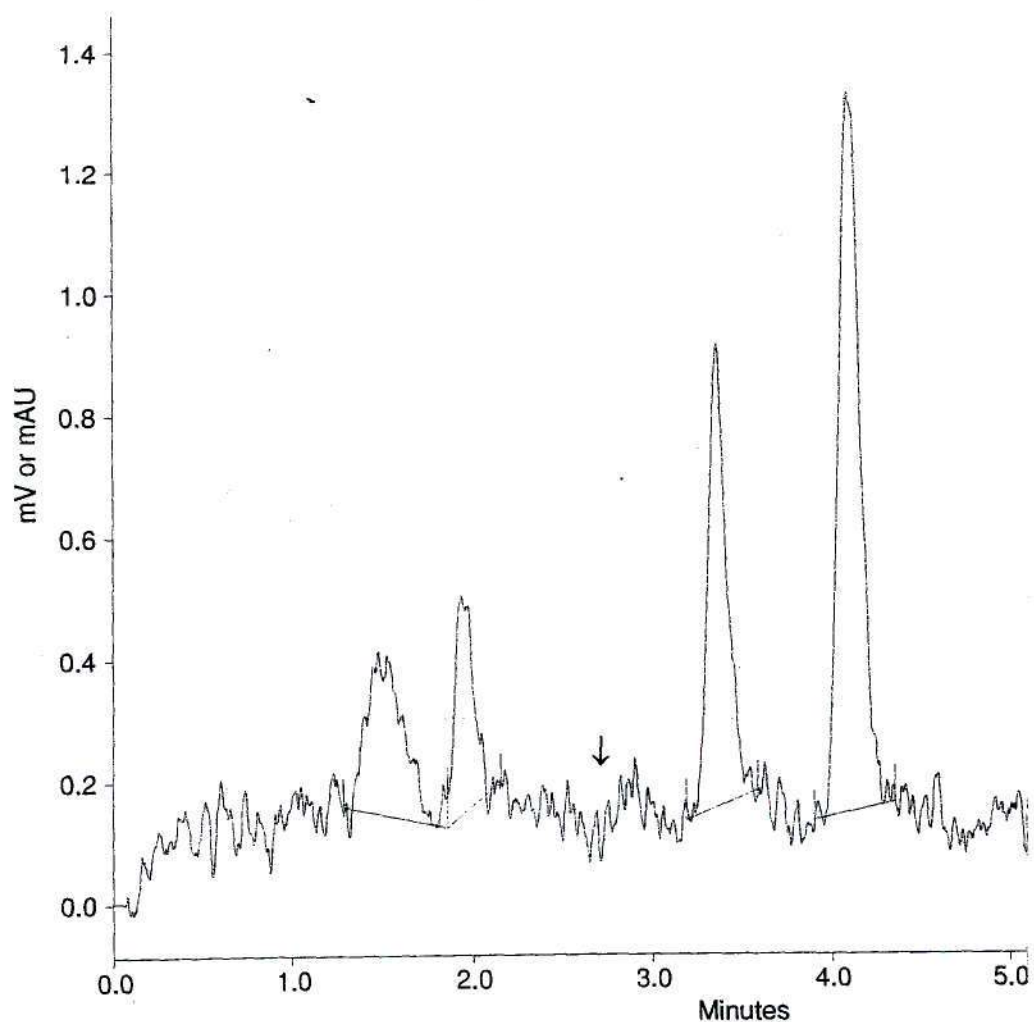


Figure 15. A high performance liquid chromatogram of an aflatoxin B₁-spiked fermented maize meal sample. The detection limit was 10 ng/ml. → = where AFB_{2a} would have occurred. The unidentified peaks represent other compounds associated with the lactate fermentation of maize meal.

