

**FUMONISIN TOXICITY, BIOACCESSIBILITY AND CONTROL OF *FUSARIUM*
SPP. USING ESSENTIAL OILS**

by

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DECLARATION BY CANDIDATE

“I hereby declare that the thesis submitted for the degree of PhD: Science, at Tshwane University of Technology, is my own original work and has not previously been submitted to any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references.”

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Signature:

October 2018

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ABSTRACT

Maize is a major staple food and the largest grain crop in South Africa. The mycotoxin, fumonisin B (FB), a Group 2B carcinogen produced by *Fusarium* spp., is a common contaminant in South African maize. The mode and mechanism whereby FB exerts toxicity is multifaceted and seems to vary in different cells and animal species. A major difficulty in the determination of FB in food is the occurrence of various forms of cryptic or masked FB (including modified and matrix-associated forms). These are not detected by routine analytical methods, which may result in an underestimation of the contamination levels and the associated health risk. The broad aim of the study was to gain a better understanding of the risks associated with fumonisins in South African maize. This was achieved by determining the toxicity of fumonisins, evaluating the interactions of fumonisins with the macrocomponents of maize, and determining their bioaccessibility from maize porridge. In addition, the *in vitro* potential of selected plant essential oils (EOs) as natural fungicides towards *Fusarium* spp., isolated from maize and soil, was investigated.

In this study, the zebrafish embryo test (FET) was used to complement cell-based assays for the determination of the cytotoxicity, teratogenicity and acute toxicity of FB. The reaction between FB and starch, glucose, zein, amino acids and maize meal, respectively, were studied under different pH and temperature processing conditions. To evaluate the risk associated with the consumption of fumonisin-contaminated maize porridge, the bioaccessibility of FB from in stiff maize porridge was determined in the TNO Gastrointestinal Model of the stomach and small intestine (tiny-TIM). Soil and maize samples were collected from the two major maize-producing provinces in South Africa. The soil was characterised by determining the soil texture, moisture content, pH, conductivity and trace element composition. Fungi in the soil and maize samples were isolated, characterised and identified. The potential of lemongrass, spearmint and thyme EOs for the biocontrol of *Fusarium* spp. isolated from soil and maize was determined using an *in vitro* toxic medium assay. Furthermore, the effect of the lemongrass EO on *FUM1* and *FUM21* gene expression and FB production was assessed using two fumonisin-producing *F. verticillioides* strains.

The results demonstrated that FB₁ exhibited a very low cytotoxicity towards the RTL-W1 trout liver cell line when tested without pH adjustment (IC₅₀ 1 260 µg/mL), but no cytotoxicity when the pH was adjusted to physiological conditions. The mycotoxins

FB₁, FB₂, FB₃ and HFB₁ were not genotoxic towards the RTL-W1 cell line. The zebrafish FET also yielded high LC₅₀ values for FB₁ (765 and 277 mg/L after 48 and 96 h exposure, respectively), indicating a low acute toxicity. After adjusting the pH of FB₁ solutions to physiological conditions, the LC₅₀ could only be obtained after 96 hpf using the concentrations tested. The LC₅₀ for pH-adjusted FB₁ (682 mg/L) was 2.5 times higher than the corresponding concentration without pH adjustment (277 mg/L). This indicates that the acute toxicity towards zebrafish embryos was reduced, but not entirely eliminated when adjusting the pH. In the zebrafish FET assay, HFB₁ proved to be less toxic than FB₁. The LC₅₀ values for HFB₁ at 48 and 96 h were 1 090 and 471 mg/L, respectively. The teratogenicity index for FB₁ (with and without pH adjustment) and for HFB₁ (without pH adjustment) were <2, indicating a lack of teratogenicity towards zebrafish embryos. The current study quantified the pH shifts caused by high concentrations of FB₁ and HFB₁ and proved that the pH shifts contribute greatly to their toxicities. It also confirmed that the FET is a useful toxicity screening tool that may account for effects not observable in cell cultures.

None of the processing treatments (pH 4, 7 and 10) or temperatures (30 and 90 °C) resulted in a significant decrease in the recovery of FB₁ from starch, glucose, zein or maize meal spiked with FB. These results confirm the stability of FB₁ in maize porridge and the absence of formation of modified forms of FB. Although the recovery of FB₁ from a mixture of amino acids and from glutamic acid alone was low, the ultra-performance liquid chromatography-quadrupole time-of-flight-high-definition mass spectrometry analysis demonstrated that no modified FB was formed in the presence of glutamic acid at ambient temperature. This highlights the challenges associated with the determination of FB in different food matrices, and the importance of validating the methods applied to each matrix to promote evidence-based decisions regarding the risk of FB to food safety.

Stiff maize porridge was prepared from maize meal naturally contaminated with fumonisins, as well as from fumonisin-free maize meal. To determine the bioaccessibility of the mycotoxins, the porridge was digested using the tiny-TIM *in vitro* dynamic model with parameters simulating human digestion. The results proved that FB₁, FB₂ and FB₃ are released rapidly from stiff maize porridge made from contaminated maize meal. After 360 min, 68%, 94% and 87% of FB₁, FB₂ and FB₃, respectively, were bioaccessible, which confirms that the low bioavailability of FB is not due to strong association of the toxin with the intestinal content. The fact that the fumonisins in

contaminated maize porridge become available rapidly for absorption during digestion, imply that it is likely that the intestine could be exposed to high concentrations of FB after consuming porridge prepared from contaminated maize meal.

A total of 25 *Fusarium* cultures were isolated from soil and maize samples. The soil did not contain any fumonisin-producing fusaria, as only *F. equiseti*, *F. solani* and *F. brachygibbosum* were detected. Soil analysis confirmed that the samples represented a variety of textural classes, and that there were differences in the pH and the trace mineral composition of the soil samples collected from the different locations. Two species of *Fusarium* were isolated from maize, namely *F. oxysporum* and *F. verticillioides*; with *F. verticillioides* being the most prevalent. Lemongrass EO was more efficient than spearmint and thyme in inhibiting the growth of the *Fusarium* spp. *in vitro* when tested in the toxic medium assay. At a concentration of 400 $\mu\text{L/L}$, lemongrass oil was able to prevent the growth of 18 *Fusarium* cultures isolated from maize and soil, as well as that of the reference culture MRC 826. The expression of *FUM1* and *FUM21* genes, which are involved in the biosynthesis of fumonisins, were also prevented at the minimum inhibitory concentration (MIC). Concentrations lower than the MIC (100 $\mu\text{L/L}$ and 200 $\mu\text{L/L}$) resulted in an increase in *FUM1* and *FUM21* gene expression, and after 28 days, the highest fumonisin concentration (28.6 ng/mL) was detected in the MRC 826 culture treated with 200 $\mu\text{L/L}$ lemongrass EO. The results obtained are of concern, since they imply that EO treatments aimed at controlling pathogenic fungi may actually stimulate mycotoxin production in fungi that survive. This could pose health risks for consumers.

This study was the first to use the RTL-W1 trout liver cell line and zebrafish FET to determine FB toxicity and has broadened the existing scientific knowledge concerning the toxic effects of FB. The stability of FB during the preparation of stiff maize porridge was confirmed and there was no evidence of the formation of modified FB under typical conditions used for the preparation of porridge. The current project presented new insights into the kinetics of FB release from porridge during simulated human digestion. The rapid release of FB from cooked porridge, thereby exposing the intestinal mucosa to FB, suggest that consumers using maize as a staple may be at risk. The inhibitory effects of lemongrass EO on *FUM* gene expression demonstrates potential for it to be used as a fungicide above the MIC. However, reported for the first time, is the potential risk of increased FB production by the fungus when exposed to concentrations below the MIC.

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LIST OF ABBREVIATIONS

AFB ₁	Aflatoxin B ₁
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
CLA	Carnation Leaf Agar
CONTAM	Panel on contaminants in the food chain
Cps	Counts per second
C _q	Quantification cycle
C _t	Cycle threshold
CTN	Citrinin
3D	Three-dimensional
DAFF	Department of Agriculture, Forestry and Fisheries
DCA	3,4-Dichloroaniline
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
dpf	Days post fertilisation
D3G	Deoxynivalenol-3-glucoside
EC	European Commission
EC ₅₀	Concentration to cause effects in 50% of the test organisms
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EO	Essential oil
EpH ₅₀	pH at which effects are caused in 50% of fish embryos
EU	European Union
ESI	Electrospray ionization
FAO	Food and Agriculture Organization of the United Nations
FB	Fumonisin B
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
FB ₃	Fumonisin B ₃

FDA	Food and Drug Administration (United States of America)
FET	Fish Embryo Toxicity
GIT	Gastrointestinal tract
Glu	Glutamic acid
HDMS	High definition mass spectrometry
HFB ₁	Hydrolysed Fumonisin B ₁
HFB ₂	Hydrolysed Fumonisin B ₂
HFB ₃	Hydrolysed Fumonisin B ₃
hpf	Hours post fertilisation
HPLC	High performance liquid chromatography
IAC	Immunoaffinity columns
IARC	International Agency for Research on Cancer
IC ₅₀	Concentration to inhibit 50% growth
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
ITS	Internal transcriber spacer
JECFA	Joint Expert Committee on Food Additives
LC ₅₀	Concentration to kill 50% of test organisms
LOD	Limit of detection
LOQ	Limit of quantification
LpH ₅₀	pH at which 50% of fish embryos are killed
LSD	Least Significant Difference
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTL	Maximum tolerated levels
NC	Negative control
OTA	Ochratoxin A
PAT	Patulin
PBS	Phosphate-buffered saline
PC	Positive control
PRC	Polymerase Chain Reaction
PDA	Potato dextrose agar
PHFB ₁	Partially hydrolysed Fumonisin B ₁
PHFB ₂	Partially hydrolysed Fumonisin B ₂
PHFB ₃	Partially hydrolysed Fumonisin B ₃

PMTDI	Provisional Maximum Tolerable Daily Intake
qPCR	Quantitative Polymerase Chain Reaction
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SAGL	Southern African Grain Laboratory
SAX	Strong anion exchange
SDS	Sodium dodecylsulfate
SLE	Solid-liquid extraction
SPE	Solid phase extraction
TCA	Tricarballic acid
TI	Teratogenicity index
TQD	Triple quadrupole detector
TUT	Tshwane University of Technology
QToF	Quadrupole time-of-flight
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
WHO	World Health Organisation
ZEN	Zearalenone

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND AND MOTIVATION

Maize (*Zea mays* L.) is the most widely cultivated cereal with a worldwide production of 1 034 million tons in 2017 (FAO, 2017). It is a major staple food worldwide (Nuss and Tanumihardjo, 2010) and is the largest grain crop in South Africa (Du Plessis, 2003). In the 2016/2017 season, commercial maize production in South Africa reached an all-time high of 16 820 000 tons (SAGL, 2018). This record harvest was more than double that of the 2015/2016 crop, which was affected by severe droughts. Most maize (about two thirds) is consumed by the local market and of that, about 50% is intended for human food, 40% for animal feed and 10% is used for seed and industrial purposes (SA, 2013). According to the Southern African Grain Laboratory's (SAGL) annual crop quality survey, fumonisin mycotoxins are common contaminants of South African maize (SAGL, 2018). This group of structurally-related compounds are produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum* (Beukes *et al.*, 2017).

Fumonisin cause agriculturally important acute toxicity syndromes, including equine leucoencephalomalacia and porcine pulmonary oedema (Shephard, 2005). The International Agency for Research on Cancer (IARC) classified fumonisin B₁ (FB₁) as a Group 2B carcinogen, categorising it as possibly carcinogenic to humans (IARC, 2002). The Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO), as well as the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) recently reassessed the risk associated with fumonisins in food (FAO/WHO, 2017; EFSA, 2018). The current opinion is that, although diets high in fumonisins have been associated with oesophageal cancer, liver cancer, neural tube defects and growth impairment, causality has not been proven. However, several studies confirmed that FB₁ causes toxicity and tumours in the livers and kidneys of rodents following long-time

exposure. There is a need for more research on fumonisin toxicology and in this regard, the zebrafish embryo model may be useful.

Masked or cryptic fumonisins are not detected by conventional analytical methods, but may contribute to the total fumonisin content and associated health risk (Braun and Wink, 2018). This category includes modified fumonisins, which are represented by covalently bound and non-covalently bound fumonisins, referred to as hidden fumonisins, probably resulting from physical entrapment by matrix macroconstituents. Modified fumonisins may be formed in the maize plant by enzymes involved in detoxification. A considerable portion of fumonisins may also be converted to other substances during food processing, as described in detail in the review by Shier *et al.* (2003).

To date, there are limited reports on systematic studies to establish the stabilities of FB₁, FB₂ and FB₃ under various pH and temperature conditions in the presence of maize matrix macrocomponents (e.g. starch/sugars and protein/amino acids). Extensive alterations in the structures of fumonisins are possible, without the complete loss of *in vitro* toxicity (Shier *et al.*, 2003). Although maize porridge is a staple food in southern Africa and is consumed by a broad spectrum of the community (Louw *et al.*, 2010), there is currently not sufficient information available to establish to what extent masked/cryptic fumonisins are formed in maize porridge and whether they are released during digestion in the body. In addition, new strategies should be developed to reduce maize fumonisin contamination. Plant essential oils (EOs) have great potential as natural, eco-friendly fungicides (Dwivedy *et al.*, 2016). Understanding and managing the risks associated with fumonisins in maize will benefit the entire food and animal feed industry.

1.2 PROBLEM STATEMENT

Fumonisin produced by *Fusarium* spp., are common contaminants of South African maize. More research is required into the toxicity of fumonisins, to understand the full extent of the threat that fumonisins pose to food safety and security. Shephard *et al.* (2002) reported that the preparation of traditional South African maize porridge resulted in a 23% reduction of the FB levels. However, a follow-up study by Shephard *et al.* (2012) indicated only a relatively small decrease (11%) in the total amount of fumonisins after cooking. It is not known whether these reductions could be ascribed to thermal decomposition of the FB, or to binding with food matrix components (e.g. starch and protein). Hence, there is a need for further investigation to determine whether the fumonisins in maize porridge are simply masked, rather than detoxified. The toxicological impact of fumonisins does not only depend on their concentrations in food, but also on their bioavailability within the body (De Saeger and Van Egmond, 2012). There is a need to understand the effect of digestion on fumonisins in maize porridge, to determine whether they are released and absorbed in the gastrointestinal tract (GIT).

Sustainable, eco-friendly strategies should be developed to reduce the fumonisin contamination levels in maize. Plant EOs have the potential to control fungal growth and mycotoxin production in maize, but up to now most studies have focussed mainly on mycelial growth inhibition or prevention of spore germination. The effect of EOs on the expression of genes coding for mycotoxin production, especially at concentrations lower than the minimum inhibitory concentration (MIC), is often neglected. It is less problematic if the fungus grows, but does not produce the toxins.

The project was designed to systematically obtain more information on the binding of fumonisins B₁, B₂ and B₃ to maize starch and its predominant monomer (i.e. glucose), and to maize protein (zein) and its predominant monomers (i.e. the amino acids glutamic acid, proline, alanine and leucine). Different temperature and pH conditions were evaluated for their role in fumonisin binding interactions. This was done to gain a better understanding of how fumonisins bind to complex macrocomponents in maize, namely starch and protein. The project also generated data on the *in vitro* bioaccessibility, as well as the *in vivo* toxicity of fumonisins and

was aimed at investigating the potential of plant EOs to control *Fusarium* spp. isolated from maize and soil.

1.3 RESEARCH AIM

The broad aim of the study is to gain a better understanding of the risks associated with fumonisins in South African maize. This was achieved by evaluating the interactions of fumonisins with the macrocomponents of maize, determining their bioaccessibility and toxicity and investigating the potential of selected plant EOs as natural fungicides towards *Fusarium* spp. isolated from maize and soil.

1.4 OBJECTIVES

The following objectives were identified to achieve the aim of the study:

- evaluate the RTL-W1 cell line and the zebrafish embryo as *in vivo* models to assess fumonisin toxicity,
- investigate the interaction of fumonisin B with maize and maize macrocomponents (starch, protein and their respective monomers), and the formation of cryptic fumonisins under different pH and temperature conditions,
- assess the *in vitro* bioaccessibility of fumonisins in South African stiff maize porridge using a dynamic model of the human stomach and small intestine; and
- evaluate the potential of selected EOs for the biocontrol of soil and maize *Fusarium* spp. by testing *in vitro* in a toxic medium and determining the effect of the most efficient EO on the expression of the fungal genes responsible for fumonisin production.

1.5 FORMAT OF THE STUDY

This thesis consists of seven chapters. In Chapter 1, a general introduction, which provides the background and motivation for the project, as well as the research aim and objectives, is provided. A review of the literature relevant to the project and current knowledge on the chemistry, toxicity, bioavailability and control of fumonisins in maize is presented in Chapter 2. In Chapters 3, 4, 5, and 6 the research dealing with fumonisin toxicity, the interaction of fumonisins with maize and its macrocomponents, fumonisin bioaccessibility, and control of *Fusarium* spp. by means of selected EOs, respectively, is outlined and the results are discussed in detail. Chapter 7 is a summative chapter and contains the consolidated conclusions and recommendations for further investigations.

CHAPTER 2

LITERATURE REVIEW

This literature review provides an overview of fumonisins, including the chemical, toxicological and analytical aspects, with a specific focus on South African maize. The disease cycle of *Fusarium* and strategies to control the pathogen and reduce fumonisins in maize are also discussed.

2.1 CHEMICAL STRUCTURES OF FUMONISINS

In 1970, there was an outbreak of equine leukoencephalomalacia in South Africa (Marasas, 2001). The fungus *F. verticillioides* was isolated from the mouldy maize associated with the outbreak and subsequent studies confirmed that the fungus caused the disease. However, the isolation and identification of the specific metabolites responsible for equine leukoencephalomalacia was a great challenge. It took 18 years of intensive research (1970-1988) by a multi-disciplinary team to characterise the chemical properties of fumonisin mycotoxins. The fact that fumonisin molecules lack a suitable chromophore or fluorophore and therefore do not have ultraviolet (UV) or fluorescence absorption contributed to the difficulty with the isolation and identification (Ndube-Tsolekile *et al.*, 2009). Structure elucidation was achieved by mass spectrometry ^1H and ^{13}C nuclear magnetic resonance spectroscopy (Bezuidenhout *et al.*, 1988).

The chemical structures of fumonisins (Figure 2.1) are based on a 20-carbon aminopolyhydroxy-alkyl chain, which is diesterified with propane-1,2,3-tricarboxylic acid (tricarballic acid, TCA) (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988).

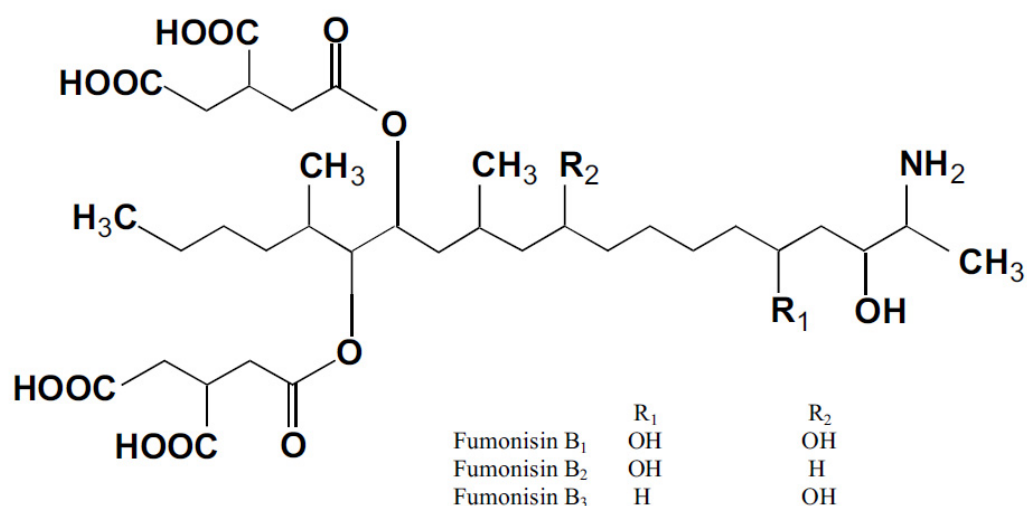


Figure 2.1: Chemical structures of fumonisins B₁, B₂ and B₃ (Shephard, 2005)

Several fumonisin analogues have been characterised and these are grouped into the A, B, C or P-series (Rheeder *et al.*, 2002). Fumonisin B (FB) is the toxicologically important component and also the most abundant naturally occurring fumonisin. For this reason, this study focussed on fumonisins from the B-series and exclude the other analogues. Fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) are structurally related to fumonisin B₁ (FB₁) and differ only with regard to the type and position of side chains attached to the fumonisin backbone (Scott, 2012). They usually co-occur in lower concentrations than FB₁ (FB₁ typically comprises 70-80% of the total fumonisins present) (Rheeder *et al.*, 2002).

Free fumonisins can occur in food products as the parent fumonisin (e.g. FB₁, FB₂ or FB₃), in a partially hydrolysed form (e.g. PHB₁, PHB₂ or PHB₃) or as a totally hydrolysed form (HB₁, HB₂ or HB₃) (Falavigna *et al.*, 2012). The chemical structures of FB₁ and HFB₁ are illustrated in Figure 2.2. In partially hydrolysed forms, one, and in hydrolysed fumonisins, both, of the TCA groups are absent (De Girolamo *et al.*, 2011). Hydrolysis can be facilitated by alkaline treatment or by treatment with the enzyme fumonisin carboxylesterase (Heinl *et al.*, 2010; Grenier *et al.*, 2012).

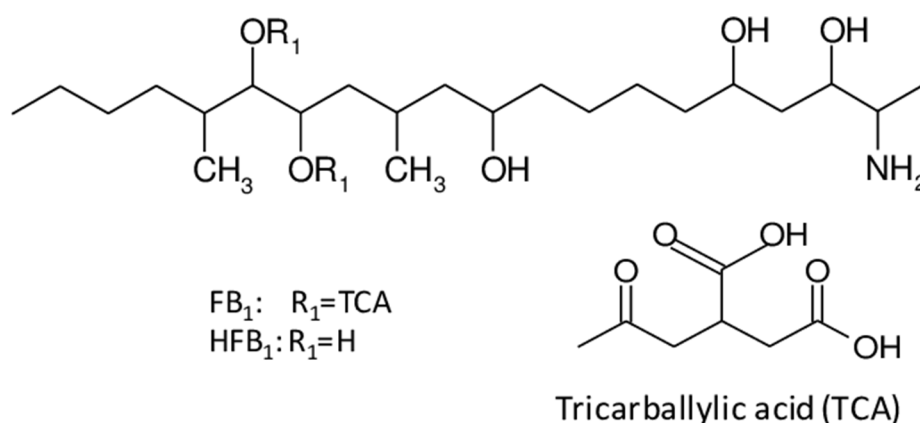


Figure 2.2: Chemical structures of fumonisin B₁ (FB₁) and hydrolyzed fumonisin B₁ (HFB₁) (Mendez-Albores *et al.*, 2014)

All forms of free fumonisins can be detected by conventional analytical methods if the appropriate standards are available (Berthiller *et al.*, 2013).

2.2 TOXICITY OF FUMONISINS

Mycotoxins are secondary fungal metabolites that are toxic to humans and animals (Rheeder *et al.*, 2009; Berthiller *et al.*, 2013). The toxicological effects of mycotoxins on animals are well researched and include the following (Rheeder *et al.*, 2009; Da Rocha *et al.*, 2014):

- carcinogenicity (ability to cause cancer),
- genotoxicity (causing genetic damage),
- teratogenicity (interfering with embryo or foetal growth and development),
- oestrogenicity (duplicating the function of female hormones),
- immunotoxicity (damages the immune system); and
- nephro- and hepatotoxicity (kidney and liver damage, respectively).

Fumonisin are known to exhibit toxicity towards a variety of animal species including horses, pigs, cattle, poultry, fish and laboratory rodents (Voss *et al.*, 2007). Horses seem to be the species most sensitive to fumonisin toxicosis and quite low levels of exposure cause equine leucoencephalomalacia, which targets the brain. In pigs, fumonisins affect the heart and lungs, causing porcine pulmonary oedema.