The HPLC chromatogram (Figure 18) shows that neither α - nor β -ZEA (Figures 16 and 17) was detected in ZEA-spiked fermented maize meal. This means that these ZEA derivatives are not produced by subjecting ZEA to lactic acid fermentation, but it is known that they can be produced during alcoholic fermentation (Scott and Lawrence, 1988).

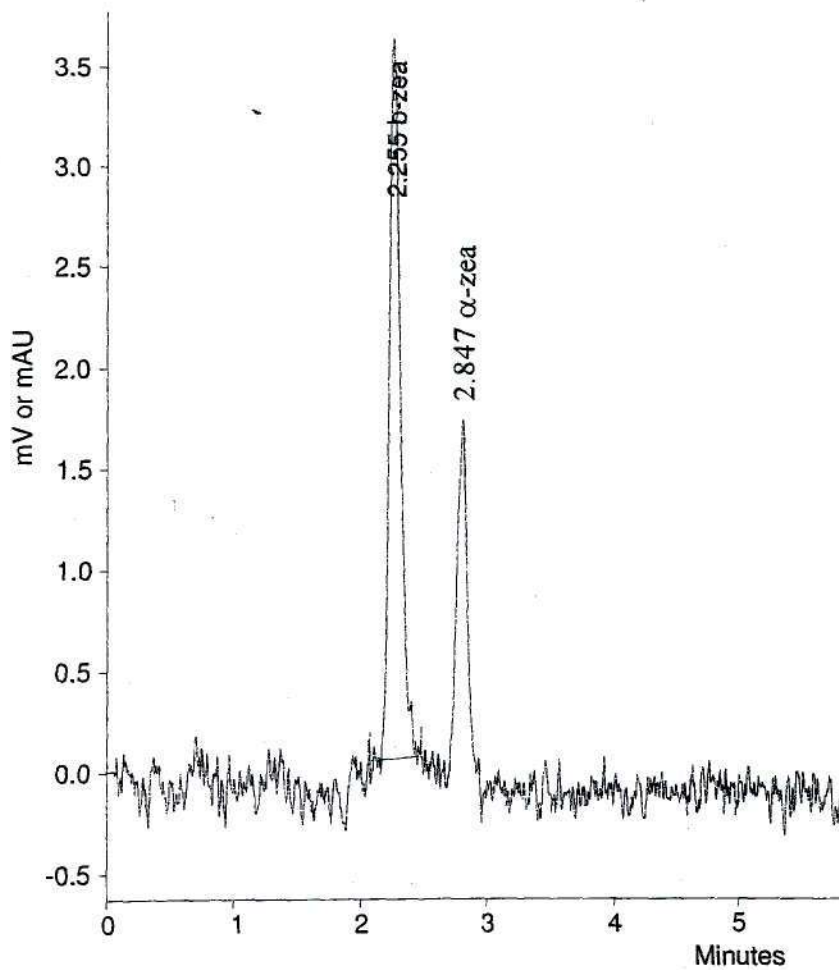


Figure 16. A high performance liquid chromatogram of a mixture of a 1.0 μg α -zearalenol and a 1.0 μg β -zearalenol standard. The detection limit was 100ng/ml.