The liver and kidneys of these animals are also affected. In addition, fumonisins can affect the immune system. Not only does this increase susceptibility to infections, but it can interfere with the efficacy of vaccinations and increase the tendency to develop cancer. It has been suggested that the intestine (especially the small intestine) is a primary site for fumonisin toxicity (Bouhet and Oswald, 2007). Despite FB being poorly absorbed and metabolised in the intestine, it can bring about intestinal disturbances (abdominal pain or diarrhoea) and may cause extra-intestinal organ pathologies (Bouhet and Oswald, 2007; Grenier *et al.*, 2012). Fumonisins are also known to initiate and promote liver cancer in rats (Gelderblom *et al.*, 1988; Gelderblom *et al.*, 2001a).

Fumonisins, particularly the hydrolysed forms, are structurally similar to the cellular sphingolipids sphinganine and sphingosine (Figure 2.3) (Smith, 2012). Both contain a long chain carbon backbone with a primary amino group. Sphingolipids play an important role in eukaryotic cells, where they are structural components of cell membranes and regulate certain cell functions (e.g. cell growth, differentiation, morphology, apoptosis, endothelial permeability) (IARC, 2002; Soriano *et al.*, 2005; Stockmann-Juvala and Savolainen, 2008). The alteration of sphingolipids and the expression of sphingolipid-metabolising enzymes have been associated with a variety of diseases, including cancer (Kolter, 2011). Fumonisins disrupt sphingolipid metabolism by inhibiting the enzyme ceramide synthase (IARC, 2002; Voss *et al.*, 2007; Stockmann-Juvala and Savolainen, 2008). This leads to an accumulation of sphinganine in cells and tissues. The disruption of sphingolipid metabolism by FB₁ is correlated with cardiovascular, liver and kidney dysfunction (IARC, 2002). Fumonisins also induce oxidative stress in mammalian cells, leading to clastogenic effects (EFSA, 2018). The mode and mechanism whereby FB₁ exerts toxicity is complex and seems to vary in different cells, tissues and animal species. In general, the biological activity of FB₁ seems to be induced by its free amino group (Soriano *et al.*, 2005).

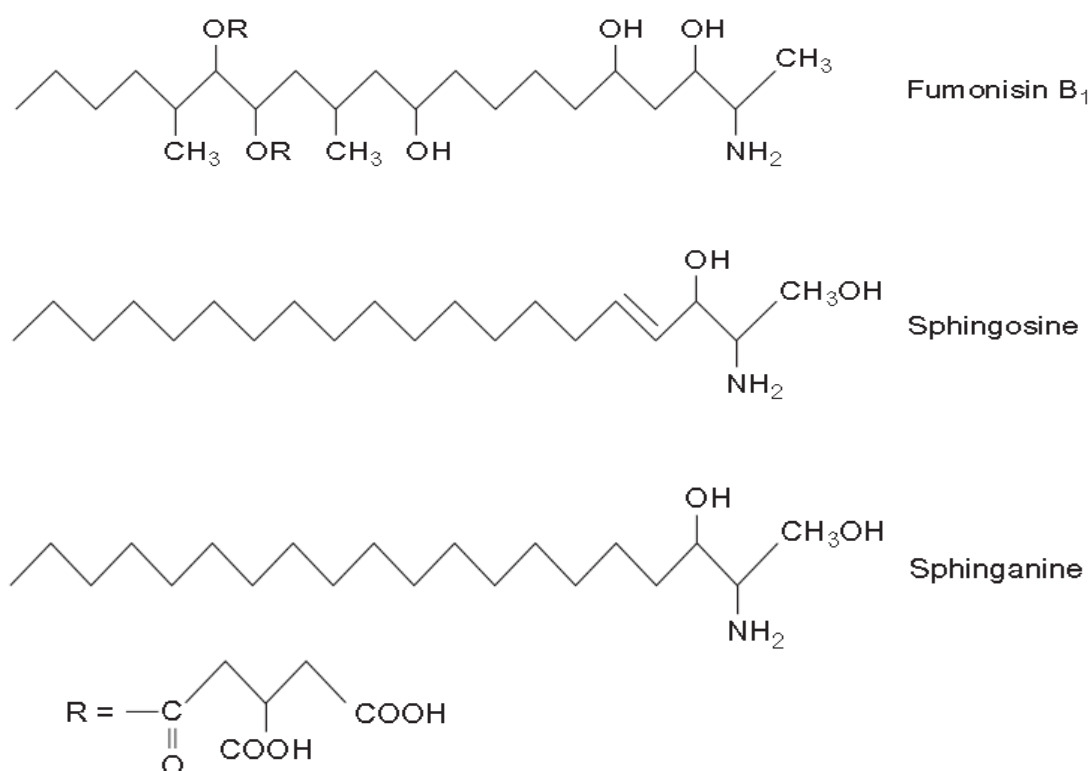


Figure 2.3: Chemical structures of sphinganine, sphingosine and fumonisin B₁ (Smith, 2012)

Providing causal evidence for exposure to fumonisins and the development of human disease is problematic, because it is not possible to conduct toxicological studies on humans as is carried out on laboratory animals. Apart from diet, there are also several other factors that contribute to human disease, such as genetics, lifestyle and environmental factors. Based on the current knowledge, the International Agency for Research on Cancer (IARC) classified fumonisin B₁ as a Group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2002). Although diets high in fumonisins have been associated with oesophageal cancer, primary liver cancer, neural tube defects and idiopathic congestive cardiopathy in humans, the evidence up to date is not sufficient to demonstrate causation (EFSA, 2018).

2.3 FUMONISIN LEGISLATION AND REGULATION

Health authorities worldwide regulate mycotoxin levels in human food and feed (Van Egmond *et al.*, 2007; Kubo, 2012). Maximum limits for fumonisins are established

for the European Union (EU), Turkey, Bosnia and Herzegovina, Norway, Switzerland and Brazil (Kubo, 2012). In the United States of America (USA), the Food and Drug Administration (FDA) has also issued an advisory with maximum limits for fumonisins. Until recently, South African national regulations (Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972) and regulations published under Government Notice No. R. 1145), only considered three types of fungus-produced toxins namely aflatoxin, patulin and *Ergot sclerotia* (SA, 2004). An amendment to Government Notice No. R. 1145, which introduced maximum levels for deoxynivalenol and fumonisins, was published in Government Notice No. 987 in September 2016 (SA, 2016). Table 2.1 provides a comparison of the EU, USA and South African specifications for maximum allowed fumonisin levels, confirming that the local limits are aligned to international regulations.

Table 2.1: Summary of international and national limits for fumonisin levels in food and animal feed

Legislation	Food products	Animal feed
European Union (EC, 2006a; EC, 2006b; EC, 2007)	Sum of fumonisins B ₁ and B ₂ : 4 000 µg/kg for unprocessed maize 1 000 µg/kg for maize-based foods for direct consumption 800 µg/kg for maize-based breakfast cereals and snacks 200 µg/kg for processed maize-based foods and baby foods	Sum of fumonisins B ₁ and B ₂ : 60 000 µg/kg in maize and maize products 5 000-50 000 µg/kg in complete and complementary feeding stuffs, depending on class and age of animal
United States of America (FDA, 2001)	Sum of fumonisins B ₁ , B ₂ and B ₃ : 4 000 µg/kg for whole or partially de-germed dry milled maize products 2 000 µg/kg for de-germed dry milled maize products	Sum of fumonisins B ₁ , B ₂ and B ₃ : 5 000-100 000 µg/kg in maize and maize-based products (depending on class of animal and the percentage of the diet)
South Africa (SA, 2016; SAGL, 2016)	Sum of fumonisins B ₁ and B ₂ : 4 000 µg/kg for raw maize grain 2 000 µg/kg for maize flour and maize meal, ready for human consumption	Fumonisin B ₁ : 5 000-50 000 µg/kg in feed, depending on type of animal

At present, none of the other southern African countries impose regulations or provide guidelines for fumonisins (Misihairabgwi *et al.*, 2017).

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) has a Joint Expert Committee on Food Additives (JECFA) that also sets limits for, amongst others, mycotoxins and food contaminants. Currently the Provisional Maximum Tolerable Daily Intake (PMTDI) for fumonisins B₁, B₂ and B₃ (alone or in combination) is 2 µg/kg body weight per day (FAO/WHO, 2017). However, it has been demonstrated that individuals who consume a maize-based staple diet may dramatically exceed the PMTDI when consuming maize which conforms to the legal limits for fumonisins (Shephard, 2004; Shephard, 2008). For example, a person with a body weight of 60 kg, who consumes 400 g of maize contaminated with 2 000 µg/kg fumonisin per day, would have a dietary exposure of 13 µg/kg body weight/day or 650% of the PMTDI. Exposure to fumonisins is estimated to be high in Brazil, Tanzania, Gautemala, Argentina and South Africa (Marin *et al.*, 2013). In contrast to this, a person in the developed world (e.g. Nordic countries) who typically consumes less than 10 g of maize per day, could be exposed to contamination levels as high as 12 000 µg/kg before reaching the PMTDI set by JECFA. The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) recently established a tolerable daily intake (TDI) for FB₁ at 1.0 µg/kg body weight per day (EFSA, 2018). Although this is half the PMTDI proposed by JECFA, a person in the scenario described above would still dramatically exceed the PMTDI.

Some authors proposed that the South African limit should be set lower than the FDA and EU limits, because *per capita* maize consumption is much higher in South Africa than in North America and Europe (Van Egmond *et al.*, 2007; Rheeder *et al.*, 2009; Shephard *et al.*, 2013c). Conversely, regulations that are too strict will lead to a decrease in availability and an increase in price, and since maize is a major staple in southern Africa, this will have a negative impact on food security in the region. Due to large variations in contamination and consumption levels of maize internationally, the harmonisation of regulations has proven to be a huge challenge. Since maize is a major staple food in South Africa, this underlines the importance of proper research and risk assessment related to fumonisins in the South African context.

2.4 FUMONISIN LEVELS IN SOUTH AFRICAN MAIZE

The Southern African Grain Laboratory (SAGL) conducts multi-mycotoxin determination on a subsample of the approximately 1 000 maize samples they receive annually as part of the crop quality survey (SAGL, 2014). These samples represent white and yellow maize from different maize production areas of South Africa. Based on the results acquired over the period 2000-2018, fumonisin can be regarded as a common contaminant of South African maize, with contamination levels reaching as high as 13 000 µg/kg (sum of fumonisins B₁, B₂ and B₃). Of the 2014/2015 crop, 56% of the 350 samples analysed tested positive for fumonisins (the lower limit of detection 20 µg/kg). The average concentration of the positive samples was 397 µg/kg, and the maximum was 3 382 µg/kg. Based on the national and international limit of 4 000 µg/kg for unprocessed maize (EC, 2006b; EC, 2007; SA, 2016), the average fumonisin contamination level of South African commercial maize is within the specifications, although individual samples with higher contamination levels are encountered occasionally.

Maize destined for animal feed is of a lower grade than that intended for human food and the allowed limits for fumonisin contamination are higher (EC, 2006a; EC, 2006b; EC, 2007). Apart from the potentially toxic effects on livestock, there is also a risk that these mycotoxins may be transferred back to the human food chain *via* animal products such as meat, milk and eggs (Voss *et al.*, 2007). Although most studies demonstrate that this carry-over of mycotoxins from the feed to animal food products is low, transfer remains a risk that must be assessed and managed to protect consumer health (Voss *et al.*, 2007; Coffey *et al.*, 2009; Iqbal *et al.*, 2014; Tsiplakou *et al.*, 2014; Flores-Flores *et al.*, 2015). The results from the annual Biomin worldwide survey indicated that the number of fumonisin-contaminated animal feed samples collected worldwide increased from 61% (2016) to 69% (2017) (Biomin, 2017). The prevalence of fumonisins in samples of South African maize destined for animal feed was 80%, with an average fumonisin content of 1 681 µg/kg for the positive samples. In addition, 90% of the samples contained deoxynivalenol (548 µg/kg).

There is less information available with regard to mycotoxin contamination of maize produced through subsistence farming, because there is no routine annual crop quality survey performed as is done for commercial maize. It is estimated that about four million people are engaged in subsistence or smallholder agriculture in South Africa (Du Toit, 2011), and that about 600 000 households depend on subsistence farming for their livelihood (Watkinson and Makgetla, 2002). A survey of maize samples collected during the 2006 and 2007 growing seasons indicated that fumonisin contamination occurred in all the maize subsistence farming areas in South Africa (Ncube *et al.*, 2011). More than half of the samples from Zululand (KwaZulu-Natal) and some samples from Mokopane (Limpopo) and Lusikisiki (Eastern Cape) contained fumonisins exceeding the FDA limit of 2 000 µg/kg. Another study assessed the mycotoxin contamination of home-grown maize in rural northern South Africa (Mngqawa *et al.*, 2016). Fumonisin and aflatoxin levels were significantly higher in the maize samples collected from the Vhembe District Municipality (Limpopo) than in those from the Gert Sibande District Municipality (Mpumalanga) over the seasons 2011 and 2012.

A dietary exposure assessment in the former Transkei region of the Eastern Cape, South Africa revealed that, although the mean fumonisin levels in good home-grown maize were below the FDA limit, fumonisin exposure was above the PMTDI set by JECFA (Shephard *et al.*, 2007; Shephard *et al.*, 2013b). This is due to the fact that the inhabitants of this area consume maize as their main staple. Subsistence farmers also select lower grade maize, which is not suitable for direct human consumption, for brewing traditional beer (Shephard *et al.*, 2005) and for animal feed (Shephard, 2008; Chilaka, 2012).

2.5 EFFECT OF PROCESSING ON FUMONISINS IN FOOD

2.5.1 Cleaning, milling and fractionation

Cleaning, milling and fractionation are physical methods for decreasing the fumonisin content of whole maize. Maize is normally cleaned before processing; this involves separation of any dirt, debris, and broken and infected kernels (Saunders *et al.*, 2001). Cleaning is a very important pre-processing step that can significantly decrease, but not eliminate, the concentrations of fumonisins in maize (Saunders *et al.*, 2001; Bullerman and Bianchini, 2007; Van der Westhuizen *et al.*, 2011; Generotti *et al.*, 2015). Mechanical cleaning typically make use of aspiration to remove dust, cob particles and stalk pieces (Anderson and Almeida, 2019). Coarse particles and broken kernels are removed by size separation while magnets and destoners are employed to remove metal and other heavy extraneous material, respectively.

Whole maize is converted into edible products by applying one of two main processes: dry milling or wet milling. In South Africa, dry milling is the main process, and this is used to produce samp, maize grits, maize rice, unsifted, sifted, coarse, super and special maize meal (SA, 2013). Wet milling is used to extract pure starch from maize, with maize germ (oil), husk (fibre) and gluten (protein) as by-products. The basic unit operations involved in commercial dry milling of maize are illustrated in Figure 2.4 (Anderson and Almeida, 2019). After tempering the maize, the hull (pericarp), germ and tip cap are removed (Saunders *et al.*, 2001). The endosperm is then passed through a series of mills, sifters and gravity tables to convert it into grits, meal and flour.

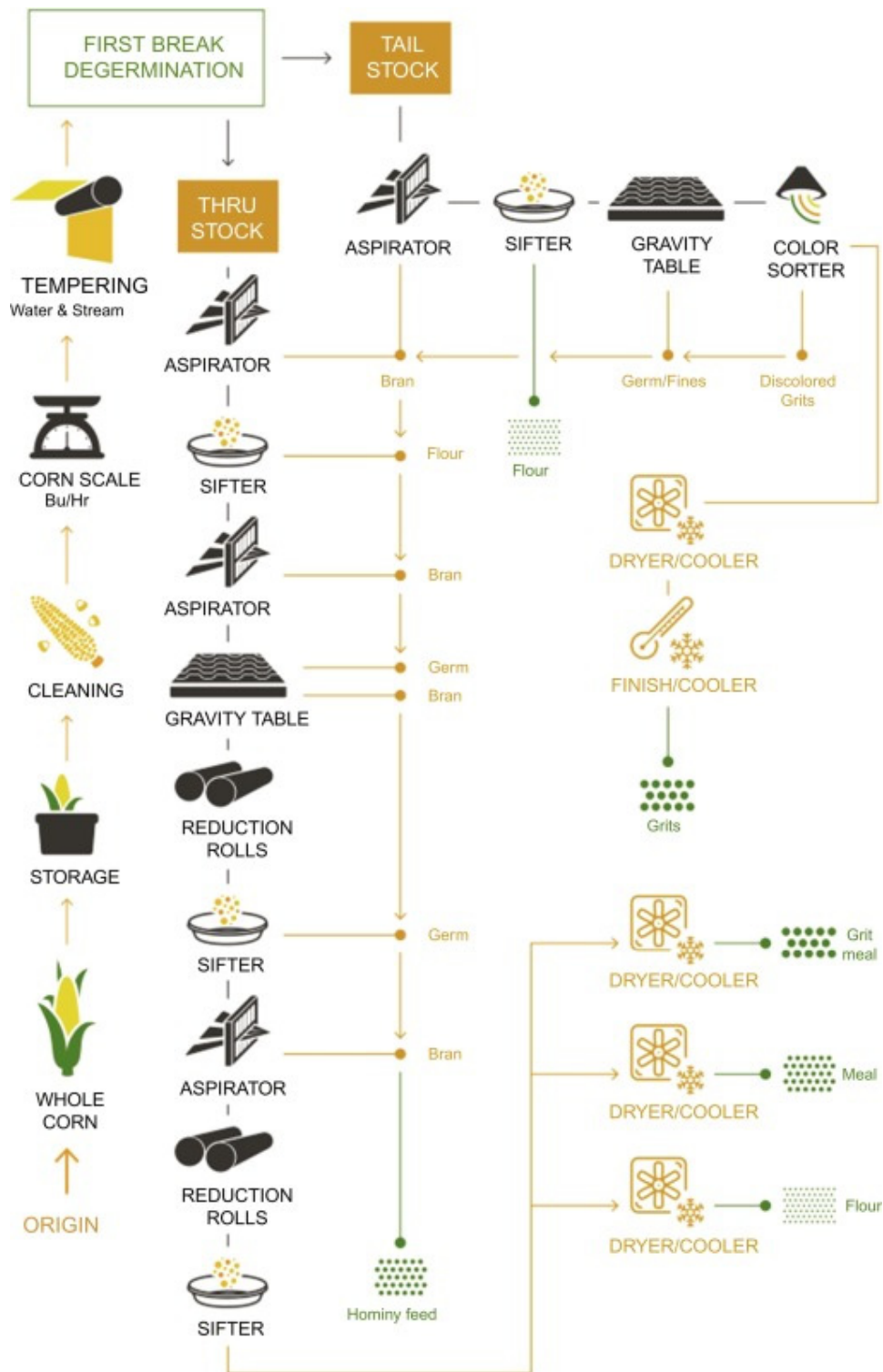


Figure 2.4: Unit operations for commercial dry milling of maize (Anderson and Almeida, 2019)

Figure 2.5 provides a comparison between two studies, where the distribution of fumonisin in dry milling fractions was studied under commercial milling (Vanara *et al.*, 2009) and experimental milling (Burger *et al.*, 2013) conditions, respectively.

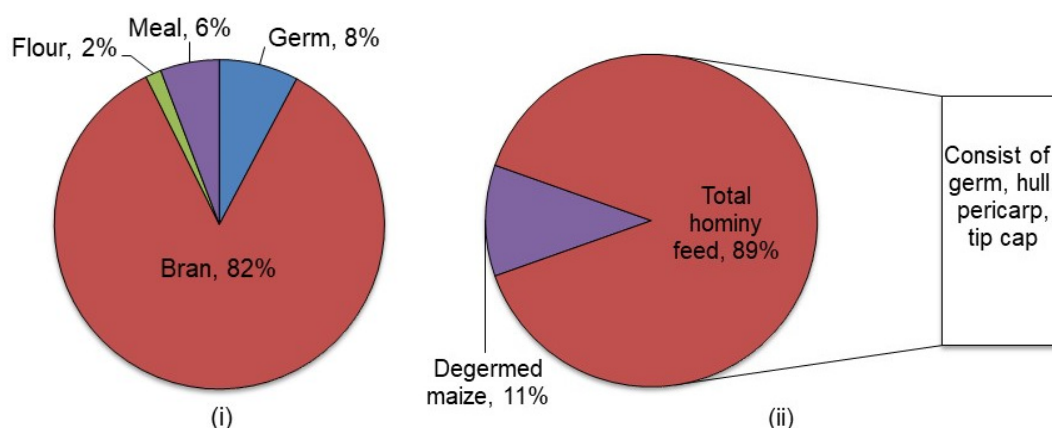


Figure 2.5: Fumonisin distribution in maize milling fractions: comparison of two studies (i) Fumonisin B₁ distribution in commercial maize milling fractions (Vanara *et al.*, 2009) and (ii) Total fumonisin B distribution in experimental maize milling fractions (Burger *et al.*, 2013)

Both studies established the same trend; more than 80% of the fumonisin content is present in the outer layers of the maize kernel, which is removed during dry milling. Only 8-11% remains in the fraction normally used for human food (flour/meal or de-germed maize). A similar trend was observed in a study that focussed on the industrial process to produce cornmeal semolina (Generotti *et al.*, 2015). Research also shows that the pure maize starch fraction, which is produced during wet milling, does not contain measurable amounts of fumonisins (Saunders *et al.*, 2001).

Fumonisin contamination occurs mainly in the field during the last part of the growing season, when high temperatures and air humidity favours the growth of *Fusarium verticillioides* (Vanara *et al.*, 2009). The outer layer of the maize kernel (pericarp) is colonised by fungi first, explaining why higher levels of fumonisin contamination is observed in the milling fraction that contains the pericarp (normally the bran or hominy grits).

2.5.2 Alkaline processing

The manufacture of tortillas from maize involves lime treatment (nixtamalisation). Cooking, steeping, and rinsing under alkaline conditions, dramatically lowers the concentrations of FB in the product (Dombrink-Kurtzman *et al.*, 2000; De Girolamo *et al.*, 2011; De Girolamo *et al.*, 2014). The steeping liquid and rinsing water, which

contains dissolved fumonisins, are usually discarded. The pericarp of the maize kernel is also partially removed during the process. Since fumonisins are concentrated in the outer layers of the kernel, this leads to a further reduction in their concentrations in the remaining masa, which is the maize dough used for making tortillas (Dombrink-Kurtzman *et al.*, 2000). Nixtamalisation also causes alkaline hydrolysis of fumonisins, removing the TCA side chains from FB to form HFB and PHFB (De Girolamo *et al.*, 2014). The majority of the FB in maize is converted to HFB during nixtamalisation (Dombrink-Kurtzman *et al.*, 2000). Currently, there is not sufficient evidence to establish whether converting FB to HFB eliminates toxicity towards humans. In a study using a short-term liver cancer initiation/promotion model, HFB₁ displayed greater cytotoxicity than FB₁ (Gelderblom *et al.*, 1993). Some researchers reported that hydrolysed fumonisins are less toxic to laboratory animals and pigs than their parent forms (Voss *et al.*, 1996; Hartl and Humpf, 2000; Grenier *et al.*, 2012). There is also evidence that HFB is acylated *in vitro* and *in vivo* and that the *N*-acyl-HFB is cytotoxic HT29, a human colonic cell line (Seiferlein *et al.*, 2007). In contrast to these studies, a more recent study found that hydrolysed and partially hydrolysed fumonisins are non-toxic to rats (Hahn *et al.*, 2015).

2.5.3 Heat treatment

Fumonisins are fairly heat stable in both dried maize culture medium and in aqueous medium (Dupuy *et al.*, 1993; Jackson *et al.*, 1996a; Jackson *et al.*, 1996b; Jackson *et al.*, 1996c). In aqueous medium, loss of FB₁ is more rapid and extensive under alkaline (pH 10) and acidic conditions (pH 4) than at neutral pH (Jackson *et al.*, 1996c). In addition, thermal decomposition, with the formation of hydrolysis products, increases with an increase in processing temperature and time. There is no information available on the thermal stability of FB₃ under similar conditions. Although there is some data available on the stability of fumonisins in food products, to date there has been only one publication concerned with the stability of FB₁, FB₂ and FB₃ under different pH and temperature conditions in the presence of a food matrix (Bryla *et al.*, 2017). This information is critical, because as described in the review by Shier *et al.* (2003), extensive alterations in the structures of fumonisins are possible, without complete loss of *in vitro* toxicity.