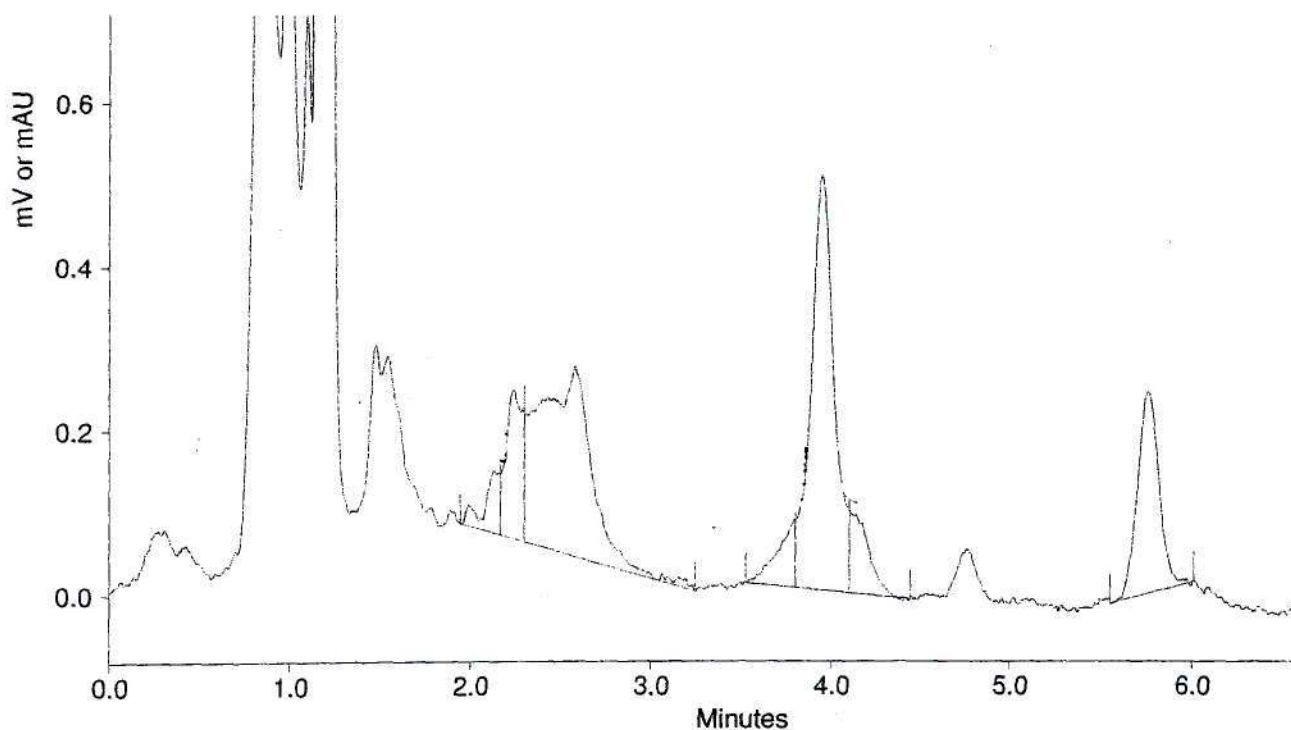


Figure 17. A high performance liquid chromatogram of a zearalenone-spiked fermented maize meal sample. The detection limit was 100ng/ml. None of the peaks matched either α -zearalenol (expected to elute at 2.8 min) or β -zearalenol (expected to elute at 2.3 min).

3.5 CYTOTOXICITY OF EXTRACTS OF FERMENTED MAIZE MEAL SAMPLES SPIKED WITH FUMONISIN B₁, AFLATOXIN B₁ AND ZEARALENONE

There was a steady increase in percent cell viability (23 – 38%) as the dilution of the three different mycotoxin standards increased (Tables 8, 9 and 10) suggesting that there was a dose-response to each toxin used. Maize meal spiked with FB₁ and inoculated with the starter culture (Table 8), was 10% less toxic than the lowest dilution of the FB₁ standard. These samples further had a higher cell viability of (36%) compared to fermented maize meal spiked with FB₁ with no starter culture (cell

viability of 30%). Fermented maize meal without FB₁ and no starter culture resulted in higher cell viability (64%).