In general, it seems that mild heat treatments, such as pasteurisation and cooking have little or no effect on fumonisins, while high temperature heat treatments (150-200 °C), including baking, frying and extrusion, reduce fumonisin concentration with time, temperature and recipe being important variables (Humpf and Voss, 2004). Preparation of South African maize porridge at 100 °C for 20 min resulted in a 23% reduction in the fumonisin levels (Shephard *et al.*, 2002). Extrusion cooking of corn grits at slow screw speed with 10% added glucose resulted in a 93% reduction in fumonisin content (Bullerman and Bianchini, 2007).

Overall, it can be noted that processing does not seem to increase or concentrate the free fumonisin content of maize-based food products. No effect, or a decrease in fumonisin content have been reported, depending on the type of processing (Saunders *et al.*, 2001). During milling, the fumonisin content of the refined maize is decreased due to physical separation of the maize kernel components. In most other studies where a reduction in fumonisin levels was observed, it was not clear whether the reduction was due to thermal decomposition of the fumonisins, or due to binding with components in the food matrix (e.g. protein, sugar, starch or lipids) (Humpf and Voss, 2004). Masked fumonisins may still pose a significant health risk and this issue is discussed in more detail in the following section.

2.6 MASKED, HIDDEN, MODIFIED AND CRYPTIC FUMONISINS

2.6.1 Definitions and classification

The term “masked mycotoxin” originated in the 1980’s when it was observed that, in some cases of mycotoxicosis, the symptoms did not correspond with the levels of mycotoxins measured in the samples (Freire and Sant’Ana, 2018). This led to the idea that there could be undetected forms of mycotoxins that contribute to their toxic effects. Masked mycotoxins are mycotoxin derivatives that are not detectable by conventional routine analysis (De Boevre *et al.*, 2015). From literature it is evident that masked forms of many different mycotoxins exist, including fumonisin, deoxynivalenol, alternariol, zearalenone, ochratoxin A, aflatoxin B₁ and T2 toxin (Freire and Sant’Ana, 2018). However, the term “masked mycotoxin” is non-specific

and has been used ambiguously (Rychlik *et al.*, 2014). Some authors suggest that the term “masked mycotoxins” be used only for metabolites formed following the metabolism of mycotoxins by plants, while others include derivatives formed during processing (Berthiller *et al.*, 2013). For example, one definition of masked fumonisins includes covalently bound fumonisins (bound fumonisins) and non-covalently bound fumonisins (hidden fumonisins, probably through physical entrapment by matrix macroconstituents) (Falavigna *et al.*, 2012). Figure 2.6 provides a summary of the different forms of fumonisins in food using this definition.

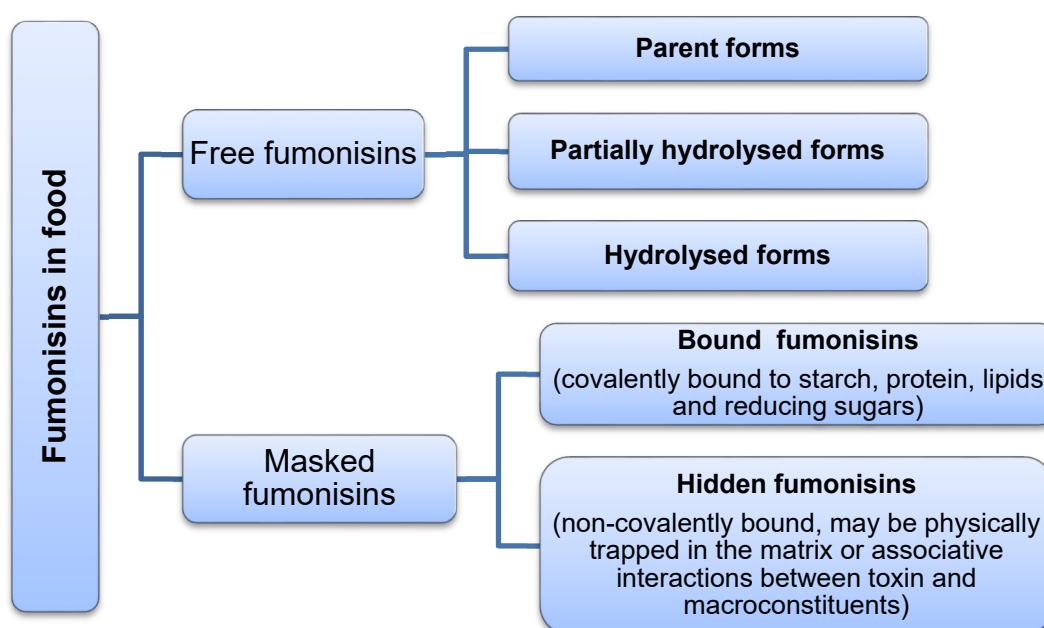


Figure 2.6: Schematic indicating the various forms of fumonisins in food (Falavigna *et al.*, 2012)

The term “masked mycotoxin” is broad and it does not provide any information regarding the nature of the masking. It was subsequently proposed that a new systematic definition with four hierarchic levels be used to distinguish between free and the different forms of modified mycotoxins (Rychlik *et al.*, 2014). As illustrated in Figure 2.7, the updated definition includes other relevant forms of mycotoxins, such as mammalian metabolites and thermally generated modifications. Masked fumonisins may be formed in the maize plant by enzymes involved in detoxification. A considerable portion of fumonisins may also be converted to other substances during typical thermal food processing (Shier *et al.*, 2003).

The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) recently published a report on health-based guidance values for fumonisins and their modified forms (EFSA, 2018). In this context, modified, mycotoxins are defined as “*all forms that differ in their chemical structure from the parent toxin*”, including fungal and plant metabolites, compounds formed during processing and covalent adducts with matrix constituents. However, in this definition, fumonisins physically trapped in the matrix are not considered to be a modified form, since there is no covalent bond or change in the chemical structure. Although physical entrapment in the matrix is of importance and can lead to considerable underestimation of FB levels, this is considered to be an analytical issue. Furthermore, Braun and Wink (2018) introduced the term “cryptic” fumonisins to include all routinely non-detectable fumonisins.

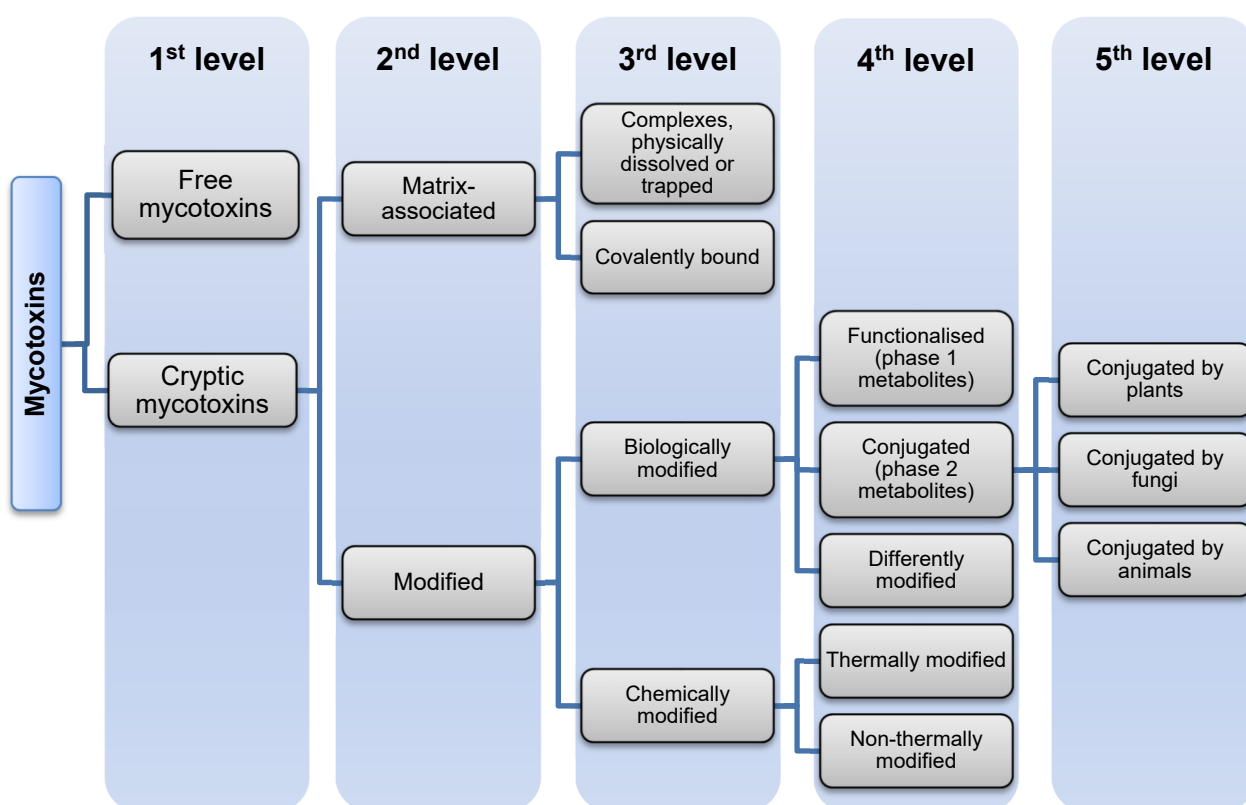


Figure 2.7: Schematic of the updated systematic definition of modified mycotoxins (Rychlik *et al.*, 2014; Braun and Wink, 2018)

The formation of modified fumonisins by plants and during food processing are discussed in more detail in the next sections.

2.6.2 Formation of modified fumonisins in plants

Plants protect themselves from phytotoxins, such as mycotoxins, by three main biochemical mechanisms (Pereira *et al.*, 2014), which are summarised in Figure 2.8 below.

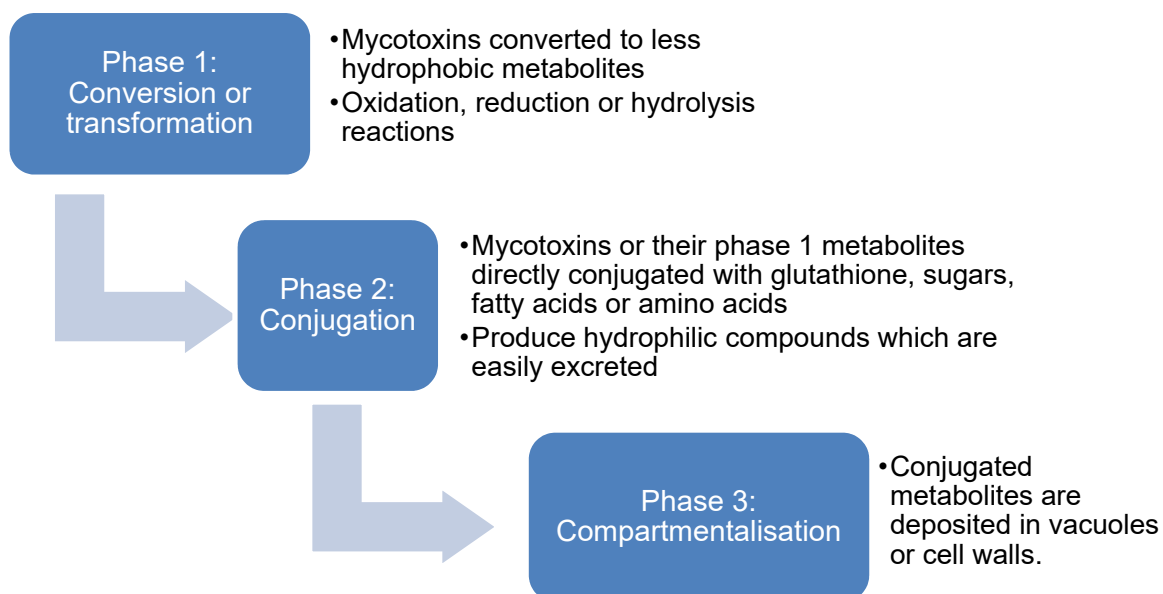


Figure 2.8: Plant biochemical processes for protection against phytotoxins (Pereira *et al.*, 2014)

Due to these biochemical conversions, conjugated or masked fumonisins may occur in unprocessed cereal grains. The presence of such masked fumonisins, which are released through alkaline hydrolysis, has been reported in literature (Dall'Asta *et al.*, 2008; Dall'Asta *et al.*, 2009; Dall'Asta *et al.*, 2010).

2.6.3 Formation of modified fumonisins during food processing

Fumonisins may form covalent bonds with several food components. The current knowledge on the interaction of fumonisins with carbohydrates, proteins and lipids are reviewed below, with specific reference to maize.

2.6.3.1 Fumonisin interaction with carbohydrates

The reaction of fumonisin B₁ with reducing sugars occurs *via* the C2-amino group and results in the formation of *N*-(carboxymethyl) fumonisin B₁ (Howard *et al.*, 1998). Fructose reacts with FB₁ in a Maillard-type reaction (Lu *et al.*, 1997). It is suggested that a Schiff's base forms when the fructose ketone combines with the FB₁ amine. This reaction blocks the amine group of FB₁ and eliminates the toxicity of FB₁ as demonstrated in rats (Lu *et al.*, 1997; Hahn *et al.*, 2015). In a follow-up study (Lu *et al.*, 2002), the reaction products formed when FB₁ is heated in the presence of glucose were determined. In addition to the *N*-(carboxymethyl) fumonisin B₁ previously detected (Howard *et al.*, 1998), a number of other Maillard reaction products were identified including the fumonisin B₁-glucose Schiff's base *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₁ (Figure 2.9) (Lu *et al.*, 2002). The rate and complexity of the reaction was found to increase with an increase in temperature. A similar reaction takes place when FB₂ and FB₃ are heated in the presence of glucose (Matsuo *et al.*, 2015). Solid glucose also interacts with fumonisins (FB₁ and FB₂) at room temperature (Kim *et al.*, 2002). Only 20% of fumonisins spiked in solid glucose could be recovered after 24 h storage at room temperature. However, after three months storage, only a small amount of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₁ and no *N*-(carboxymethyl) fumonisin B₁ was detected, with the fate of the unrecoverable fumonisins unknown.

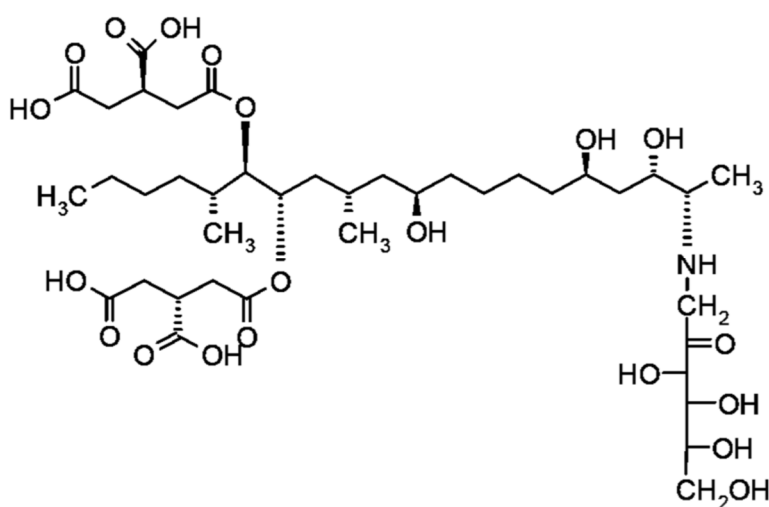


Figure 2.9: Chemical structure of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₁ (Falavigna *et al.*, 2012)

Whole maize contains only a small amount of free sugars (1.61-2.22% on a dry weight basis) (Kent and Evers, 1994), therefore the reaction between reducing sugars and fumonisins is unlikely to play an important role in raw maize. However, glucoside derivatives of the trichothecene mycotoxins T-2 toxin and HT-2 toxin have been identified in naturally contaminated wheat and oats (Lattanzio *et al.*, 2012). These are formed by plant metabolism as a natural mechanism to reduce mycotoxin accumulation. Currently, little information is available on naturally occurring fumonisin glucoside derivatives in unprocessed maize. Only one study reported the presence of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₂ and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₃ in naturally contaminated corn (maize) flour (Matsuo *et al.*, 2015). Low levels of *N*-(carboxymethyl) fumonisin B₁ have been detected in some samples of commercially processed food products (tortilla chips, nachos chips and corn flakes) (Seefelder *et al.*, 2001).

The main carbohydrate in maize is starch, which is a glucose polymer. Whole maize kernel and maize endosperm contain about 62% and 87% starch on a dry weight basis, respectively (Shukla and Cheryan, 2001). Fumonisins (FB₁ and FB₂) seem to be unstable in Thai white rice flour, with spiked fumonisins apparently disappearing after 10 h storage at room temperature (Kim *et al.*, 2002). The same study also demonstrated that fumonisins spiked into corn starch seemed to disappear after 5.5-day storage at room temperature, as it was not possible to recover them from the spiked samples. In addition, for both the Thai white rice flour and corn starch, the recovery of fumonisins directly after spiking was poor with only 50-60% being recoverable. This could indicate some kind of interaction between the fumonisins and the matrix of the white rice flour and corn starch, or it could be an analytical problem.

Seefelder *et al.* (2003) demonstrated, using a model system, that the reaction of FB₁ with methyl α -D-glucopyranoside (representing starch) occurs *via* the TCA side chains of FB₁. However, the extent to which this reaction takes place in food products during processing is not known (Humpf and Voss, 2004).

2.6.3.2 Fumonisin interaction with proteins

In a model system, it was demonstrated that the reaction of FB₁ with amino acid derivatives (representing proteins) occurs *via* the TCA side chains of FB₁ (Seefelder *et al.*, 2003). Under baking or roasting conditions, FB₁-anhydride is expected to react with proteins at the ϵ -amino moiety of lysine, or the thiol function of cysteine, to form covalently bound products (Shier *et al.*, 2003).

Whole maize kernel and maize endosperm contain about 7.8% and 8% protein, respectively (Shukla and Cheryan, 2001). The distribution of the protein fractions in maize is listed in Table 2.2.

Table 2.2: Distribution of protein fractions in maize (Shukla and Cheryan, 2001)

Protein fraction	Solubility	Distribution (% dry weight basis)		
		Whole kernel	Endosperm	Germ
albumins	water	8	4	30
globulins	salt	9	4	30
glutelin	alkali	40	39	25
zein	alcohol	39	47	5

The main amino acids in maize protein are glutamic acid, proline, alanine and leucine (Sodek and Wilson, 1971; Shukla and Cheryan, 2001). In a laboratory model of a cornflake manufacture process, FB₁ bound predominantly to the ϵ -amino group of lysine in non-zein proteins (Shier *et al.*, 2003). The binding position is illustrated in Figure 2.10. Maize zein is deficient in lysine (Shukla and Cheryan, 2001) and the other Osborne protein fractions of maize contain very low concentrations of lysine (Sodek and Wilson, 1971).

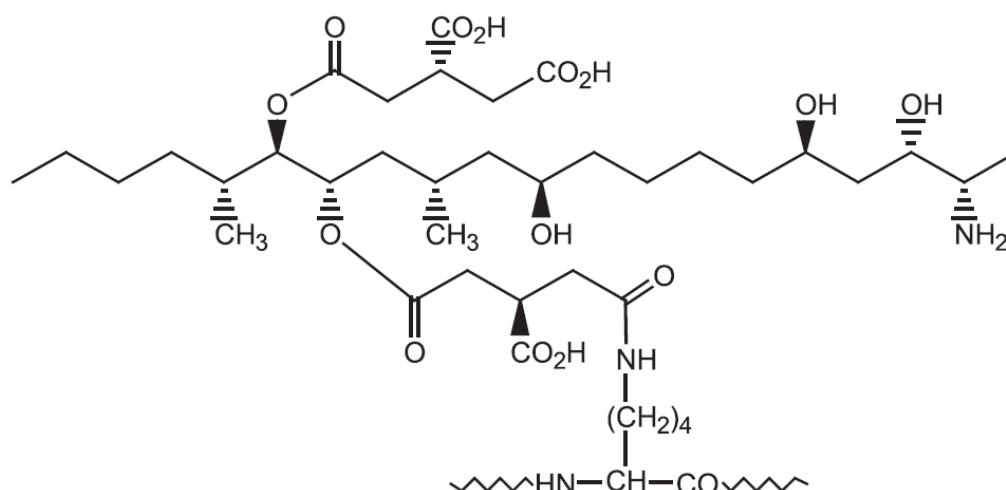


Figure 2.10: Fumonisin B₁ bound to non-zein maize protein (Shier *et al.*, 2003)

Fumonisin B₁ may also bind to maize prolamins and globulins. Antibodies against FB₁ were able to recognise protein-bound fumonisins in a model system, as well as in naturally contaminated maize-based food product samples (Dall'Asta *et al.*, 2008). This may explain the systematic “over-estimation” of FB₁ levels obtained when using enzyme-linked immunosorbent assays (ELISA) when compared to results from high performance liquid chromatography (HPLC) analysis. It is possible that free FB₁ may be released from protein-bound FB₁ during digestion in the GIT (Shier *et al.*, 2003).

Protein-bound FB₁ may be extracted with sodium dodecylsulfate (SDS). It has been suggested that there are also other types of bound fumonisins, besides protein conjugates, present in heat-processed corn foods, since the total amount of bound fumonisins in samples were not completely recovered during SDS extraction (Park *et al.*, 2004).

2.6.3.3 Fumonisin interaction with lipids

Fumonisin B₁ can bind to fatty acids during deep fat frying of products including tortilla chips, to form *N*-fatty acyl fumonisins (Park *et al.*, 2013). An example of this type of compound is *N*-palmitoyl hydrolysed fumonisin B₁, which is illustrated in Figure 2.11. This is a cause for concern, because *in vitro* studies suggest that some *N*-acylated HFB₁ derivatives could be more toxic than FB₁ (Harrer *et al.*, 2013).

Optimal *in vitro* toxicity was reported when fumonisin derivatives contained fatty acyl chain lengths of 8-12 carbons (Shier *et al.*, 2003).

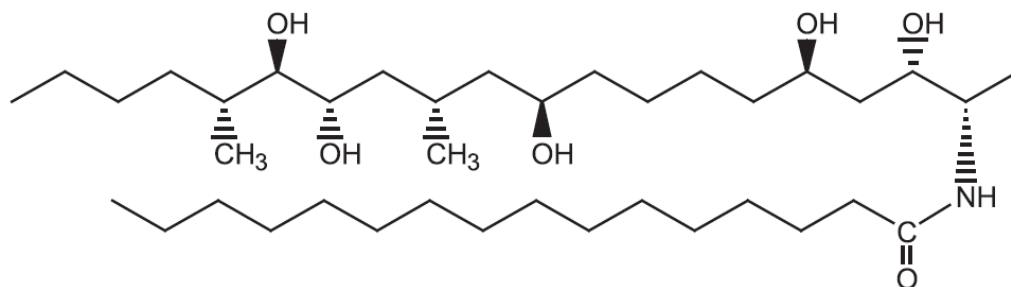


Figure 2.11: Chemical structure of N-palmitoyl hydrolysed fumonisin B₁ (Shier *et al.*, 2003)

Whole maize contains about 3.8% oil (Shukla and Cheryan, 2001). The main fatty acids in maize oil are linoleic (49%), oleic (33.4%) and palmitic acid (14%), with minor amounts of stearic (2%) and linolenic acid (1.5%) (Kent and Evers, 1994). There is no information available on the occurrence of *N*-fatty acyl fumonisins in raw, unprocessed maize. Instead, research has been focussed on high heat processing, such as deep fat frying of tortilla chips (Shier *et al.*, 2003).

2.7 DETERMINATION OF FUMONISINS

2.7.1 Determination of free fumonisins

High performance liquid chromatography (HPLC) is widely used for the determination of fumonisins, but ultra-performance instruments (UHPLC) are becoming increasingly important (Shephard *et al.*, 2013a). Fumonisins lack a good chromophore and therefore HPLC methods usually rely on mass spectrometry (MS) for detection, or alternatively derivatisation and detection by fluorescence or UV absorbance. Although methods involving derivatisation are used extensively, LC-MS/MS and high-resolution MS are gaining importance. These methods have low limits of detection (LOD), well below the levels needed to monitor for regulatory purposes and can be used to detect single mycotoxins or any combination of mycotoxins in multi-mycotoxin determination.

Maize samples that are contaminated with a particular mycotoxin often also contain other mycotoxins. This is due to the fact that one fungal species can produce several toxic metabolites (Pereira *et al.*, 2014). For example, *Fusarium* fungi are able to produce trichothecenes, zearalenone (ZEN), fumonisins, moniliformin and fusaric acid (D'Mello *et al.*, 1999). In addition, maize samples may be contaminated with different types of fungal species, which produce different toxins (Pereira *et al.*, 2014). For this reason, multi-mycotoxin determination methods are useful for screening samples for different mycotoxins in a single analysis. Figure 2.12 provides a summary of the basic steps in the determination of mycotoxins in food samples.

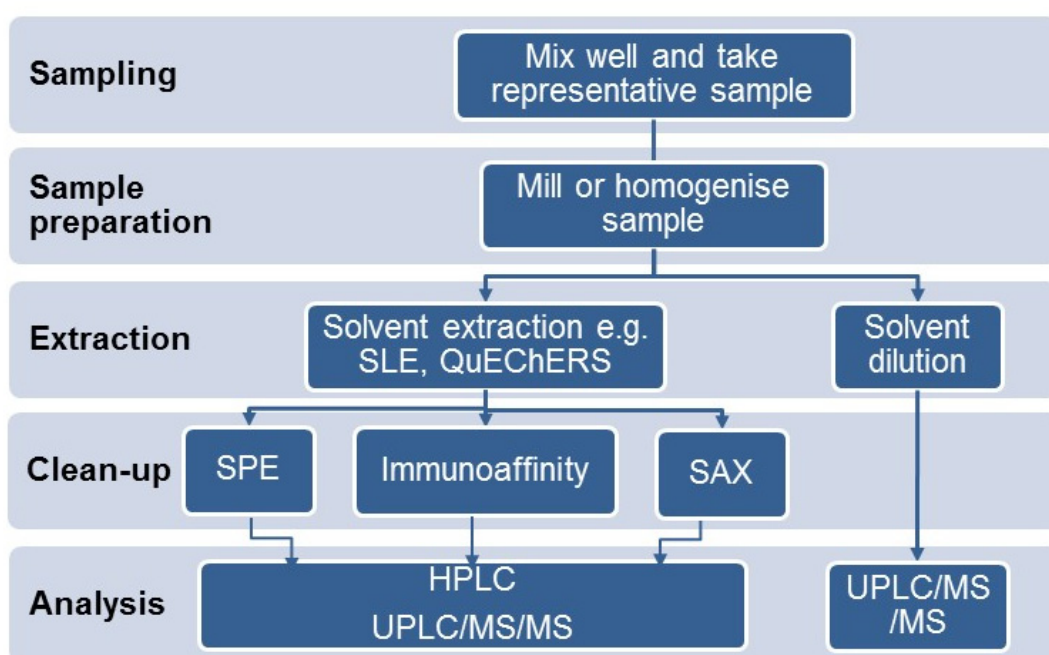


Figure 2.12: General procedures for mycotoxin determination in food samples (Marschik *et al.*, 2013; Shephard *et al.*, 2013a; Pereira *et al.*, 2014) (SLE = solid-liquid extraction, QuEChERS = quick, easy, cheap, effective, rugged and safe, SPE = solid phase extraction, SAX = strong anion exchange, HPLC = high performance liquid chromatography, UPLC = ultra-high performance liquid chromatography, MS = mass spectrometry)

Samples are usually extracted before mycotoxin determination, mostly with aqueous methanol, aqueous acetonitrile, or an aqueous mixture of methanol and acetonitrile (Marschik *et al.*, 2013). The following are examples of extractants used for fumonisins in published methods: aqueous phosphate buffer (pH 7.5), methanol:water (3:1, v/v) with ultrasonic agitation and methanol:water (3:1, v/v), using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe)

method (Shephard *et al.*, 2013a). The extraction step is usually followed by a clean-up step, employing solid-phase extraction (SPE), strong anion exchange (SAX) cartridges or immunoaffinity columns (IAC) (Marschik *et al.*, 2013). It is also possible to analyse extracts without clean-up, using the so-called “*dilute and shoot*” method (Beltran *et al.*, 2013). Matrix effects cause suppression or enhancement of the MS signal and depend on the method and the type of matrix (Scott, 2012). For this reason, matrix-matched standards are often used (Dall’Asta *et al.*, 2010; Burger *et al.*, 2013). However, since maize is processed into a wide range of products, each product would require its own set of matrix-matched standards, which increases the cost and number of analyses to be performed dramatically. Another approach is the use of [¹³C]-labelled internal standards to compensate for matrix effects (Varga *et al.*, 2012; Varga *et al.*, 2013). It is possible to distinguish between stable isotopically labelled standards and the naturally occurring fumonisins in food samples, due to the difference in molecular mass. Nevertheless, they share the same chemical and physical properties as the target analyte.

2.7.2 Determination of masked/cryptic fumonisins

Unlike free fumonisins, masked/cryptic fumonisins are not detected by conventional analytical methods. Binding with the food matrix may change the fumonisin structure in such a way that the amino group, essential for detection by derivatisation, is blocked. The result of this is that the fumonisins are not detectable by conventional HPLC analysis (Park *et al.*, 2013). Bound mycotoxins are not extractable by conventional analytical methods, and must therefore be liberated from the matrix by chemical or enzymatic treatment before chemical analysis (Berthiller *et al.*, 2013).

Total fumonisins are often determined by hydrolysing the sample residue left over after extraction of the free fumonisins, with KOH. Alkaline treatment causes hydrolysis of one or both of the TCA side chains, yielding hydrolysed (HFB) or partially hydrolysed (PHFB) fumonisins (Maragos *et al.*, 1997). The amount of fumonisins released after alkaline hydrolysis of contaminated maize products is often higher than the stoichiometrically expected value (Berthiller *et al.*, 2013). This indicates the presence of masked/cryptic fumonisins. If reference standards are

available, LC-MS/MS is the method of choice for targeted determination of modified mycotoxins (De Saeger and Van Egmond, 2012). The toxicological importance of masked/cryptic fumonisins depend not only on the concentration of these bound and hidden forms in food products, but also on the amount that becomes bioavailable during digestion (Humpf and Voss, 2004).

2.8 BIOAVAILABILITY OF FUMONISINS

2.8.1 Introduction and definitions

When assessing dietary fumonisin exposure, it is important that not only the amount of fumonisins ingested be considered, but also the bioavailability thereof (Gonzalez-Arias *et al.*, 2013). The term “bioavailability” is often used with some ambiguity. From a nutritional perspective, bioavailability refers to “*the fraction of the food nutrient/contaminant ingested that is available for use in the physiologic functions or to be stored in body*” (Cardoso *et al.*, 2015). Bioavailability is also often described as “*the proportion of an ingested contaminant in food that reaches the systemic circulation*” (Versantvoort *et al.*, 2005; Brandon *et al.*, 2006). Other definitions consider the oral bioavailability of a compound to be the result of three consecutive processes, as illustrated in Figure 2.13.

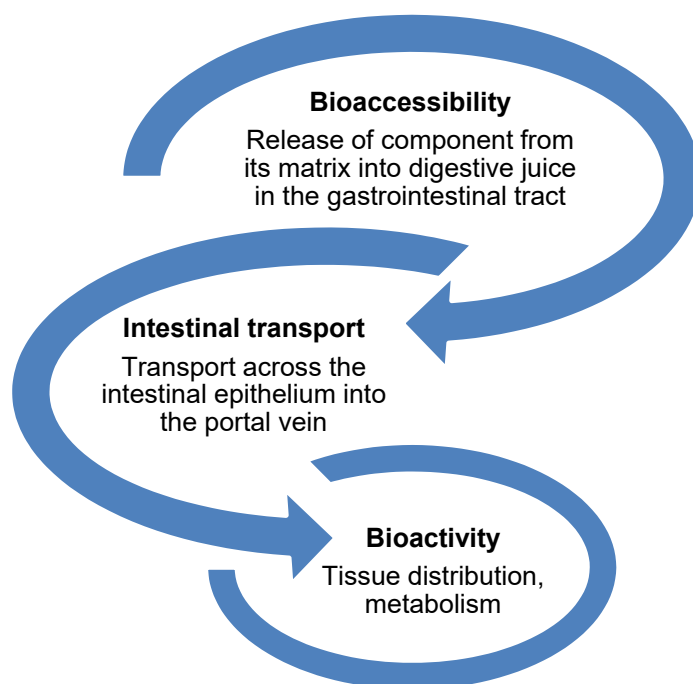


Figure 2.13: Processes involved in determining the oral bioavailability of nutrients and contaminants in food (Gonzalez-Arias *et al.*, 2013; Cardoso *et al.*, 2015)

The bioavailability of a bioactive component in food may be calculated using Equation 1 (Joye *et al.*, 2014):

$$F = F_C \times F_B \times F_A \times F_M \quad \text{.....Equation 1}$$

where F is the overall bioavailability, F_C is the fraction of the substance in active form within the food prior to ingestion, F_B is the fraction bioaccessible, F_A is the fraction absorbed and F_M is the fraction not metabolised

The determination of the bioactivity of a compound requires *in vivo* methods and is difficult to quantify (Cardoso *et al.*, 2015). For this reason, many researchers only consider bioaccessibility and intestinal transport when predicting bioavailability. This section of the literature review provides an overview of human digestion and *in vitro* methods to predict bioaccessibility.

2.8.2 Overview of human digestion

Human digestion involves a series of highly complex physicochemical and physiological events. The upper tract of the GIT consists of the oral cavity, oesophagus, stomach and small intestine (duodenum, jejunum and the ileum), whereas the lower tract consists of the large intestine (cecum, colon, rectum and anal canal) (Verhoeckx and Cotter, 2015a). Each part of the GIT plays an important role in the digestion and absorption of food. These processes are summarised in Figure 2.14.

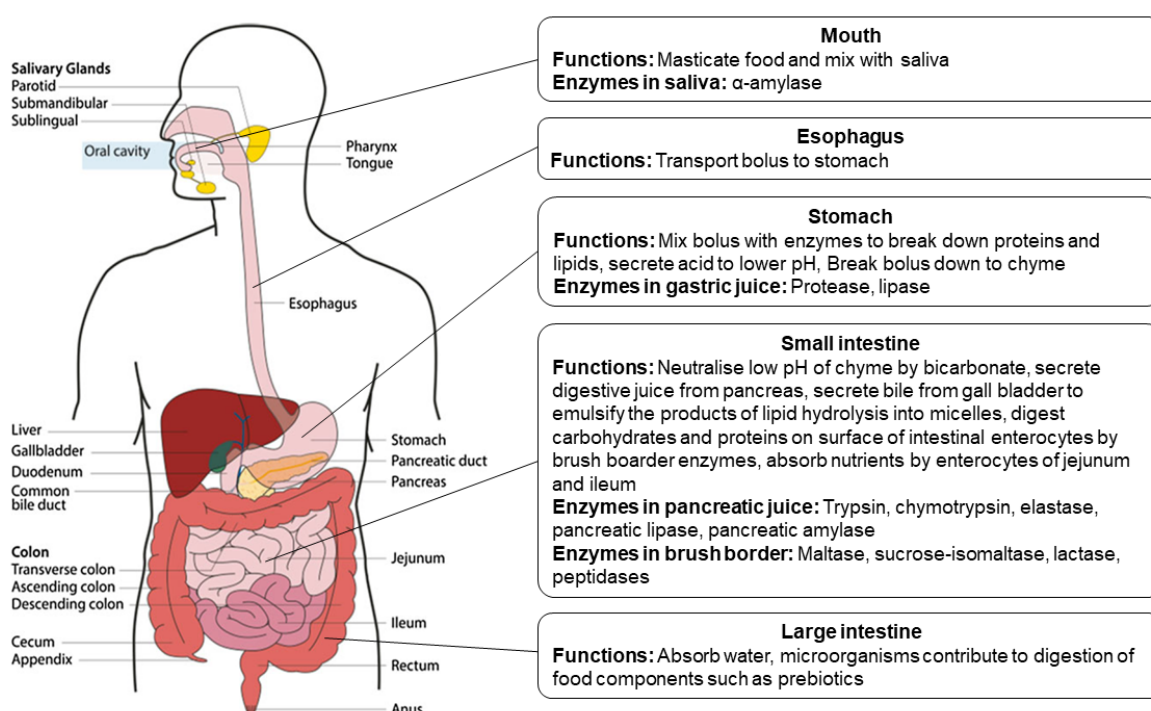


Figure 2.14: Schematic of the human GIT and its functions (DeSesso and Williams, 2008; Verhoeckx and Cotter, 2015a)

Absorption of ingested substances (including food and toxins) takes place mainly in the small intestine, which is the longest section of the GIT (Cencic and Langerholc, 2010). It has a large surface area (estimated 252 m² for an adult human) due to the projections of the mucosa, called intestinal villi (DeSesso and Williams, 2008). The wall of the GIT consists of several layers that forms a selective barrier between the luminal contents and the systemic circulation. The gastrointestinal mucosa consists of sheets of epithelial cells connected laterally by tight junctions. Transport of nutrients is facilitated across the epithelium, while toxins and pathogens should be

excluded. Molecules can be transported across the intestinal epithelium *via* three mechanisms: transcellular (across the cells), carrier mediated or paracellular (between the cells) (Balimane and Chong, 2005). These mechanisms, as well as mechanisms that can prevent absorption, are illustrated in Figure 2.15.

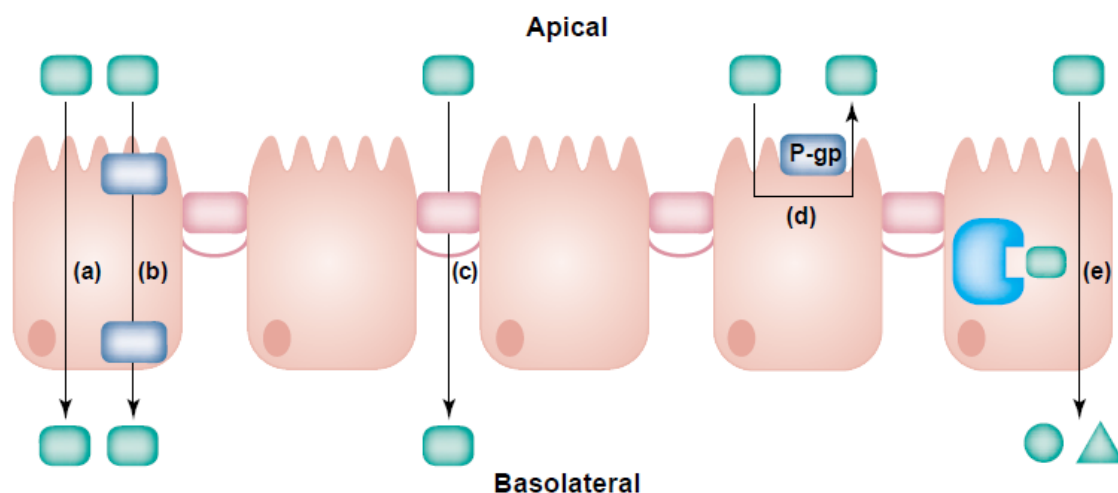


Figure 2.15: Different pathways for intestinal absorption of a compound (Balimane and Chong, 2005) The intestinal absorption of a compound can occur *via* several pathways: (a) transcellular passive permeability; (b) carrier-mediated transport; and (c) paracellular passive permeability. However, there are also mechanisms that can prevent absorption: (d) intestinal absorption can be limited by P-gp, which is an ATP-dependent efflux transporter; and (e) metabolic enzymes in the cells might metabolise the compound

Substances that are absorbed through the intestinal cells pass into the interstitial fluid, and from there they may enter the local tissue cells, blood capillaries or lymphatic system¹. Toxicants are often stored in adipose tissue, bone, the liver or kidneys. The liver and kidneys are also two important routes for eliminating toxins. Small toxin molecules are filtered out by the kidneys and excreted into the urine. The main route for eliminating toxins in the faeces is *via* the biliary route. Substances excreted by the liver into the bile are primarily large, ionised molecules. Once in the intestinal tract, the toxin can be eliminated with the faeces, or it may be reabsorbed and returned to the liver by the portal circulation (enterohepatic circulation). Although most substances are not reabsorbed, some (especially smaller non-polar molecules) may enter the enterohepatic circulation, resulting in an increase of the half-life of the toxin in the body.

¹ <https://toxictutor.nlm.nih.gov/index.html> [Accessed 01/03/2018]

In vivo human and animal studies are expensive, time consuming and must adhere to stringent ethical requirements; therefore *in vitro* models are often used to simulate human digestion. These models are aimed at mimicking the physiological conditions in the human GIT as closely as possible (Verhoeckx and Cotter, 2015a), which is quite challenging considering the intricacies of the processes involved with digestion and absorption.

2.8.3 *In vitro* methods to determine bioavailability

The bioaccessibility of a food component or contaminant from its matrix can be used as an indicator of its bioavailability, because release in the GIT is a prerequisite for uptake in the body (Versantvoort *et al.*, 2005). Since the early 1990's, research on *in vitro* gastrointestinal models increased dramatically, and a large number of models have been developed (Lucas-González *et al.*, 2018). Most of these involve static models, imitating oral, gastric and small intestinal digestion. The bioavailability of a wide range of food and pharmaceutical products have been established. Important parameters to consider when developing an *in vitro* gastrointestinal model include the specific digestive enzymes used and their concentrations, pH, digestion time and concentrations of electrolytes and bile (Minekus *et al.*, 2014). However, in the past there have been considerable variation in the parameters used by different research groups, which hampers direct comparisons between the results from different studies. To address this problem, the COST Action Infogest network developed a standardised protocol for simulating digestion *in vitro* under static conditions, which is now internationally accepted (Minekus *et al.*, 2014; Dupont, 2016).

The static method of Versantvoort *et al.* (2005) was used in many of the investigations into the *in vitro* bioaccessibility of fumonisins (Motta and Scott, 2007; Motta and Scott, 2009; Dall'Asta *et al.*, 2010; Falavigna *et al.*, 2012; Cirlini *et al.*, 2015; Szabó-Fodor *et al.*, 2015). The main steps and experimental conditions used in this method are summarised in Table 2.3, and are compared to the harmonised protocol developed by Minekus *et al.* (2014).

Table 2.3: Summary and comparison of the main steps and experimental conditions of two validated *in vitro* digestion models to determine the bioavailability of compounds

	Versantvoort <i>et al.</i> (2005)	Minekus <i>et al.</i> (2014)
Oral phase:		
Dilution	Food to salivary fluid 4.5 g: 6 mL	Food to salivary fluid 1:1 (w/v))
pH	6.8	7
Enzymes	α -amylase 0.58 g/L	α -amylase 75 U/mL
Duration	5 min	2 min
Gastric phase		
Dilution	12 mL gastric fluid	1:1 (v/v) with gastric fluid
pH	2-3	3
Enzymes	Pepsin 5 g/L	Pepsin 2 000 U/mL
Duration	120 min	120 min
Intestinal phase		
Dilution	12 mL intestinal fluid	1:1 (v/v) with intestinal fluid
pH	6.5-7	7
Enzymes	Pancreatin 18 g/L Lipase 3 g/L	Pancreatin (based on trypsin activity of 100 U/mL) or Trypsin 100 U/mL Chymotrypsin 25 U/mL Pancreatic lipase 2 000 U/mL Colipase [2:1 (w/w) ratio with lipase] Pancreatic amylase 2 000 U/mL
Bile salts	6 mL, 60 g/L	10 mM
Duration	120 min	120 min

Although the two methods (Versantvoort *et al.*, 2005; Minekus *et al.*, 2014) simulate the same digestive phases, make use of similar enzymes and have a similar duration, the differences in the units for expressing the concentrations of the reagents make it extremely difficult compare the methods directly. Furthermore, static models are overly simplified compared to human digestion and do not mimic dynamic processes such as the secretion of digestive fluids over time, gradual changes in pH and gastric emptying (Guerra *et al.*, 2012). These factors affect the kinetics of macronutrient hydrolysis and absorption (Dupont, 2016).

Dynamic models simulate the physiological conditions during digestion better, including more realistic transit times and simulation of the complex peristaltic movements in the digestive tract. The most well-known dynamic models are the TNO Gastrointestinal Model (TIM), the Dynamic Gastric Model (Institute of Food Research, United Kingdom), the Human Gastric Simulator (Riddet Model, University of California, Davis) and DIDGI® system (French National Institute for Agricultural Research) (Dupont, 2016).

If a substance is indeed released from the matrix during gastrointestinal digestion, the next step would be transport across the intestinal epithelium into the hepatic portal vein (Versantvoort *et al.*, 2005). Trans-epithelial transport is often simulated *in vitro* by using epithelial cell models based on cell lines such as Caco-2 (Lea, 2015), HT29 (Martínez-Maqueda *et al.*, 2015) and IPEC-J2 (Vergauwen, 2015). An alternative approach is the use of *in vitro* intestinal tissue models, such as intestinal segments or rings from human or animal origin (Verhoeckx and Cotter, 2015b). Some gastrointestinal models, e.g. the TIM and tiny-TIM make use of dialysis through a semipermeable membrane to separate the digested and dissolved molecules from the intestinal lumen (Verwei *et al.*, 2016). This simplified approach allows for sampling during the digestion process and the assessment of the bioaccessible fraction in the dialysate.

2.8.4 Current knowledge on fumonisin bioavailability

Shier (2000) used the term “*fumonisin paradox*” for the observation that fumonisins cause agriculturally significant diseases, despite having an apparent low bioavailability after oral administration. Studies on rats (Shephard *et al.*, 1994a; Dantzer *et al.*, 1999; Martinez-Larranaga *et al.*, 1999) and non-human primates (Shephard *et al.*, 1994b; Shephard and Snijman, 1999) revealed that FB is mainly excreted in the faeces (60-80%) and to a lesser extent in urine (<4%) after intragastric administration, and that only traces are retained in organs, mainly the liver and kidneys. Similar results were obtained in a recent piglet feeding study (Souto *et al.*, 2017). It was found that fumonisins are mainly excreted *via* the bile and the exposure of the intestine to the toxin may be increased by enterohepatic circulation (Bouhet and Oswald, 2007). They reported that despite a bioavailability of only 3-6%, FB was cytotoxic to the intestinal epithelial cells, disrupted lipid biosynthesis and altered the barrier function of the intestine.

Human studies are extremely rare, but confirm that <1% of FB₁ is excreted *via* urine (Riley *et al.*, 2012). It is also interesting to note that, although present in the feed or food, FB₂ and FB₃ were either not detected, or detected in disproportionately low amounts in the urine and organs (Voss *et al.*, 2017).

From a food safety viewpoint, the main concerns are the contamination level of foodstuffs and whether the FB present will be released during digestion in the human body. Furthermore, bound mycotoxins may be regarded as detoxified if they are not released from the food matrix during food processing or in the human digestive system (Berthiller *et al.*, 2013). Currently there is not sufficient information available to establish to what extent FB and their modified forms are released in the human gut. According to Motta and Scott (2007, 2009), an *in vitro* digestion procedure simulating gastrointestinal conditions released only about half of the free FB₁ and half of the bound FB₁ in a contaminated cornflakes sample. On the contrary, Dall'Asta *et al.* (2010) found that the total fumonisin levels measured after digestion of a raw maize sample were higher than those measured by the standard procedure (extractable fumonisins) and were comparable to fumonisin levels measured after alkaline hydrolysis. A similar result was obtained in a follow-up study by when a variety of maize-based products were analysed (Falavigna *et al.*, 2012). The reason for the apparent disagreement in results of some of the studies is not clear, although it should be noted that the researchers mentioned above used the same *in vitro* digestion procedure (Versantvoort *et al.*, 2005) and all indicated that modified fumonisins could indeed be released from food matrices during digestion.

To assess the risk that FB poses to human health, more research is required on the effect of digestion on the release of fumonisins from different food matrices. Dall'Asta *et al.* (2010) and Falavigna *et al.* (2012) determined the bioaccessibility of fumonisin from unprepared food samples, which implies that some of the products (e.g. maize, cornmeal and pasta) were raw. The results would have been more relevant if the food products had been prepared in the same way as they would typically be consumed, before subjecting them to *in vitro* digestion.