Table 8. The effect of maize meal fermentation on the cytotoxicity of fumonisin B₁ (FB₁) to an SNO oesophageal cell culture

Treatment	FB ₁ stds, samples and controls	% Cell viability
FB ₁	50 µg/ml	26 (0.01) ^{a,b,c}
	25 µg/ml	26 (0.01) ^{a,b,c}
	12.5 µg/ml	27 (0.01) ^{a,b,c}
	6.25 µg/ml	32 (0.01) ^{a,b,c}
	3.12 µg/ml	38 (0.01) ^{a,b,c}
4-day fermented test Samples	2 samples: FB ₁ + starter culture	36 (0.03) ^{a,b}
Control sample 1	1 sample: FB ₁ + No starter culture	30. (0.01) ^{a,b}
Control sample 2	1 sample: No FB ₁ + No starter culture	64 (0.01) ^{a,b}
Control wells	Cells + Growth Medium	100 (0.03) ^a

^asignificantly different (p=0.000), ^bnot significantly different (p=0.295), ^cnot significantly different (p=0.772)

Statistical analysis showed that there was a significant difference (p = 0.000) in the toxicity of fermented maize samples, FB₁, and culture medium to the cells. However, there were no significant differences in the toxicity of experimental samples and the controls that were spiked with toxins but no starter culture was added (p = 0.295). There were also no statistically significant differences in the cytotoxicity of the different FB₁ concentrations used (p=0.772).

Maize meal samples spiked with AFB₁ and inoculated with the starter culture (Table 9) were 6% less toxic than the lowest dilution of the AFB₁ standard. These samples had a cell viability of 29% compared to the samples where AFB₁ was added but no starter culture was added (cell viability of 24%). Control samples (not spiked with AFB₁ and no starter culture was added), had a higher percent cell viability (68%), as expected.

Table 9. The effect of maize meal fermentation on the cytotoxicity of aflatoxin B₁ (AFB₁) to an SNO oesophageal cell culture

Treatment	AFB ₁ stds, samples and controls	% Cell viability
AFB ₁	50 µg/ml	23 (0.01) ^{a,b,c}
	25 µg/ml	27 (0.01) ^{a,b,c}
	12.5 µg/ml	27 (0.003) ^{a,b,c}
	6.25 µg/ml	28 (0.003) ^{a,b,c}
	3.12 µg/ml	29 (0.01) ^{a,b,c}
4-day fermented test Samples	2 samples: AFB ₁ + starter culture	29 (0.02) ^{a,b}
Control sample 1	1 sample: AFB ₁ + No starter culture	24 (0.01) ^{a,b}
Control sample 2	1 sample: No AFB ₁ + No starter culture	68 (0.01) ^{a,b}
Control wells	Cells + MTT	100 (0.03) ^a

^asignificantly different (p=0.000), ^bnot significantly different (p=0.681), ^cnot significantly different (p=0.533)

Statistical analysis showed that there was a significant difference (p = 0.000) in the toxicity of fermented maize samples, AFB₁, and culture medium to the cells. However, there were no significant differences in the toxicity of experimental samples and the controls that were spiked with AFB₁ but no starter culture was added (p = 0.681). There were also no statistically significant

differences in the toxicity of the different AFB₁ concentrations used (p=0.533).

Maize meal spiked with ZEA and inoculated with a starter culture (Table 10) were 8% less toxic than the lowest dilution of the ZEA standard. These samples had a higher cell viability (30%) compared to the control samples where ZEA was added but no starter culture added (cell viability was 24%). Fermented maize meal samples not spiked with ZEA and to which no starter culture was added, had a cell viability of 63%.

Table 10. The effect of maize meal fermentation on the cytotoxicity of zearalenone (ZEA) to an SNO oesophageal cell culture

Treatment	ZEA stds, samples and controls	% Cell viability
ZEA	50 µg/ml	24 (0.02) ^{a,b,c}
	25 µg/ml	27 (0.01) ^{a,b,c}
	12.5 µg/ml	29 (0.01) ^{a,b,c}
	6.25 µg/ml	30 (0.01) ^{a,b,c}
	3.12 µg/ml	32 (0.01) ^{a,b,c}
4-day fermented test Samples	2 samples: ZEA + starter culture	30 (0.01) ^{a,b}
Control sample 1	1 sample: ZEA + No starter culture	24 (0.03) ^{a,b}
Control sample 2	1 sample: No ZEA + No starter culture	63 (0.01) ^{a,b}
Control wells	Cells + MTT	100 (0.03) ^a