2.9 CELL CULTURE-BASED TOXICITY ASSAYS

Although *in vitro* cell culture-based toxicity assays have known limitations, they are useful in preclinical safety assessments (Astashkina *et al.*, 2012). Examples of cytotoxicity assays, measuring general cell viability or cell proliferation endpoints, include the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Alamar Blue, CellTiterGlow, ToxiLight, Lactate dehydrogenase (LDH), neutral red and CyQuant assays. The mutagenic potential of substances may also be evaluated in cell cultures using tests such as the comet assay and micronucleus assay (Araldi *et al.*, 2015). The principles of the neutral red assay and comet assay are briefly described in the next sections, because these assays were selected for the current study.

2.9.1 Neutral red assay

The neutral red uptake assay is a cytotoxicity test which provides a quantifiable estimate of the number of viable cells in a culture (Repetto *et al.*, 2008). This assay is based on the ability of viable cells to incorporate neutral red dye into their lysosomes. Cells are seeded in 96-well tissue culture plates, exposed to the test substance and then incubated with a medium containing neutral red. The dye moves freely into cells. Due to the acidic pH in the lysosomes, the dye is protonated and bound in the lysosomes. As cells lose their viability and die, their lysosomes are damaged and the ability to incorporate neutral red decreases. After incubation with neutral red, the cells are washed to remove the unbound dye. The bound dye remaining in the well is subsequently extracted with a solvent and the absorbance is measured at 540 nm. A loss of neutral red uptake corresponds to a loss of cell viability.

2.9.2 Comet assay

Genotoxicity or deoxyribonucleic acid (DNA) damage may be quantified using the comet assay, which is a single cell microgel electrophoresis technique (Singh *et al.*, 1988; Liao *et al.*, 2009; Araldi *et al.*, 2015). Damage to DNA may occur due to 1) the

direct modification of DNA by chemical agents or their metabolites; 2) from the processes of DNA excision repair, replication, and recombination; or 3) from the process of apoptosis (Ladeira, 2016). The damage causes breaks in the DNA strands; the resulting DNA segments migrate out of the cell nucleus towards the anode of the electrophoresis chamber forming what appears to be the tail of a comet (Singh *et al.*, 1988; Araldi *et al.*, 2015). Intact DNA remains in the cell nucleus and forms the head of the comet. The size of the tail is quantified by manual scoring or image analysis software and is proportional to the extent of DNA damage. This method is sensitive and can measure low levels of DNA damage. It has the added advantage that the effects are observed at single cell level. Genotoxicity assessment is performed at a concentration of the test substance where the cell culture cells are still viable.

Although results from cell culture-based assays cannot be directly extrapolated to animals and humans, these techniques are valuable for screening purposes (Das, 2018).

2.10 FISH EMBRYO TOXICITY (FET) TEST USING ZEBRAFISH

2.10.1 Introduction

As a primary vertebrate class, fish have traditionally been regarded as an indispensable component of integrated toxicity testing strategies for aquatic pollution (Braunbeck and Lammer, 2006; Lammer *et al.*, 2009). Their presence and/or absence in aquatic systems has served as an indicator of water quality. In addition, research has indicated that monitoring early life stage development of fish is an accurate predictor of long-term toxicity (McKim, 1977). As a result, fish embryo tests (FET) have been developed for assessing acute toxicity, with mortality as the primary endpoint (Embry *et al.*, 2010). Other endpoints are demonstrated by coagulation of the developing embryo, a lack of heartbeat and somite formation, and non-detachment of the tail (Braunbeck *et al.*, 2005; Braunbeck and Lammer, 2006; Lammer *et al.*, 2009). The FET has been evaluated against a large number of tested compounds and it was established that the results obtained correlate well with the

acute fish toxicity test (Lammer *et al.*, 2009) and rodent toxicity test (Ducharme *et al.*, 2013).

2.10.2 Ethical considerations

Researchers depend on animal models to understand the pathogenesis of human disease at cellular and molecular level (Lieschke and Currie, 2007). However, experimentation on animals must be justified, and harm to the animals must be reduced to a minimum. Fish acute toxicity methods are used across the world to assess the hazards and risks associated with industrial effluent, municipal wastewater, pesticides, biocides and pharmaceuticals (Embry *et al.*, 2010). The zebrafish (*Danio rerio*) is an established model in developmental biology, toxicology and genetic research (Dooley and Zon, 2000; Hill *et al.*, 2005; Busch *et al.*, 2011; Ali *et al.*, 2012). According to EU Directive 2010/63/EU, concerning the protection of animals used for scientific purposes, the earliest developmental stages of animals are not defined as protected and are therefore exempt from the regulatory frameworks dealing with animal experimentation (Strähle *et al.*, 2012). As illustrated in Figure 2.16, the use of fish embryos is not restricted before the stage of independent feeding, and is therefore gaining popularity as an alternative to animal studies in Europe (Embry *et al.*, 2010).

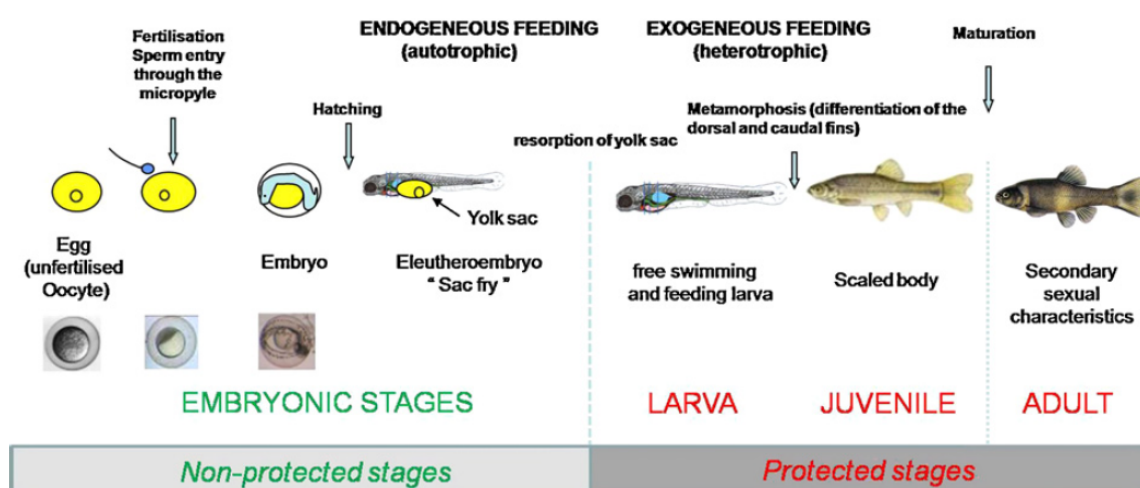


Figure 2.16: Fish life-stages and EU Directive (2010/63/EU) protection status (Embry *et al.*, 2010)

In South Africa there is no single law prescribing animal use for scientific research, however, there are several different laws and acts that must be considered. According to the South African National Standard (SANS) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008), all experimentation on live non-human vertebrates and higher invertebrates must adhere to specific requirements. This standard includes eggs and embryos from fish under the category of animals. Although the standard is voluntary, all animal facilities must be registered with the South African Veterinary Council (SAVC), who impose the SANS regulation. The Tshwane University of Technology's Animal Research Ethics Committee (AREC) is registered with the SAVC, therefore the zebrafish facility is subject to inspections by the SAVC and the National Council of the Society for the Prevention of Cruelty to Animals (NSPCA). Furthermore, the SPCA act, Animal Protection Act and Rule 32 of the Veterinary and Para-veterinary Professions Act (19 of 1982) must be adhered to.

2.10.3 Justification for choosing FET

The core ethical principles of animal research (replace, reduce and refine – known as the three R's) require that animals must be replaced with non-animal models, lower life forms or immature life stages of vertebrate where possible; that the number of animals be kept to the minimum that will produce valid results; and that the life time harm to each animal be minimised (MRC, 2004). However, *in vitro* tests have limitations and do not provide the same biological information as animal tests (De Araújo *et al.*, 2014). Due to the complexity of the zebrafish embryo, it is better suited than cellular models to research the modes of action of drugs and toxins (Busch *et al.*, 2011). Embryonic development is a resistant life stage, even more so than the developmental stages that succeed it (Woltering, 1984). The embryo has been shown to experience less pain, suffering and distress than more mature stages (Embry *et al.*, 2010), therefore it is clearly favourable to make use of fish embryos, instead of adult fish or rodents, when possible.

The normal morphology, physiology, and biochemistry of zebrafish at all stages of early development, in juveniles and adults of both sexes, are well researched (Hill

et al., 2005; Ali *et al.*, 2012). In addition, the transparent appearance of the embryo allows for unobstructed observation and easy detection of morphological disorders, such as malformation and missing organs (Hill *et al.*, 2005; Busch *et al.*, 2011; Weigt *et al.*, 2011). Unlike rodents, in which the embryos must be harvested, the embryonal development of zebrafish can be continuously followed in live individuals (Hill *et al.*, 2005). Zebrafish is an outstanding vertebrate model, because its developmental stages are very similar to that of mammals, including humans (Hill *et al.*, 2005; Wu *et al.*, 2012). This makes the zebrafish ideal for toxicology research where the objective is to identify the adverse effects of chemical exposure. This model can also provide insight into the mechanisms of toxicity of chemicals, is useful as a screening tool, and complements cell-based assays (Hill *et al.*, 2005).

2.10.4 Suitability for fumonisin toxicity testing

Fumonisin are known to be toxic to humans and a variety of animal species (Gelderblom *et al.*, 1988; Gelderblom *et al.*, 2001a; Voss *et al.*, 2007). However, the mode and mechanism whereby FB₁ exerts toxicity is complex and seems to vary in different cells, tissues and animal species (IARC, 2002). The toxin usually targets the brain, liver, heart or kidneys. It is estimated that evolutionary divergence between zebrafish and mammals occurred about 445 million years ago (Peterson *et al.*, 2004). In spite of the long evolutionary separation, there is a high degree of genetic similarity between zebrafish and humans (Harper and Lawrence, 2011). The kidneys of the embryonic zebrafish demonstrate complex renal functions similar to those of the higher vertebrate kidney (Hill *et al.*, 2005; Wu *et al.*, 2012). Zebrafish embryos are also suitable for studying cardiovascular and hematopoietic disorders (Dooley and Zon, 2000). The zebrafish embryonic heart resembles the heart of the human embryo at three weeks gestation and starts beating at approximately 22 hours post fertilisation (hpf). Blood circulation usually starts by 24 hpf.

A summary of publications on the use of the zebrafish model in mycotoxin toxicological studies is provided in Table 2.4. As far as could be ascertained using databases, no research has been published to determine the toxicity of fumonisins

using this test (literature search using Science Direct, Google Scholar and Scopus dated September 2018).

Table 2.4: Summary of publications on the use of zebrafish to assess mycotoxin toxicity

Mycotoxin	Method/conditions	Main findings
AFB ₁ (Troxel <i>et al.</i> , 1997)	Adult zebrafish injected with aflatoxin (50-400 µg/kg)	AFB ₁ rapidly metabolised and excreted DNA adducts formed
CTN, PAT (Wu <i>et al.</i> , 2012)	Zebrafish embryos exposure, up to 96 hpf	CTN-induced expression of proinflammation genes, CTN and PAT caused nephrotoxicity
ZEN (Bakos <i>et al.</i> , 2013)	Zebrafish embryos and larvae (120 hpf) Adult males	LC ₅₀ of 893 µg/L Defects in heart and eye development, abnormal fin development and estrogenic effects
DON (Sanden <i>et al.</i> , 2012)	Zebrafish larvae (30 days post hatch) fed diets spiked with DON	DON detoxified in the liver through the phase 1 system, disturbance in oxidative balance, but no effects on fish growth performance
OTA (Wu <i>et al.</i> , 2018)	Wild type and transgenic zebrafish embryos (6 hf) exposed to OTA (0.025-0.5 µM)	OTA exposure resulted in the generation of small livers that led to deficiency of coagulation factors in embryonic zebrafish
AFB ₁ , DON ZEN (Zhou <i>et al.</i> , 2017)	48-120 hpf	AFB ₁ (1.28 µM) 100% mortality at 48 hpf ZEN (18.85 µM) high mortality at 48 hpf DON no mortalities up to 135 µM
T-2 toxin (Yuan <i>et al.</i> , 2014)	Zebrafish embryos exposure, up to 144 hpf	Exposure to 0.2 µM or higher increased mortality and malformation Oxidative stress and cell apoptosis observed

AFB₁ = Aflatoxin B₁, CTN = Citrinin, DON = Deoxynivalenol, PAT = Patulin, OTA = Ochratoxin A, ZEN = Zearalenone, hpf = hours post fertilisation, LC₅₀ = concentration to kill 50% of test organisms

Since the zebrafish embryo model has been used successfully to study the toxicity of other mycotoxins, and the organism possesses the organ systems commonly affected by fumonisins, it has great potential for fumonisin toxicology research and was identified as the toxicity model of choice for this study.

2.11 *FUSARIUM* DISEASE CYCLE AND MYCOTOXIN PRODUCTION

The genus *Fusarium* is a complex polyphyletic grouping which is distributed worldwide and flourishes in many different environments (Summerell *et al.*, 2010). The genus encompasses a variety of fungal plant pathogenic species, which are responsible for enormous production losses in agriculture and horticulture, including food crops such as wheat and maize. There are two key diseases of maize ears that are caused by *Fusarium* spp., namely Fusarium ear rot (primarily due to *F. verticillioides*, *F. subglutinans* and *F. proliferatum*) and Gibberella ear rot (primarily due to *F. graminearum*) (Munkvold, 2003). In addition to being plant pathogens of agricultural importance, these fungi produce mycotoxins, which are toxigenic secondary metabolites that are of major concern to the human food and animal feed industries (Ncube *et al.*, 2011). *Fusarium verticillioides* and *F. proliferatum* are producers of fumonisins, whereas *F. subglutinans* produces moniliformin, and *F. graminearum* produces zearalenone and deoxynivalenol.

Fusarium verticillioides is the most common maize pathogen. It is ubiquitous and infects the plant at all stages of development, typically by airborne conidia *via* the silk channel or wounds, and systemically, due to infected seeds (Munkvold and Desjardins, 1997; Munkvold and White, 2016). The disease cycle of *Fusarium* in maize has a saprophytic, as well as a pathogenic stage, and is illustrated in Figure 2.17.

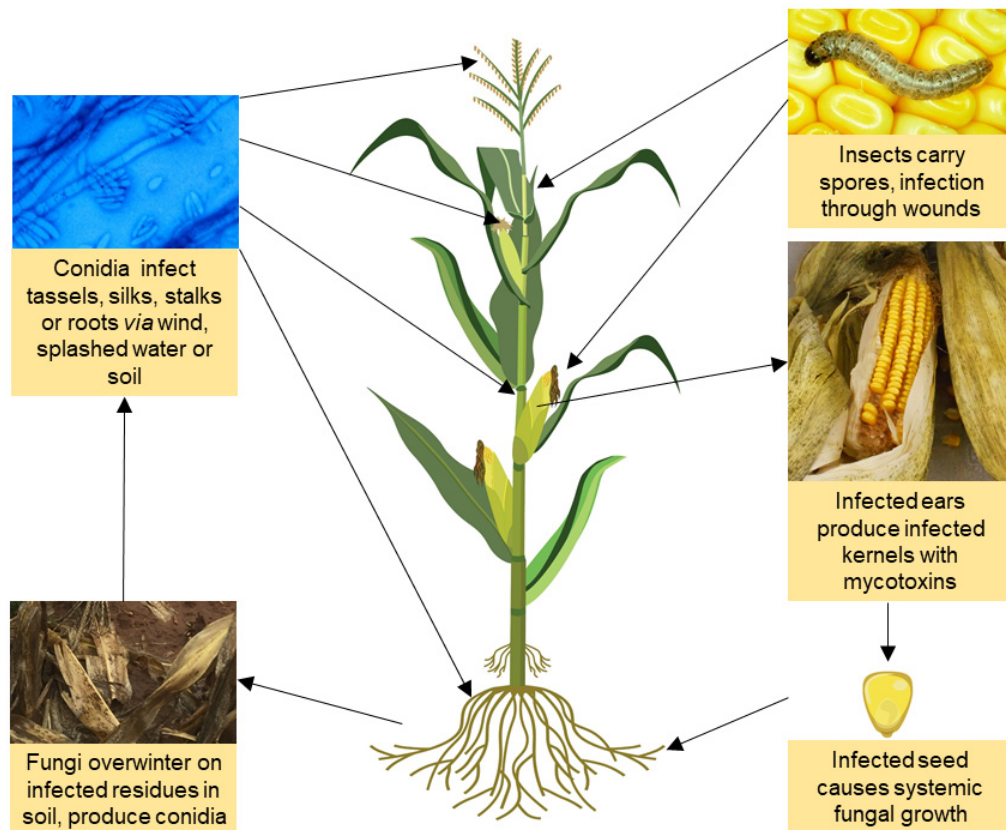


Figure 2.17: Disease cycle of *Fusarium verticillioides* in maize²

Fusarium verticillioides overwinters in maize debris and soil, infecting germinating seeds and roots in the next season, and moving into the plant through systemic growth (Battilani *et al.*, 2003; Munkvold and White, 2016). Airborne micro- and macroconidia are also produced from sporulation on the tassels of the previous crop residue. Susceptibility to infection is increased by moisture on the silks, damage by other ear rot infections, and injuries caused by insects (Munkvold and Desjardins, 1997). Some insects, such as the European corn borer (*Ostrinia nubilalis* Hübner) and African maize stalk borer (*Busseola fusca* Fuller), can also act as vectors for the spread of *F. verticillioides*.

Fumonisin production by *Fusarium* spp. is regulated by the fumonisin biosynthetic gene cluster (*FUM*) (Proctor *et al.*, 2013), which is illustrated in Figure 2.18.

² Image of maize plant purchased from iStock (order number 2053995147). Photo of corn borer from <https://www.ent.iastate.edu/pest/cornborer/media-gallery/detail/192/378> [Accessed 13/03/2018]. Disease cycle (Battilani *et al.*, 2003; Munkvold and White, 2016)

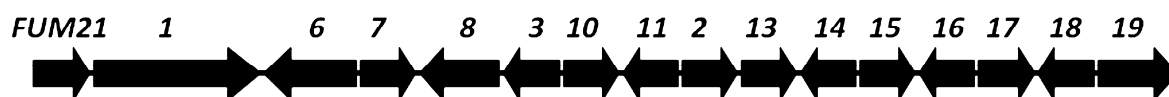


Figure 2.18: Organisation of the fumonisin biosynthetic gene cluster (Proctor *et al.*, 2013)

Fusarium verticillioides and *F. proliferatum*, as well as some strains of *F. oxysporum*, share the same number, order and orientation of genes within the *FUM* cluster, although the genomic location is different in the three species (Waalwijk *et al.*, 2004). The 16 genes in the fumonisin biosynthetic gene cluster encode biosynthetic enzymes, as well as regulatory and transport proteins (Proctor *et al.*, 2013). Figure 2.19 provides an overview of the role of *FUM* genes in fumonisin biosynthesis.

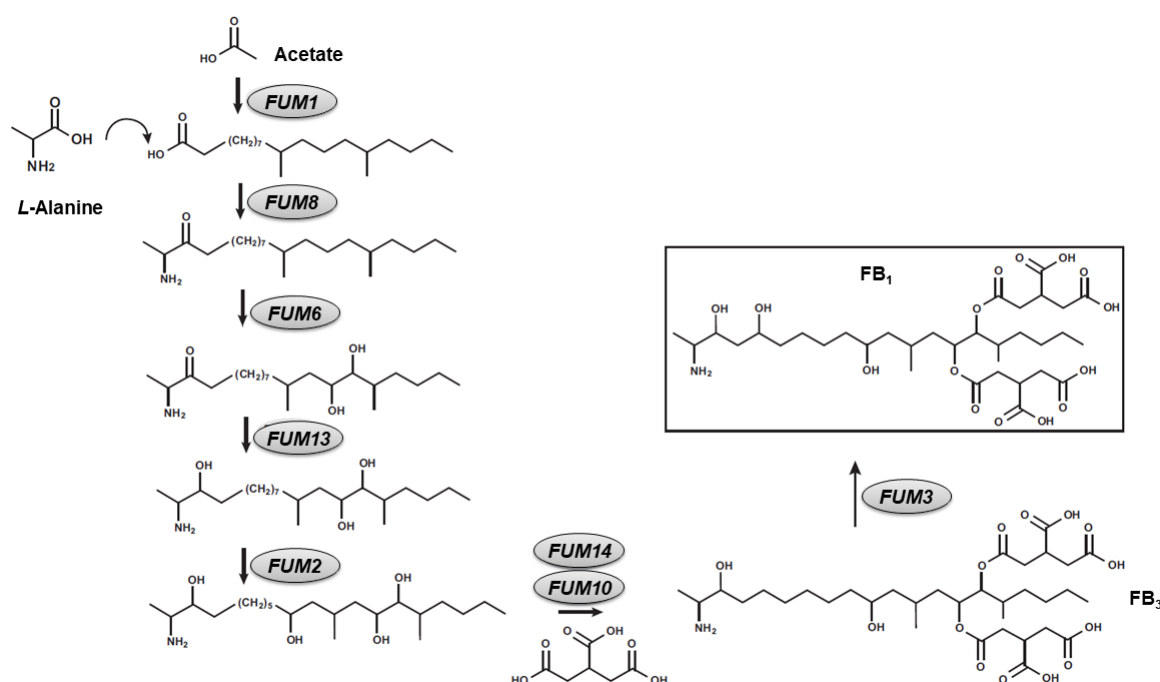


Figure 2.19: The role of *FUM* genes in fumonisin biosynthesis (De la Torre-Hernández *et al.*, 2014)

The first step involves polyketide synthase, which synthesises the backbone of the fumonisin molecule, which is subsequently modified by other enzymes (Rocha *et al.*, 2015; Medina *et al.*, 2016). The role of the *FUM* genes in fumonisin biosynthesis may be summarised as follows:

- synthesis of the linear polyketide backbone structure (*FUM1*), amino transferase (*FUM8*), C-3 carbonyl reductase (*FUM13*) and cytochrome P450 oxygenase (*FUM2*, *FUM3*, *FUM6* and *FUM15*),
- tricarballic acid ester esterification (*FUM7*, *FUM10*, *FUM11*, *FUM14* and *FUM16*),
- longevity assurance (*FUM17*, *FUM18* and *FUM19*); and
- DNA-binding transcription factor (*FUM21*) which regulates *FUM* gene expression.

The growth of toxigenic *Fusarium* spp. and the production of fumonisin mycotoxins is a complex biological process that is affected by complex interactions between several abiotic and biotic factors (Gong *et al.*, 2015), some of which will be discussed in the next section.

2.12 STRATEGIES USED TO CONTROL FUSARIUM AND REDUCE FUMONISINS IN MAIZE

Since maize is a staple food in South Africa, it is crucial that strategies are developed to reduce fumonisin contamination, because the quality and safety of the crop and the health of the consumers depend on it. Prevention is better than cure, therefore the first focus is to reduce infection by *F. verticillioides* and prevent the production of fumonisins in maize in the field. Luo, Liu and Li (2018) recently published a comprehensive review on strategies for pre- and postharvest control of mycotoxins. In this section of the literature review, the following preharvest strategies are briefly discussed: crop production practices, commercial fungicides, transgenic maize, biocontrol microorganisms and the application of EOs. Selected methods for postharvest remediation and detoxification are also summarised, highlighting methods suitable for food production.

2.12.1 Pre-harvest mycotoxin control

2.12.1.1 Crop production practices

The growth of *F. verticillioides* and the production of mycotoxins are affected by a complex interaction of biotic and abiotic factors. Environmental factors have an enormous effect on the accumulation of mycotoxins in food crops (Patriarca and Fernández Pinto, 2017). Hot and dry growing conditions are known to increase fumonisin contamination levels (Ahangarkani *et al.*, 2014). A recent study found that temperatures of 29-32 °C and relative humidities of 27-32% favour fumonisin production in South African commercial maize (Janse van Rensburg *et al.*, 2017). The advent of global warming is expected to change the geographical distribution of mycotoxigenic fungi, and increase the risk of mycotoxin contamination in staple crops such as maize (Medina *et al.*, 2017).

Climatic conditions cannot be controlled, but the following cropping practices contribute to creating less favourable conditions for fumonisin production (Luo *et al.*, 2018)³:

- nitrogen fertilisation,
- timing of sowing and harvesting,
- insect control,
- correct plant density; and
- crop rotation with plants that do not host *Fusarium* spp.

2.12.1.2 Commercial fungicides and insecticides

Currently there are no fungicides registered for the control of ear rots in South Africa⁵. In Europe, foliar fungicide sprays are not used, although it is common practice for maize seeds to be treated with fungicides containing amide, dithiocarbamate or pyrrole as active ingredients (Meissle *et al.*, 2010). Studies on the use of fungicides to control pure cultures of pathogens in the laboratory, as well as on field trials involving crops, seem to produce contradictory results (D'Mello *et*

³ <http://www.grainsa.co.za/how-to-manage-fusarium-ear-rot-and-fumonisin-production>
[Accessed 01/03/2018]

al., 1998). Sub-lethal doses of fungicides (carbendazim, tridemorph, difenoconazole and tebuconazole with triadimenol) resulted in an increase in mycotoxin production by *Fusarium* spp. Furthermore, maize ears are covered by tight husks, which prevent contact of fungicides with the ear rot pathogens and increase their susceptibility to infection (Beukes *et al.*, 2017).

Insecticides have been used with success to reduce fumonisin contamination of maize. The European corn borer is a common maize pest. Its larvae tunnel into the stalks and ears of the plant and cause damage that increases susceptibility to *F. verticillioides* and subsequent fumonisin contamination (Blandino *et al.*, 2009; Folcher *et al.*, 2009). Optimal application of insecticides can therefore reduce mycotoxin contamination. For example, the carbamate insecticide, benfuracarb, is effective to control African maize stalk borer and reduces *Fusarium* ear rot as well as fumonisin contamination levels (Ncube *et al.*, 2017). Although carbamates have a relatively short biological half-life and therefore a low bioaccumulation potential, they are not only toxic to insects, but also to mammals, birds, bees and aquatic organisms (Eren *et al.*, 2016).

2.12.1.3 Transgenic maize

Maize insect resistance may be increased by genetic modification, for example by inserting genes from the bacterium *Bacillus thuringiensis*, which encodes for the expression of insecticidal crystal proteins (Munkvold and White, 2016). These hybrids, called *Bt* maize, also contain high concentrations of lignin, which contributes to the plant's defence against insects and pathogens (Ncube *et al.*, 2018). Better resistance to pests, such as the corn borer, is associated with less damage to the plant, which reduces fungal infections and fumonisin contamination (Alberts *et al.*, 2017). However, the degree of infestation by the African maize stalk borer and infection by *Fusarium* ear rot is unpredictable and is affected by climatic conditions, causing inconsistencies in the efficiency of *Bt* maize to resist *Fusarium* infection from season to season (Ncube *et al.*, 2018). Subsistence farmers in South Africa are not likely to purchase the expensive transgenic maize hybrid seeds (Alberts *et al.*, 2017), making them vulnerable to fumonisins. Genetic control is also

not a universal solution to control fumonisins, because the use of genetically modified maize is forbidden in some European countries (Scarpino *et al.*, 2018).

2.12.1.4 Biocontrol microorganisms

Alberts *et al.* (2016) reviewed biologically-based strategies to control fumonisin-producing *Fusarium* species. Biocontrol organisms usually inhabit the same ecological niche as the pathogens. The mechanism of action involves a three-way interaction between the host plant, the pathogen and the antagonistic microorganism. This may involve, amongst others, competition for nutrients and space, parasitism of the pathogen, secretion of antifungal compounds and induction of systemic resistance. The biocontrol organisms are usually applied to seed or soil, but may also be applied to crops after harvest (Alberts *et al.*, 2017). The following are examples of organisms that have been found to have potential for controlling *Fusarium verticillioides*: *Trichoderma* spp., *Bacillus subtilis*, *Bacillus mojavensis* and *Pediococcus pentosaceus* (Alberts *et al.*, 2016). Non-pathogenic strains of pathogenic species are often applied for biocontrol.

Although there has been success with biocontrol, its potential for application in rural subsistence farming communities is questionable and there are a number of other factors that must be considered (Alberts *et al.*, 2017), which include the following:

- gene transfer between toxigenic and non-toxigenic strains,
- production of other mycotoxins by the biocontrol agent,
- damage to crops by non-toxigenic strains under favourable climatic conditions; and
- potential health risks to field workers and communities.

2.12.1.5 Essential oils

Public concern over the health and environmental hazards associated with synthetic pesticides is on the increase. Essential oils (EOs) are natural volatile organic compounds that have been isolated from aromatic plants using steam distillation (Saad *et al.*, 2013). These volatile metabolites protect the plant by functioning as antibacterial, antiviral, antifungal and insecticidal agents (Bakkali *et al.*, 2008). Since EOs are biodegradable and relatively non-toxic to mammals, birds and fish, they are considered to be eco-friendly (Dwivedy *et al.*, 2016) and are gaining popularity as green pesticides (Koul *et al.*, 2008).

Essential oils act as antifungal agents *via* two main mechanisms, namely disruption of the structure and function of the cell membranes/walls of the pathogen, and by causing dysfunction within the cells (Nazzaro *et al.*, 2017). Cell membranes may be disrupted when EO components bind to ergosterol, which is essential for the integrity and function of the cell membrane, while cell walls are disrupted by blocking the formation of β -glucans. Within the fungal cells, the mitochondrial electron transport chain, proton pumps or efflux pumps may be inhibited. The activity of enzymes, such as pectin methyl esterase and cellulase may also be inhibited (Marei *et al.*, 2012). The mode of action is broad due to the complex chemical composition of the EOs. This complexity is a distinct advantage, because fungi are less likely to develop resistance towards compounds with multifaceted bioactivities (Koul *et al.*, 2008).

Essential oils consist mainly of aliphatic hydrocarbons and terpenes, although some volatile phenolic compounds may be present. The terpenic fraction represents various compound classes including alcohols, ketones, aldehydes and ethers (Saad *et al.*, 2013). Monoterpenes usually make up the largest proportion of most EOs (Nazzaro *et al.*, 2017). The antifungal activity of an EO depends on its chemical composition. The activity of the individual components has been reported to increase in the following order: hydrocarbons < ethers < ketones $\leq$ aldehydes < alcohols < cinnamic aldehydes < phenols. Optimal antifungal activity is obtained with phenolic compounds and aromatic aldehydes that possess hydrophobic

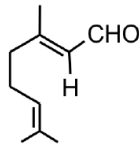
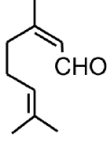
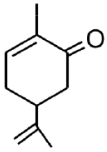
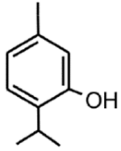
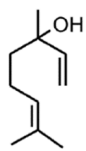
properties. Selected publications demonstrating the potential of EOs to control *Fusarium* spp. are summarised in Table 2.5.

Table 2.5: Summary of selected publications demonstrating the potential of essential oils to control *Fusarium* spp.

<i>Fusarium</i> spp.	Effective essential oils and minimum inhibitory concentrations
<i>F. graminearum</i>	citronella (1 500 µL/L), clove (1 500 µL/L), lemongrass (1 000 µL/L) and peppermint (500 µL/L) (Sreenivasa <i>et al.</i> , 2011) clove, cinnamon, lemongrass, oregano and palmarosa (1 000 mg/L) (Velluti <i>et al.</i> , 2004a) holy basil (1 500 mg/L) (Kalagatur <i>et al.</i> , 2015)
<i>F. oxysporum</i>	citronella (2 000 µL/L) and peppermint (2 500 µL/L) (Sreenivasa <i>et al.</i> , 2011) clove (500 µL/L), lemongrass (300 µL/L) and thyme (500 µL/L) (Seseni <i>et al.</i> , 2015) eucalyptus (7 000 µL/L) (Gakuubi <i>et al.</i> , 2017) lemongrass (50 µL/L) (Linde <i>et al.</i> , 2010)
<i>F. proliferatum</i>	citronella (1 500 µL/L), clove (1 500 µL/L), lemongrass (1 500 µL/L) and peppermint (2 500 µL/L) (Sreenivasa <i>et al.</i> , 2011) clove, cinnamon, lemongrass, oregano and palmarosa (1 000 mg/L) (Velluti <i>et al.</i> , 2004a) eucalyptus (8 000 µL/L) (Gakuubi <i>et al.</i> , 2017)
<i>F. solani</i>	citronella (1 500 µL/L), lemongrass (500 µL/L) and peppermint (1 500 µL/L), (Sreenivasa <i>et al.</i> , 2011) eucalyptus (8 000 µL/L) (Gakuubi <i>et al.</i> , 2017)
<i>F. subglutinans</i>	eucalyptus (8 000 µL/L) (Gakuubi <i>et al.</i> , 2017)
<i>F. verticillioides</i>	citronella (1 500 µL/L), clove (2 500 µL/L) and peppermint (2 500 µL/L) (Sreenivasa <i>et al.</i> , 2011) clove, cinnamon, lemongrass, oregano and palmarosa (1 000 mg/L) (Velluti <i>et al.</i> , 2004a) eucalyptus (8 000 µL/L) (Gakuubi <i>et al.</i> , 2017) ginger (2 000 mg/L) (Yamamoto-Ribeiro <i>et al.</i> , 2013) rosemary (300 mg/L) (Da Silva Bomfim <i>et al.</i> , 2015) turmeric (300 mg/L) (Avanço <i>et al.</i> , 2017)

It is evident that EOs have the potential to control fungal growth and mycotoxin production in agricultural crops such as maize both preharvest, postharvest and during storage. In Table 2.6 an overview of the main chemical components of lemongrass, spearmint and thyme EOs is provided.

Table 2.6: Main chemical constituents (Combrinck *et al.*, 2011) and approximate price of lemongrass, thyme and spearmint essential oils

Essential oil	Main chemical components	Chemical structures of main components		Approximate Price (US\$/L) ⁴
Lemongrass (<i>Cymbopogon citratus</i>)	geranial (42.5%) neral (31.7%) limonene (8.9%) α -terpineol (5.2%) citronellol (2.8%) linalool (2.8%)			\$74
Spearmint (<i>Mentha spicata</i>)	R-carvone (87.9%) limonene (2.6%) dihydrocarvone (2%) dihydrocarveol acetate (1.9%) dihydrocarveol (1.1%)			\$112
Thyme (<i>Thymus vulgaris</i>)	thymol (63.1%) linalool (21.3%) benzyl alcohol (4.3%) β -caryophyllene (1.7%) α -terpineol (1.6%)			\$88

These EOs were selected for the current study based on price, availability, accepted use in consumer products and fungicidal potential.

2.12.2 Postharvest mycotoxin control

At harvest maize typically contains 17 to 25% moisture (Bern *et al.*, 2019). The maize is dried to <13% moisture to lower the water activity and prevent the growth of fungi and production of mycotoxins during storage. The first step in postharvest mycotoxin control, is to ensure that the moisture content of the grain, as well as the temperature and humidity during storage, are regulated to prevent fungal growth (Luo *et al.*, 2018). Although fumonisin contamination occurs mainly on the field and not during storage, contamination levels may increase during storage if conditions are favourable.

⁴ <http://www.holisticemporium.co.za/> [Accessed 22/03/2018]

Physical methods of postharvest processing, e.g. sorting, washing and separation are effective to reduce fumonisin contamination levels (Karlovsky *et al.*, 2016; Alberts *et al.*, 2017). Some of these aspects have already been discussed (Section 2.5.1). In addition, adsorbent binders, e.g. charcoal, hydrated sodium calcium aluminosilicates, have been used with success in animal feed (Luo *et al.*, 2018).

Chemical methods for reducing mycotoxins include treatment with bases, oxidising agents and organic acids. These methods are not all suitable for detoxifying fumonisins in food, although alkaline processing (e.g. during the manufacture of tortillas; Section 2.5.2) is known to reduce fumonisin concentrations.

Lately there has been an increased interest in the use of biological methods to mitigate mycotoxins (Patriarca and Fernández Pinto, 2017). Some bacteria, moulds and yeasts have the ability to reduce fumonisin content, either by enzymatic detoxification (e.g. *Exophiala spinifera* and *Sphingomonas spp.*) or by adsorbing to the cell walls (e.g. lactic acid bacteria and *Saccharomyces cerevisiae*) (Karlovsky *et al.*, 2016; Luo *et al.*, 2018). Fumonisins may also be detoxified enzymatically using two enzymes successively: first a carboxylesterase to remove the TCA groups from FB and then an aminotransferase to de-amine the hydrolysed FB (Alberts *et al.*, 2017). Although these enzymes are utilised in commercial animal feed additives (e.g. Mycofix^{®5}) they have not been authorised for reduction of mycotoxin contamination in food (Karlovsky *et al.*, 2016).

When developing fumonisin mitigation strategies, the focus should be on affordable techniques that are effective on an industrial scale, but they should also be practical and acceptable to communities in developing regions (Patriarca and Fernández Pinto, 2017). Currently, physical techniques are the most efficient, but biological, environmentally friendly methods hold great potential for future developments (Karlovsky *et al.*, 2016).

⁵ <http://www.biomin.net/en/products/mycofix/> [Accessed 20/03/2018]

2.13 CONCLUDING REMARKS

Research on fumonisins in maize remains a priority, because it is a staple food that is consumed in large amounts by vulnerable communities. More research is needed on fumonisin toxicity, the interactions of the fumonisins with maize and its macrocomponents, as well as their bioaccessibility when contaminated maize porridge is consumed. Fumonisin contamination is best reduced by applying all available technologies from good agricultural and storage practices to the application of food processing technologies. Essential oils have potential for use as eco-friendly fungicides and their ability to control *Fusarium* infection and fumonisin production should be further investigated.

CHAPTER 3

IMPLEMENTATION OF MODELS TO ASSESS FUMONISIN TOXICITY

3.1 INTRODUCTION

Fumonisin is known to be toxic to a large number of animal species (Gelderblom *et al.*, 1988; Gelderblom *et al.*, 2001a; Voss *et al.*, 2007). However, the mode and mechanism whereby FB₁ exerts toxicity is multifaceted and seems to vary in different cells and species (IARC, 2002). The toxin usually targets the brain, liver, heart or kidneys. Controversies regarding the toxic and carcinogenic properties of fumonisins complicates human risk assessment (Gelderblom and Marasas, 2012) and therefore, more research is required to understand the full extent of the threat that fumonisins pose to human and animal health.

In this study, fumonisin toxicity was assessed using the zebrafish embryo test (FET) in conjunction with cell-based assays. The RTL-W1 trout liver cell line was utilised to assess the cytotoxicity and genotoxicity of FB using the neutral red assay and comet assay, respectively. Since the RTL-W1 cell line is also of fish origin, it was used in the neutral red and comet assays and performed in parallel with the FET test to establish the effective fumonisin concentration range. Although it is not a commercial cell line, it is useful in cytotoxicity and genotoxicity testing (Kosmehl *et al.*, 2008; Kienzler *et al.*, 2012; Zeng *et al.*, 2016). The neutral red cytotoxicity test provides a quantifiable estimate of the number of viable cells in a culture (Repetto *et al.*, 2008), while the comet assay is a single cell microgel electrophoresis technique that provides a quantitative measure of the genotoxicity by evaluating DNA damage (Singh *et al.*, 1988; Liao *et al.*, 2009; Araldi *et al.*, 2015). The zebrafish (*Danio rerio*) is an established model in toxicology research where the objective is to identify adverse effects and provide insight into the mechanisms of toxicity after chemical exposure (Dooley and Zon, 2000; Hill *et al.*, 2005; Busch *et al.*, 2011; Ali *et al.*, 2012). The platform is a useful screening tool and complements cell-based assays (Hill *et al.*, 2005; Wu *et al.*, 2012).

3.2 MATERIALS AND METHODS

3.2.1 Chemical reagents

Fumonisin standards (FB₁ batches A/10-2014, A/19-2016 and A/20-2016, FB₂ batch A/14-2015, FB₃ batch A/07+16-2015 and hydrolysed fumonisin B₁ (HFB₁) batch A09-2014), with purities >96%, were obtained from the Institute of Biomedical and Microbial Biotechnology (IBMB), Cape Peninsula University of Technology (CPUT). All other reagents, cell culture media, and buffers were obtained from Sigma Aldrich/Merck, Darmstadt, Germany.

3.2.2 Cell culture

A rainbow trout (*Oncorhynchus mykiss*) liver cell line, RTL-W1, was used for the cytotoxicity and genotoxicity assays. This cell line was previously supplied to the Aquatic Ecology and Toxicology Center for Organismic Studies (University of Heidelberg, Germany) by the Department of Biology, University of Waterloo (Ontario, Canada). The permanent cell line was cultivated at 20 °C under normal atmosphere in Leibovitz L15 medium, supplemented with 8% foetal calf serum, 100 U penicillin and 100 µg streptomycin. Cells with passage numbers <100 were sub-cultured by trypsinisation once a week with a splitting factor of 1:2.

3.2.3 Neutral red assay

The neutral red assay was performed at the Aquatic Ecology and Toxicology Department, Center for Organismic Studies (University of Heidelberg, Germany). RTL-W1 cells were grown and maintained in cell culture flasks and seeded in 96-well microtiter plates at a density of 2×10^4 cells per well in 100 µL culture medium and left to attach for 24 h. Before exposure, the medium was completely removed and replaced with 100 µL of the test solution according to the procedures described below (Klee *et al.*, 2004).

3.2.3.1 Preliminary evaluation of FB_1 , FB_2 , FB_3 and HFB_1

The cell culture was exposed to the test substances for 24 h at 20 °C. The experimental design (Table 3.1) included 3,5-dichlorophenol (DCP) at a concentration of 80 µg/mL as the positive control (PC), as well as a negative control (NC) consisting of cell culture medium. For the fumonisin standards, a portion of the stock solution (1.00 mg/mL dissolved in deionised water) was freeze-dried and re-dissolved in the same volume of cell culture medium to avoid dilution of the nutrients in the cell culture medium.

Table 3.1: Design of the preliminary fumonisin neutral red assay experiment

Sample	Concentration (µg/mL)						
Negative control (cell culture medium)	-	-	-	-	-	-	-
Fumonisin B ₁	400	200	100	50.0	25.0	12.5	6.25
Fumonisin B ₂	400	200	100	50.0	25.0	12.5	6.25
Fumonisin B ₃	400	200	100	50.0	25.0	12.5	6.25
Hydrolysed Fumonisin B ₁	400	200	100	50.0	25.0	12.5	6.25
Positive control (3,5-dichlorophenol)	80.0	-	-	-	-	-	-

The layout of the 96-well microtiter plate for the neutral red assay, which included positive and negative controls (n=6 and 12, respectively), is illustrated in Figure 3.1. Following exposure to the test substances, cells were stained with culture medium containing 0.005% (w/v) neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) for 3 h. After washing with phosphate-buffered saline (PBS, 200 µL per well), the dye was extracted with deionised water:ethanol:glacial acetic acid (50:49:1, v/v/v), by incubating the plates on a rotary shaker (Gerhardt Analytical Systems, Königswinter, Germany) at 200 rpm for 20 min. The optical density of the neutral red extract was measured in a microtiter plate reader spectrophotometer (Tecan GENios, Crailsheim, Germany) at 540 nm, with 690 nm as the reference. The viability was calculated as the average optical density of each test substance as a percentage of the negative control. For quality control purposes, the experiment was only regarded as valid if the viability of the positive control <20%.

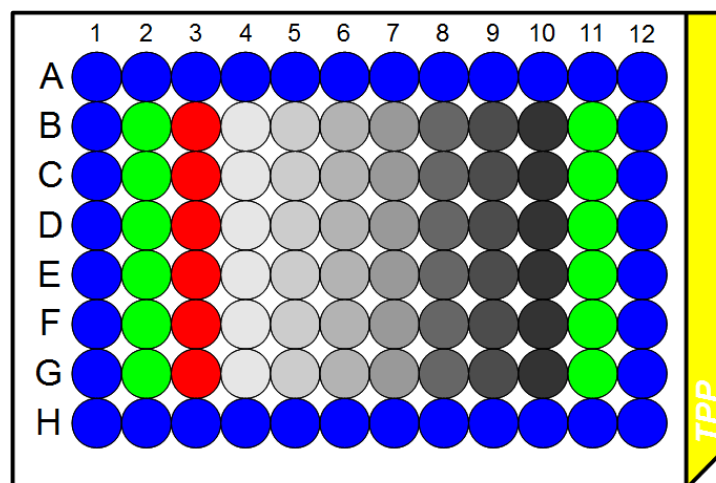


Figure 3.1: Layout of 96-well microtiter plate for the neutral red assay Green = negative control, red = positive control, grey = serial dilutions, blue = blanks⁶

3.2.3.2 Follow-up experiment with increased concentrations of FB₁

In the follow-up experiment, FB₁ was tested at higher concentrations and the exposure time was increased to 48 h (Table 3.2). A fumonisin stock solution (16 mg/mL) was prepared in PBS and serially diluted to concentrations ranging from 8 000 to 125 µg/mL (11 100 to 173 µM). All controls and treatments were performed in cell culture medium:PBS (50:50, v/v), to match the dilution of the medium in the treatment with the highest concentration of fumonisin. A preliminary experiment confirmed that this dilution of the cell culture medium did not alter the viability of the cells. The layout of the microtiter plate was as previously illustrated in Figure 3.1, and the staining and subsequent steps were performed as described in Section 3.2.3.1. Three independent replicates were conducted at separate occasions.

Table 3.2: Design of the follow-up fumonisin neutral red assay experiment

Sample	Concentration (µg/mL)						
Negative control (cell culture Medium:PBS, 50:50)	-	-	-	-	-	-	-
Fumonisin B ₁	8 000	4 000	2 000	1 000	500	250	125
Positive control (3,5-dichlorophenol)	80.0	-	-	-	-	-	-

PBS = phosphate buffered saline

⁶ Standard Operating Procedure for Neutral Red Cytotoxicity Assay, Aquatic Ecology and Toxicology, Center for Organismic Studies, University of Heidelberg, Germany

3.2.3.3 pH control and pH-adjusted experiments

Due to the risk of contamination, pre-exposure pH measurement of the fumonisin solutions was not possible. However, from the colour change in the cell culture medium, it was evident that FB₁ caused an acidic pH shift in the medium. To quantify this shift, the pH of the medium was measured at the end of the experiment described in Section 3.2.3.2. The media from six replicate wells per treatment were collected, pooled and the post-exposure pH measured using a micro-electrode (WTW, Weilheim, Germany).

For the pH control experiments, pH values were adjusted to those previously recorded for media containing different concentrations of FB₁. This was achieved by addition of 1 M HCl to cell culture medium:PBS, 50:50 (v/v). The medium was filter sterilised (0.22 µm cellulose acetate membrane; GE Healthcare, Freiburg, Germany) and the neutral red assay was performed using the same procedure as described in Section 3.2.3.2. After 48 h, the media of six replicate wells per treatment were collected, pooled and the pH measured.

For the pH-adjusted runs, the pH of FB₁-containing media was adjusted to 7.4 (physiological pH) with sterile 100 mM NaOH and phenol red as pH indicator.

3.2.4 Comet assay

The comet assay on the RTL-W1 cell line was carried out under alkaline conditions (pH 13), according to the method previously adapted by the Aquatic Ecology and Toxicology Center for Organismic Studies (University of Heidelberg, Germany) (Kosmehl *et al.*, 2004). Briefly, the cells were grown and maintained in cell culture flasks and seeded in 6-well plates at a density of 2×10^5 cells per well. Cells were exposed to two concentrations (200 and 400 µg/mL) of FB₁, FB₂, FB₃ and HFB₁ for 24 h at 20 °C. Negative controls (cell culture medium) and a positive control (methyl methane sulfonate, 40.0 µg/mL, w/v) were also included in the experimental design. Cells were washed with PBS and detached with a solution containing trypsin (0.05%, w/v) and ethylenediaminetetraacetic acid (0.02%, w/v). Frosted slides were

coated with normal melting point agarose, followed by low melting point agarose containing the cells, and finally an additional layer of normal melting point agarose (Figure 3.2).