^asignificantly different (p=0.000), ^bnot significantly different (p=0.395), ^cnot significantly different (p=0.656)

Statistical analysis showed that there was a significant difference (p = 0.000) in the toxicity of fermented maize samples, ZEA, and culture medium to the cells. However, there were no significant differences in the toxicity of experimental samples and the fermented maize meal samples

that were spiked with ZEA without a starter culture ($p = 0.395$). There was also no statistically significant difference in the toxicity of the different ZEA concentrations used ($p = 0.656$).

These results are consistent with those of Castelo *et al.* (2001), who reported that processing of mycotoxins (especially FB₁) does result in significant ($p < 0.05$) reductions in concentration. Murphy *et al.* (1996) suggested that this reduction of mycotoxins during thermal treatment could be due to the reaction of a reducing sugar with amino or other groups present in the mycotoxins. Such modifications of mycotoxins with sugars could eliminate the carcinogenicity of the toxins to experimental animals (Lu *et al.*, 1997).

Kazanas *et al.* (1984) studied the effects of toxicity of fermented and unfermented sorghum meal diets, naturally contaminated with Ochratoxin A and ZEA, on rats and found no significant differences in the toxic effects of both diets in the animals. They found a significant difference ($p < 0.05$) in body weight gain between sorghum-based diets (fermented and unfermented) and a casein based diet. Mean changes in body weight for rats fed fermented and unfermented sorghum meal and casein based diet were -2.8 , $+13.8$, and $+100.5$ g, respectively. That attributed the differences to a possible synergistic effects of the mycotoxins combined with low nutrient quality (particularly protein) of sorghum. This study also established a significant difference in the toxicity to SNO cells, of maize meal (fermented and unfermented) and cells containing growth medium alone. It is possible that the toxicity observed is a combination of trace amounts of toxins in maize meal as well the poor nutritional quality of maize to the cells. Additionally, it is possible that trace amounts of these toxins in fermented and unfermented maize meal could lead to chronic conditions and will continue to be a cause for concern. The results of Kazanas *et al.* (1984) and those of this study strongly suggest that it is advisable not to consume mycotoxin-contaminated meals, even

though fermentation reduces their concentration.

CHAPTER 4 GENERAL CONCLUSION AND RECOMMENDATIONS

This study has shown that:

1. With respect to the three mycotoxins used in this study, lactic acid fermentation resulted in a significant decrease in the concentration of each toxin ($p = 0.000$).
2. Lactic acid bacteria were responsible for the decrease in mycotoxin concentration and pH of the culture medium.
3. The use of starter culture for fermentation doubles the decrease in mycotoxin concentration.
4. The experimental samples were less toxic compared to controls where no starter culture was used in the presence of toxins (5 – 6% difference in cell viability). However, the observed difference in percent cell viability was not statistically significant ($p > 0.05$).
5. The control samples which were never spiked with toxins were twice less toxic than the experimental samples. Therefore, it will serve consumers well to obtain maize that is not contaminated with toxins and store it properly until consumption.

It is therefore recommended that:

1. In areas where there is a high infection rate of maize by mycotoxin-producing fungi, it would be useful to ferment maize and consume it as amahewu or sour porridge rather than making phuthu out of it.
2. *Amahewu* or *incwancwa* containing a starter culture and fermented for a maximum of three days will have a significant reduction in mycotoxin levels and hence promote health of consumers of these products.
3. The mechanism of reduced cytotoxicity by the fermentation broth/medium needs to be evaluated.
4. The nature of how mycotoxins are reduced or bioconverted during fermentation requires investigation.
5. The microbiology of lactic acid bacteria (i.e. which specific bacterial species contribute to the mycotoxin reduction) needs attention.

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