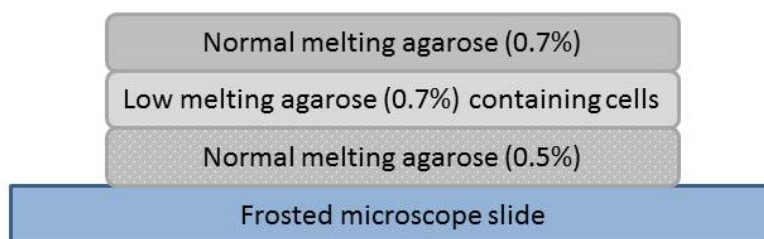


Figure 3.2: Layers of agar on slides prepared for the comet assay

The chambers containing the slides were covered with aluminum foil for protection from light and lysis was performed overnight at 4 °C. Electrophoresis was performed under alkaline conditions (pH 13) in an ice bath for 20 min at 25 V in the dark (Bio-Rad electrophoresis power supply model 200/2.0, Richmond, USA). Slides were neutralised, stained with ethidium bromide and then examined using a fluorescent microscope (Leitz Aristoplan, Leica, Germany), with a grey-scale CCD camera (Pulnix TM-765E, Kinetic Images, Liverpool, UK), and equipped with an image analysis system (Optilas, Munich, Germany) and Comet 5.5™ software (Kinetic Images, Liverpool, Germany). Duplicate slides were prepared for each sample and the olive tail moments of 100 randomly selected cells were measured on each slide.

3.2.5 Fish embryo toxicity (FET) assay

The acute toxicity and teratogenicity of FB towards the embryonic stages of zebrafish were determined on the basis of the OECD Test Guideline No. 236 (OECD, 2013).

3.2.5.1 Ethical statement

The project was approved by the Tshwane University of Technology (TUT) Animal Research Ethics Committee (AREC2015/05/001). The TUT Research Ethics Committee is registered with the South African National Health Research Ethics

Council (REC-160509-21) and has the status of an Institutional Review Board (IRB 00005968) with the US Office for Human Research Protections (IORG# 0004997).

3.2.5.2 *Experimental animals, housing and husbandry*

Adult, wild type zebrafish (*Danio rerio*) were purchased from a wholesale supplier (Daro, Pretoria, South Africa). The fish populations were housed in glass tanks (30 L capacity) filled with deionised water:borehole water (50:50, v/v) at 25 ± 1 °C, equipped with charcoal filters, heaters and thermometers. The water was aerated continuously, and water quality was maintained by replacing 10% of the water volume every second day. The population density did not exceed one fish/L and consisted of males and females, aged 4-24 months. Fish were exposed to 12 h photoperiods and fed three times per day with a varied diet, which included dry flake food (TetraMin tropical flakes, Tetra®), freeze-dried *Tubifex* worms (Today's®) and frozen *Artemia* (RUTO®). Breeding stock used for egg production was free of any observable disease or abnormalities and was not subjected to any pharmaceutical treatment for a period of two months prior to spawning.

3.2.5.3 *Collection of eggs*

Zebrafish eggs were obtained by random pairwise mating of adult male and female fish placed together in small breeding tanks (Figure 3.3). Three hours before the onset of the dark phase of the photoperiod, the day before the eggs were required, spawning groups (2:1 male to female ratio) were placed in breeding tanks filled with reconstituted water at 26 ± 1 °C. The breeding tanks were equipped with sloped, slotted spawning trays (Labotec, Johannesburg, South Africa) containing artificial aquarium plants to stimulate spawning. Three breeding groups were set up in parallel to compensate for spawning failure and to prevent genetic bias. Collection trays were removed carefully, 30 min after the onset of light the next morning, and the eggs were rinsed with reconstituted water. Any dead or abnormal embryos were removed.

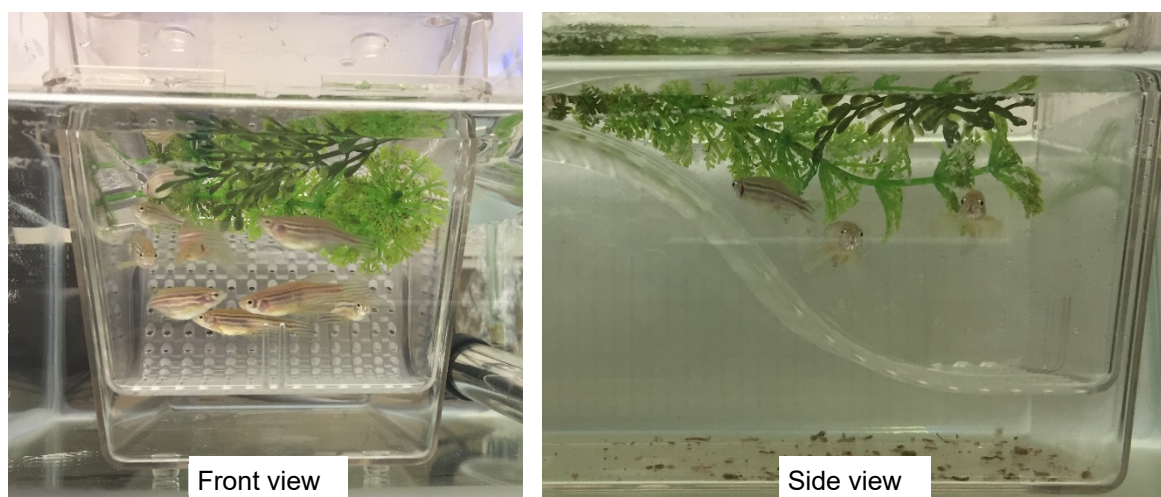


Figure 3.3: Zebrafish breeding tank with spawning tray, fish and artificial plants

3.2.5.4 Chemicals and test media

Reconstituted water was prepared by adding 20.0 mL each of KCl (0.050 g/L), CaCl_2 (29.4 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (12.3 g/L) and NaHCO_3 (6.30 g/L) to 1920 mL deionised water as specified in ISO 7346-1 (ISO, 1996). The solution was mixed, aerated and the pH was adjusted to 7.7-7.8 using 0.1 M NaOH or HCl. The reconstituted water was used as a solvent to dissolve the fumonisin standards (Section 3.2.1) and to prepare dilutions. Fresh fumonisin solutions were prepared on the day of the experiment for each independent replicate of the assay. A 3,4-dichloroaniline (DCA, purity $\geq 98\%$) stock solution was prepared in dimethyl sulfoxide (DMSO, purity $\geq 99.9\%$) and diluted with reconstituted water. The final concentration of DMSO in the diluted solutions did not exceed 0.1%.

3.2.5.5 Study design

Healthy, normally developing embryos (2-16 cell stage) were selected with the aid of a stereomicroscope and transferred to the wells of a 24-well microtiter plate (one embryo per well) containing 1.00 mL of the test substance or controls. The experimental design included DCA, at a concentration of 4.0 mg/L, as a positive control (PC), and reconstituted water as a negative control (NC). For each concentration or control, 10 embryos were exposed, and each plate contained internal negative controls (4 embryos in reconstituted water). All experiments were

performed under static conditions. The plates were covered with an adhesive film and lid and kept in an incubator at 26 ± 1 °C for up to 96 hours post fertilisation (hpf).

Preliminary range-finding experiments were performed at the Aquatic Ecology and Toxicology department, Center for Organismic Studies (University of Heidelberg, Germany) and included testing of FB₁, FB₂, FB₃ and HFB₁ at the following concentrations: 15.0, 30.0, 60.0, 90.0 and 150 mg/L. The concentration range was subsequently extended for FB₁ and concentrations of 125, 250, 500, 1 000, 2 000 and 4 000 g/L were evaluated.

Follow-up experiments were conducted at the Tshwane University of Technology zebrafish laboratory. To validate the local facility, an assay with a full range of concentrations of the PC (DCA at 0.500, 1.00, 2.00, 4.00 and 8.00 mg/L) was performed. The standards of FB₁ and HFB₁ were tested at concentrations of 125, 250, 500, 1 000 and 2 000 g/L. These ranges met the requirement of no observable effect at the lowest concentration and 100% lethality at the highest concentration (OECD, 2013). The pH of the test solutions was measured before and after the assay using a Ross glass combination micro pH electrode (Labotec, Midrand, South Africa). A control experiment was set up by adjusting the pH of reconstituted water to a range of values between 2 and 12 using 0.1 M HCl or NaOH (in the absence of fumonisins). Finally, an experiment was performed in which the pH of FB₁ solutions (125, 250, 500, 1 000 and 2 000 g/L) were adjusted to 7.8 ± 0.1 using 1 M NaOH. For each experiment three independent replicates were conducted at separate occasions.

3.2.5.6 Evaluation of developmental effects

Developmental and teratogenic effects (Table 3.3) were evaluated microscopically (Olympus IX71 inverted microscope, Wirsam Scientific, Johannesburg, South Africa) at 24, 48, 72 and 96 hpf. The test was only regarded as valid if >90% of the embryos in the NC developed normally and at least 30% mortality was achieved in the PC at 96 hpf (OECD, 2013).

Table 3.3: Zebrafish embryo developmental lethal endpoints and sublethal effects that were evaluated at 24-96 hpf

	Exposure time (hpf)			
	24	48	72	96
Lethal endpoints:				
Lack of heartbeat		*	*	*
Lack of somite formation	*	*	*	*
Non-detachment of tail	*	*	*	*
Coagulated embryos	*	*	*	*
Sublethal effects:				
Heartbeat slow		*	*	*
No blood circulation		*	*	*
Slow blood circulation		*	*	*
Blood stasis		*	*	*
Blood in yolk sack		*	*	*
No spontaneous movement	*	*	*	*
Underdeveloped embryo	*	*	*	*
Malformation of notochord	*	*	*	*
Heart deformed		*	*	*
Head malformed	*	*	*	*
Eyes malformed	*	*	*	*
Ears malformed	*	*	*	*
Scoliosis		*	*	*
Tail bent	*	*	*	*
Tail or tail tip malformed	*	*	*	*
Tail short	*	*	*	*
Oedema of yolk sack	*	*	*	*
Pericardial oedema	*	*	*	*
Oedemoa of body cavity	*	*	*	*
Lack of pigmentation	*	*	*	*
Balance problem		*	*	*
Not hatched				*

3.2.6 Statistical analysis

The data from the preliminary neutral red experiment and comet assay was statistically analysed with SigmaPlot 11.0 software (SyStat Software Inc, San Jose, CA, USA). For the neutral red assay, a pairwise t-Test was performed. The ANOVA on ranks calculation was used for the comet assay, due to the non-normal distribution of the results. Dunn's post-hoc test was done to identify groups that differed significantly.

The IC₅₀ (concentration to inhibit 50% growth) values for the follow-up neutral red assay experiments, as well as the LC₅₀ (concentration to kill 50% of test organisms) and EC₅₀ (concentration to cause effects in 50% of the test organisms) for the FET were calculated with Dr Fit (Di Veroli *et al.*, 2015). Dose-response graphs were

constructed, and the Hill model was fitted to the data using the Trust region-reflective algorithm and weighting according to the standard deviation. For the FET, a teratogenic index (TI) was calculated as the ratio LC_{50}/EC_{50} and values ≥ 2 were considered to predict teratogenicity (Selderslaghs *et al.*, 2012). All experiments were conducted as three independent replicates, unless stated otherwise.

3.3 RESULTS AND DISCUSSION

3.3.1 Fumonisin cytotoxicity

3.3.1.1 Preliminary evaluation of FB₁, FB₂, FB₃ and HFB₁

The effect of fumonisins B₁, B₂, B₃ and HB₁ on the viability of RTL-W1 cells is illustrated in Figure 3.4 in the form of dose-response curves. None of the fumonisin standards exhibited any cytotoxicity at the concentrations tested (up to 400 µg/mL). It was therefore not possible to calculate the concentration of fumonisins reflecting a 50% inhibition of the neutral red uptake (IC_{50}). The only treatment that caused a small significant decrease in cell viability compared to the negative control, was the 400 µg/mL HFB₁.

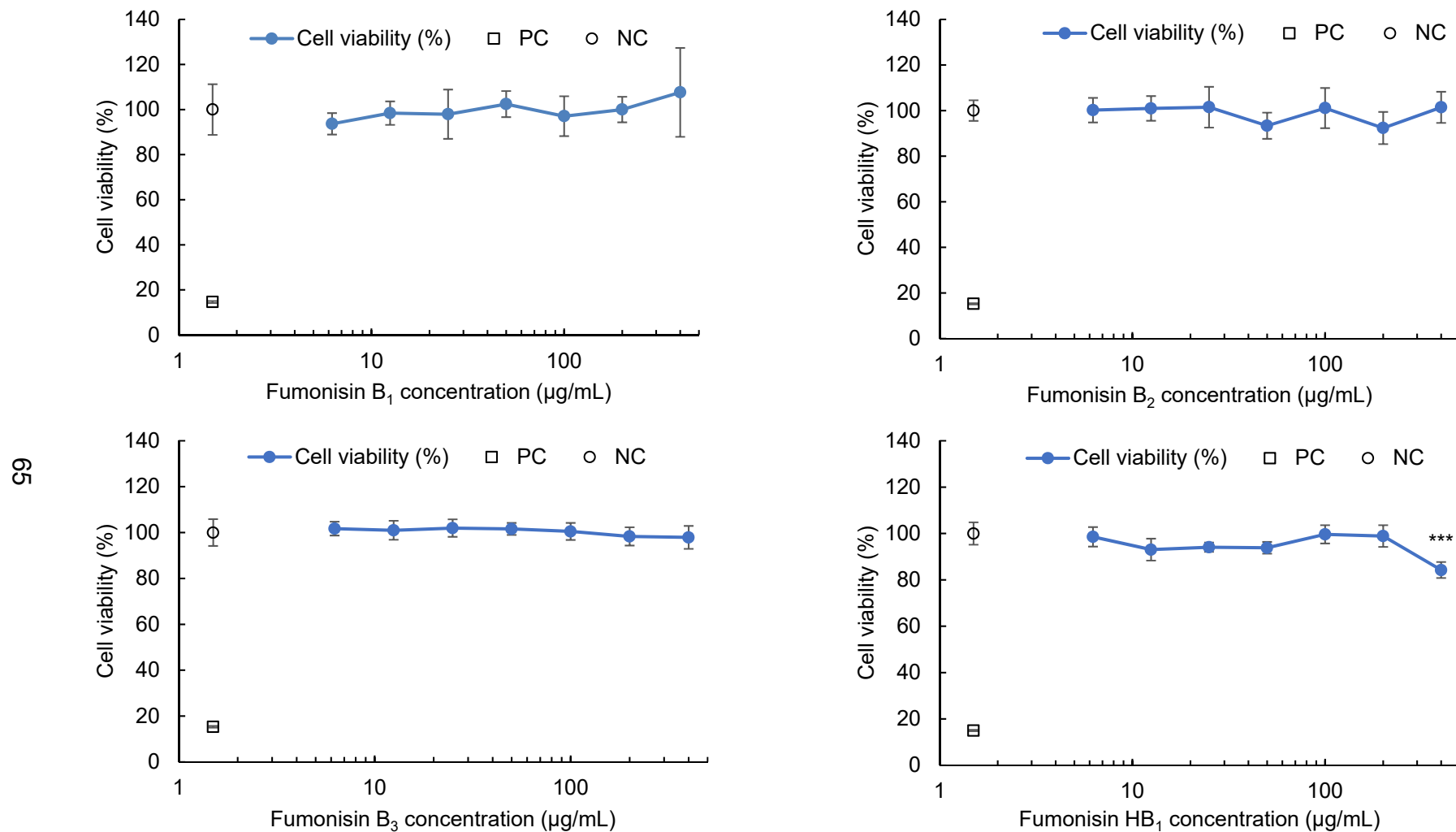


Figure 3.4: Cytotoxicity of FB₁, FB₂, FB₃ and HFB₁ as determined by the neutral red assay using the RTL-W1 cell line with 3,5-dichlorophenol as the positive control (PC) and cell culture medium as the negative control (NC). Significant differences from the negative control (pairwise t-Test) are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.3.1.2 Follow-up experiment with increased concentrations of FB₁

To obtain a toxic endpoint for fumonisin towards the RTL-W1 cell line, a follow-up experiment was performed over a higher concentration range, up to 8 000 µg/mL (11 100 µM) and the exposure time was increased from 24 to 48 h (Figure 3.5). Due to the limited availability and high cost of fumonisin standards, it was not financially feasible to test all of the standards at such high concentrations. Therefore, FB₁ was selected for this experiment, because it typically comprises 70-80% of the total fumonisins present in maize (Rheeder *et al.*, 2002).

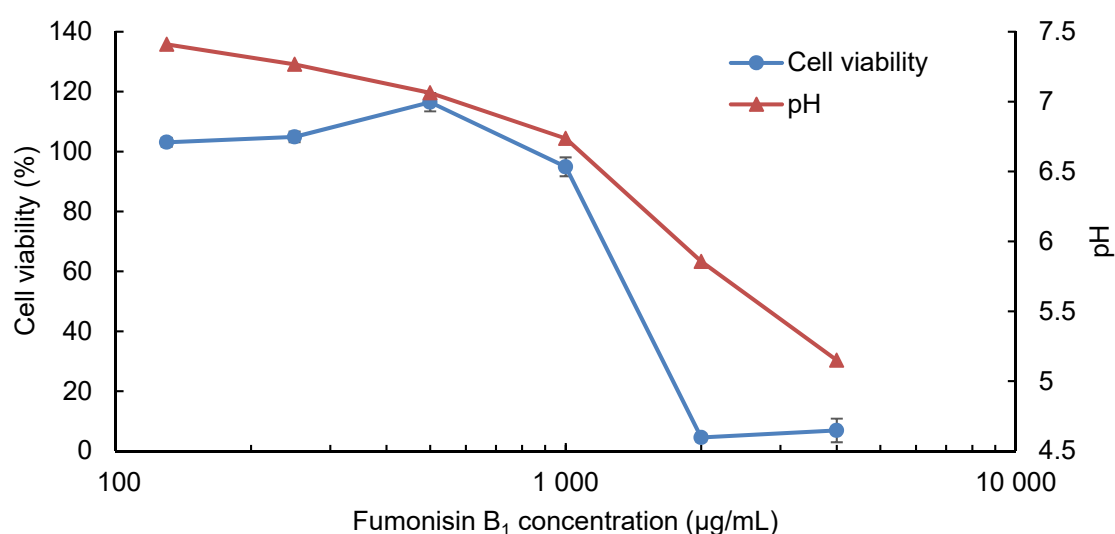


Figure 3.5: Cytotoxicity of FB₁ determined by the neutral red assay using the RTL-W1 cell line and the effect of FB₁ on the pH of the cell culture medium

The IC₅₀ (concentration where a 50% reduction of neutral red uptake was observed, indicating a 50% loss in viable cells) was calculated to be 1 260 µg/mL (1 746 µM). According to the researcher's knowledge, there is currently no literature available on the cytotoxicity of fumonisins towards the RTL-W1 cell line. However, this IC₅₀ is in accordance with the value reported previously for primary rat hepatocytes (1 444 µg/mL or 2 000 µM) (Gelderblom *et al.*, 1993). In addition, there is some evidence that FB₁ has cytotoxic effects on human HepG₂ (IC₅₀ 288 µg/mL or 399 µM) and BEAS-2B cell lines (IC₅₀ 256 µg/mL or 355 µM), although these IC₅₀ values are much lower than the value obtained for the RTL-W1 cell line in the current study (McKean *et al.*, 2006). McKean *et al.* (2006) highlighted the fact that low

concentrations of FB₁ seemed to enhance cellular proliferation; this trend is also evident in Figure 3.5. The concentrations of FB₁ that caused an increase in the viability of the RTL-W1 cells were 125 (103%), 250 (105%) and 500 µg/mL (116%).

Like most commercial culture media, the cell culture medium used in this study contained phenol red as a pH indicator. Under normal conditions (pH 7.4) the colour of the culture medium is pink-red. However, the addition of FB₁ at the high concentrations tested in this experiment caused a shift towards a more acidic pH (with the colour of the indicator changing to yellow). This acidic property is expected, because there are two TCA groups on the fumonisin molecule (Bezuidenhout *et al.*, 1988).

3.3.1.3 pH control and pH-adjusted experiments

To investigate the effect of pH on the viability of the cell line, a pH control experiment was designed in which the cell culture medium was tested without FB₁. This involved determining the toxicity of cell culture medium after pH adjustment to values comparable to those measured in the cell culture medium containing different concentrations of FB₁ (Figure 3.6). It is evident that Figures 3.5 and 3.6 display the same trend, demonstrating that the cytotoxicity of FB₁ towards the RTL-W1 cell line is associated with the acidic pH shift in the cell culture medium. From the dose-response curves (with fitting the Hill model) the pH at the IC₅₀ value of FB₁ was 6.23, which is similar to the pH in the control experiment where a 50% reduction in cell viability was observed (pH 6.14). It can therefore be recommended that pH values below 7 should be avoided when doing the assay to prevent effects caused by pH shifts.

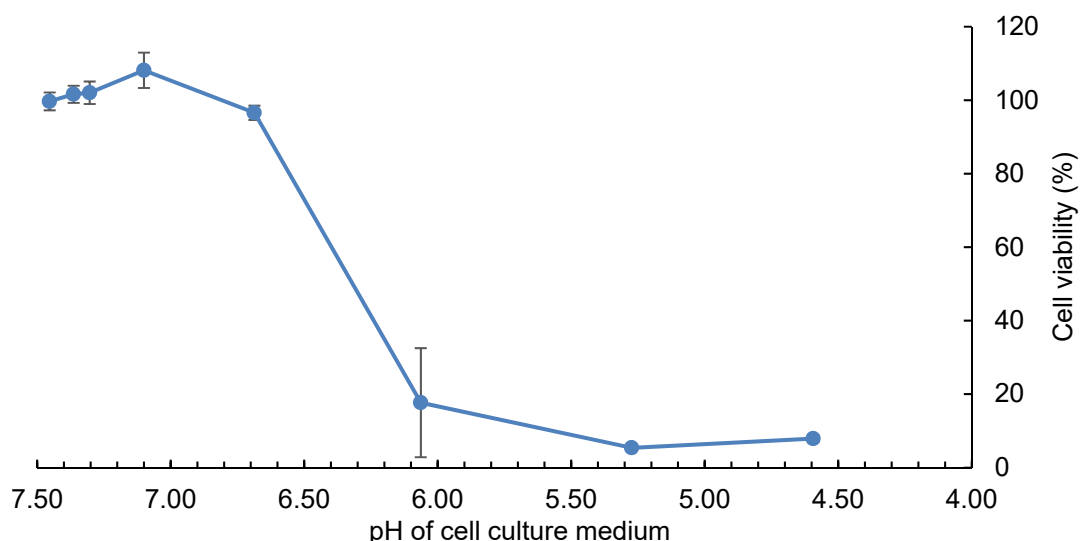


Figure 3.6: Cytotoxicity of cell culture medium with pH adjusted to values comparable to those measured in cell culture medium containing different concentrations of FB₁

To confirm the contribution of pH to the cytotoxicity of FB₁, another experiment was performed in which the pH of the cell culture medium, containing FB₁, was adjusted to physiological pH (Figure 3.7). The fact that FB₁ did not exhibit a cytotoxic effect towards the RTL-W1 cell line when the pH was adjusted to physiological pH values, emphasises the importance of considering pH shifts when performing cytotoxicity assays.

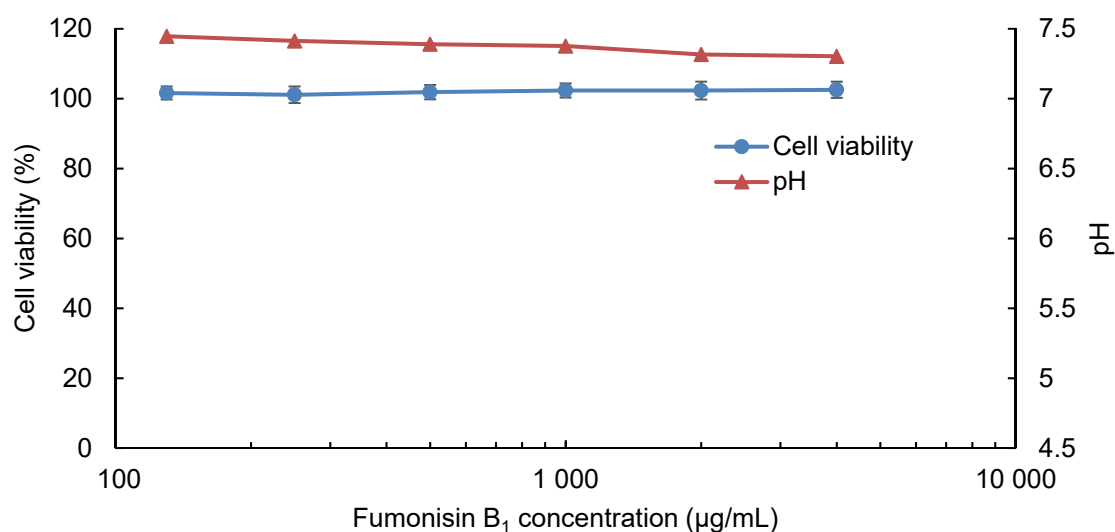


Figure 3.7: Cytotoxicity of FB₁ (pH-adjusted) as determined by the neutral red assay using the RTL-W1 cell line

Many reports in literature indicate that FB₁ do not cause cytotoxic effects. For example, no significant decrease in cell viability was observed when:

- swine jejunal epithelial cells (IPEC-J2) were exposed to FB₁ at concentrations up to 14 µg/mL (20 µM) (Wan *et al.*, 2013),
- Madine Darby Bovine Kidney (MDBK) and human intestinal Caco-2 cells exposed to 10 µg/mL (14 µM) FB₁ (Clarke *et al.*, 2014),
- rat liver cell line BRL 3A exposed to 115 µg/mL (160 µM) FB₁ (Sun *et al.*, 2015),
- the rat hepatoma cell line H4IIE exposed to up to 72 µg/mL (100 µM) FB₁ (Mary *et al.*, 2015); and
- Chang cells exposed up to 361 µg/mL (500 µM) FB₁ (Riedel *et al.*, 2016).

Other studies reported only slight decreases in cell viability, for example:

- >65% viability in porcine kidney cells (PK-15) exposed to 180 µg/mL (250 µM) FB₁ (Lei *et al.*, 2013),
- >75% viability in murine macrophage raw 264.7 cells exposed to 10 µg/mL (14 µM) FB₁ (Clarke *et al.*, 2014); and
- 77% viability in HT-29 cells after 72 h exposure to 49.8 mg/mL FB₁ (69 µM) (Minervini *et al.*, 2014).

However, the abovementioned studies investigated lower concentration ranges than the current study. In addition, although it is standard practice to evaluate cytotoxicity at physiological pH it, it was not stated clearly whether the pH of the FB₁ containing cell culture media was adjusted or not. This is the first study in which the effect of pH on the cytotoxicity of FB was assessed systematically and the concentration range was extended up to the point where FB₁ precipitated from the culture medium after 48 h (8 000 µg/mL).

In general, the fumonisins seem to be less cytotoxic than other mycotoxins (McKean *et al.*, 2006; Lei *et al.*, 2013; Clarke *et al.*, 2014). Aflatoxin B₁, deoxynivalenol and zearalenone have IC₅₀ values of 11, 16 and 170 µM, respectively (Zhou *et al.*, 2017). However, fumonisins may induce effects that promote cancer at concentrations where no cytotoxic effect is observed (Riedel *et al.*, 2016) and is known to cause

animal diseases like equine leukoencephalomalacia and porcine pulmonary edema (Voss *et al.*, 2017). Cell culture models are not complex enough to display all the effects observed in animals. Furthermore, contaminated maize often contains more than one mycotoxin and studies have shown that the toxic effect of combinations of mycotoxins may be synergistic (Grenier and Oswald, 2011; Sun *et al.*, 2015; Alassane-Kpembé *et al.*, 2016; Hove *et al.*, 2016).

3.3.2 Fumonisin genotoxicity

The effect of fumonisins on the microscopic appearance of RTL-W1 cells treated in the comet assay is illustrated in Figure 3.8.

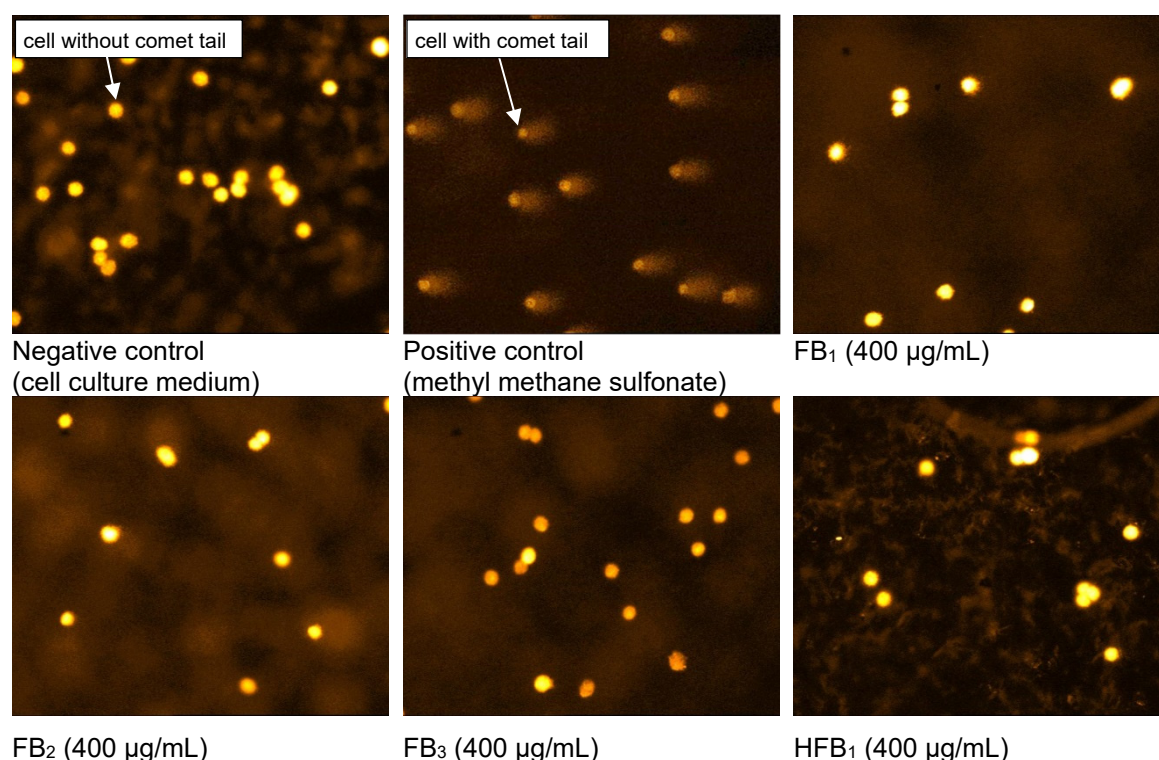


Figure 3.8: Micrographs of comet test results based on the RTL-W1 cell line to determine the genotoxicity of fumonisins FB₁, FB₂, FB₃ and HFB₁ (340x magnification viewed using a fluorescent microscope)

Even at the highest fumonisin concentration (400 µg/mL), the visual appearance of the cells was similar to that of the negative control, while typical comet tails were visible for cells treated with the positive control (methyl methane sulfonate,

40 µg/mL). This observation was confirmed with the measurement of the olive tail moments to quantify the genotoxic effect of fumonisins on the cell line (Figure 3.9).

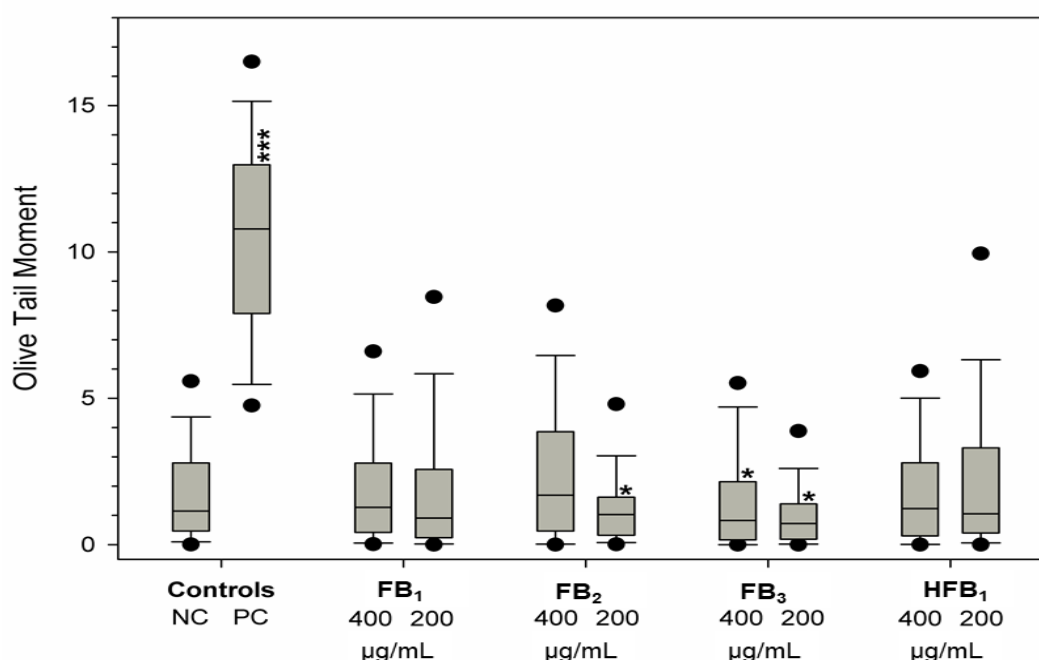


Figure 3.9: Genotoxicity of FB₁, FB₂, FB₃ and HFB₁ compared to negative (NC) and positive (PC) controls in the comet assay using the RTL-W1 cell line (Box plots show mean value (dash in box), 25th and 75th percentiles (box), standard deviation (error bars) and 95 and 5% outliers (black dots). Significant differences from the negative control are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$))

None of the fumonisin samples displayed any genotoxic effects, even at the highest concentration tested (400 µg/mL). Three samples exhibited olive tail moments that were significantly ($p < 0.05$) smaller than the negative control. That is unexpected and may be due to background interference on the microscope slides during measurement. To our knowledge, there is currently no information available in the literature pertaining to the genotoxicity of fumonisins towards the RTL-W1 cell line, however there is evidence that FB₁ induces epigenetic events, for example DNA hypomethylation in human hepatoma (HepG2) cells at a concentration of 144 µg/mL (Chuturgoon *et al.*, 2014). At present, aflatoxin is the only mycotoxin classified by the International Agency for Research on Cancer (IARC) as a Group 1 human carcinogen (Marin *et al.*, 2013; Vettorazzi and López de Cerain, 2016). Fumonisin B₁, aflatoxin M₁, ochratoxin A and sterigmatocystin are classified as Group 2B carcinogens (possibly carcinogenic to humans) (IARC, 2002; Marin *et al.*, 2013). As

reported in many publications by Gelderblom and co-workers, most animal studies point toward FB₁ being non-genotoxic and a weak cancer initiator, but they have demonstrated that the compound promotes the growth of existing tumours (Gelderblom *et al.*, 1988; Gelderblom *et al.*, 1993; Gelderblom *et al.*, 2001a; Gelderblom *et al.*, 2001b; Gelderblom *et al.*, 2002; Gelderblom *et al.*, 2008). For example, liver cancer initiation in rats was detected only at very high FB₁ concentrations (1 000 mg/kg diet fed for 21 days), whereas cancer promotion was observed at a dietary level of 50 mg/kg feed after 21 days (Gelderblom *et al.*, 1993; Gelderblom *et al.*, 1996). The results of the current study confirm that fumonisins concentrations up to 400 µg/mL, are non-genotoxic towards the trout liver cell line RTL-W1.

3.3.3 Fish embryo toxicity assay

The data reported in this section were obtained in two independent laboratories (TUT Department of Biotechnology and University of Heidelberg Centre for Organism Studies).

3.3.3.1 Normal development and method validation

As prescribed by the OECD guideline, DCA was used as a positive control and was tested in a full concentration-response range at the TUT laboratory to verify the sensitivity of the fish and demonstrate the proficiency of the laboratory (OECD, 2013). The normal development and the effects of the DCA on the development of zebrafish embryos over a period of 96 hpf are illustrated in Figure 3.10.

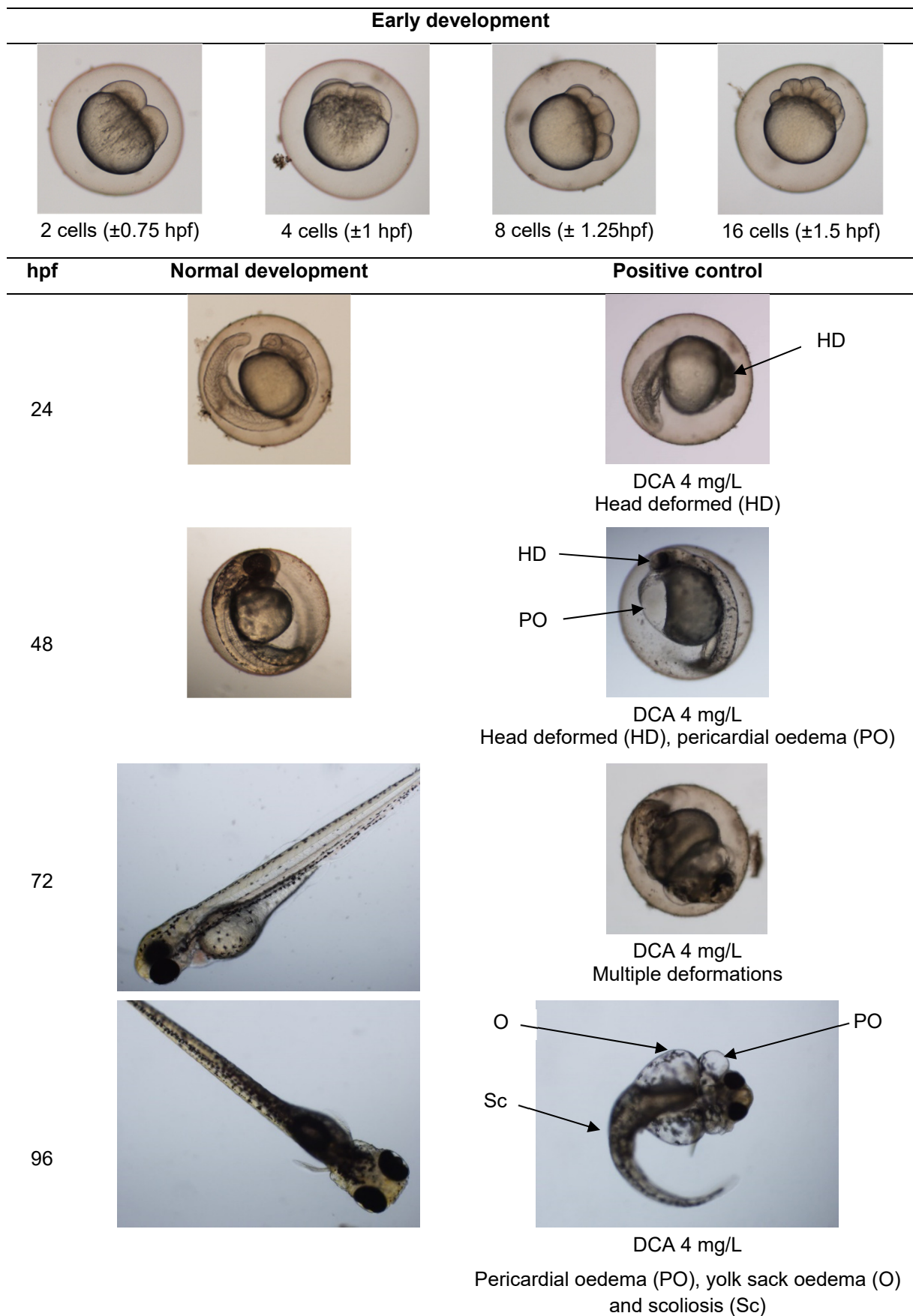


Figure 3.10: Normal development of zebrafish embryos during the first 96 hpf compared to the positive control (Positive control = 3,4-dichloroaniline (DCA))

The EC₅₀ and LC₅₀ values obtained at the TUT laboratory are presented in Table 3.4.

Table 3.4: Effective (EC₅₀) and lethal concentrations (LC₅₀) for 3,4-dichloroaniline (DCA) at 24, 48, 72 and 96 hpf

Time (hpf)	DCA (mg/L)	
	EC ₅₀	LC ₅₀
24	2.08	4.69
48	2.29	4.47
72	2.17	4.25
96	2.45	3.90

The toxicity of DCA increased with increasing exposure time. This is a typical effect that is described in literature (OECD, 2011). The EC₅₀ values ranged from 2.08 to 2.45 mg/L and the LC₅₀ values ranged from 3.90 to 4.69 mg/L. These values are in accordance with literature reporting EC₅₀ values ranging from 1.62 to 3.44 mg/L (Lange *et al.*, 1995; Lammer, 2009) and LC₅₀ values ranging from 1.2 to 4.5 mg/L for DCA (OECD, 2011).

3.3.3.2 Preliminary range-finding experiments with FB₁, FB₂, FB₃ and HFB₁

The development of embryos treated with fumonisins (FB₁, FB₂, FB₃ and HFB₁ up to a concentration of 150 mg/L) was normal and no lethal or teratogenic effects were observed. As with the cytotoxicity testing, it was not feasible to test higher concentrations of all the standards. Again, FB₁, the most common of the FB mycotoxins, was chosen for the range-finding experiment at higher concentrations. By increasing the concentration range up to 4 000 mg/L, it was possible to induce both lethal and sublethal effects in the zebrafish embryos. This data was used to construct dose-response curves and preliminary EC₅₀ and LC₅₀ values were calculated based on a single replicate performed at the University of Heidelberg (Table 3.5).

Table 3.5: Preliminary* effective (EC₅₀) and lethal (LC₅₀) concentrations for FB₁ at 24, 48, 72 and 96 hpf

Time (hpf)	FB ₁ (mg/L)	
	EC ₅₀	LC ₅₀
24	506	1 410
48	477	1 410
72	250	1 000
96	287	524

* Range finding experiment performed at the University of Heidelberg

At 24 hpf the LC₅₀ for FB₁ was 1 410 mg/L and all the embryos exposed to the 2 000 and 4 000 mg/L treatments were dead.

3.3.3.3 Follow-up experiment with FB₁ and HFB₁

The results of the preliminary experiment were verified by repeating the experiment at the TUT zebrafish laboratory (three independent replicates were performed at separate occasions). The follow-up experiment included HFB₁, which is a modified form of FB₁ present in some processed maize-based food products (Dombrink-Kurtzman *et al.*, 2000). The following concentrations of FB₁ and HFB₁ standards were tested: 25, 250, 500, 1 000 and 2 000 mg/L. Figures 3.11 and 3.12 present the dose-response curves to determine the effective and lethal concentrations for FB₁ towards zebrafish embryos, respectively.

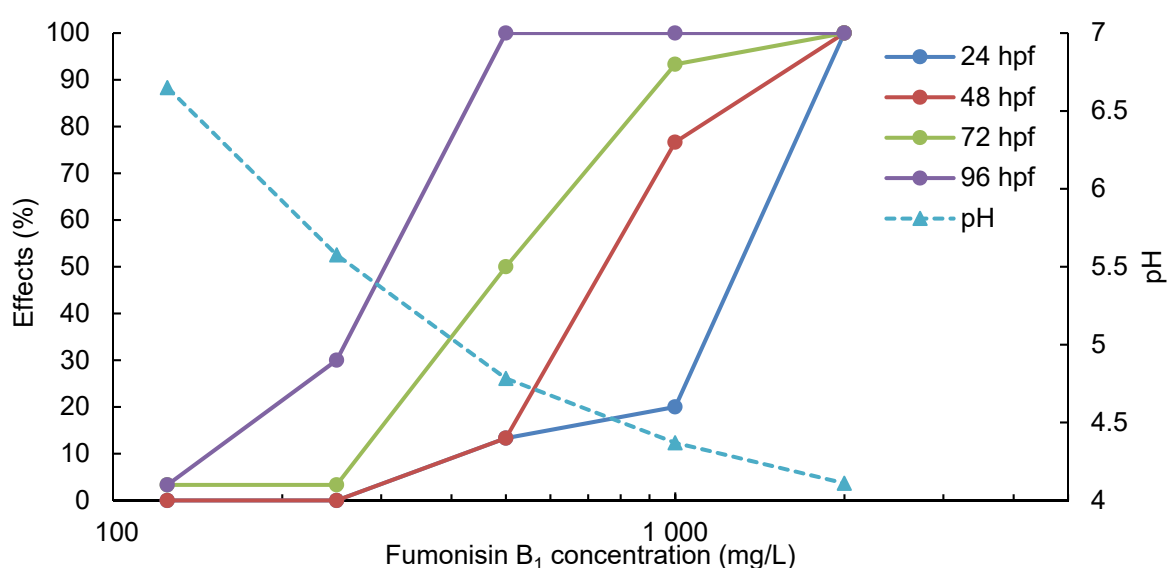


Figure 3.11: Dose response curves to determine the effective concentration for FB₁ at 24, 48, 72 and 96 hpf

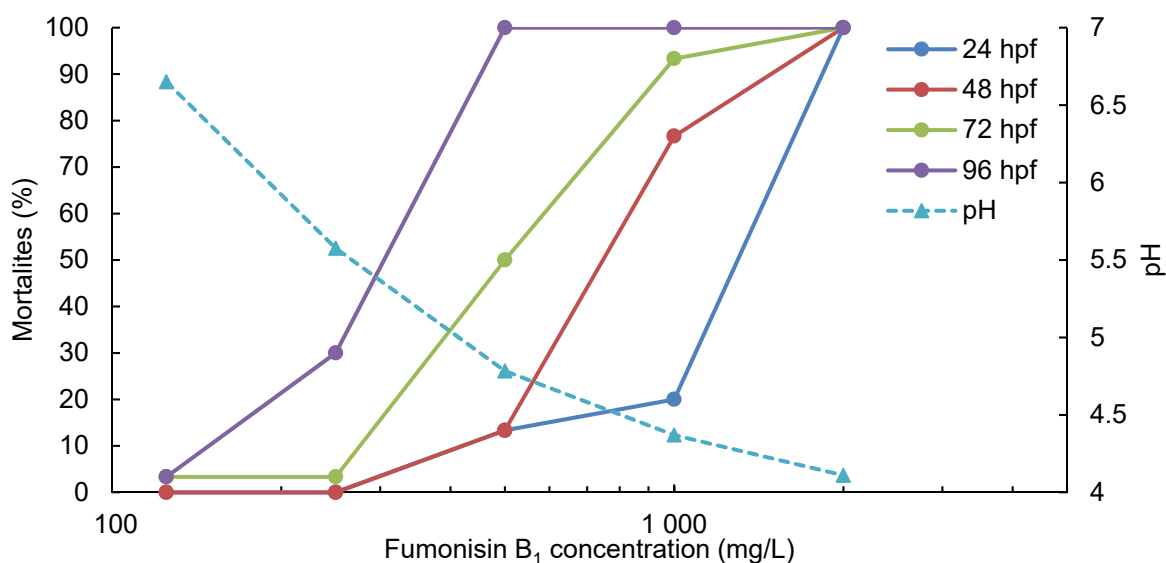


Figure 3.12: Dose response curves to determine the lethal concentration for FB₁ at 24, 48, 72 and 96 hpf

There appears to be an inverse relationship between the pH of the FB₁ solution and the percentage effects or mortalities in the zebrafish embryos. The effects and mortalities increased from <5% at pH 6.65 to 100% as the pH decreased to 4.11. The calculated EC₅₀ and LC₅₀ values (with corresponding calculated pH values), as well as the teratogenicity index (TI) for FB₁ are summarised in Table 3.6.

Table 3.6: Effective (EC₅₀) and lethal (LC₅₀) concentrations, as well as teratogenicity index (TI) for FB₁ at 24, 48, 72 and 96 hpf

Time (hpf)	FB ₁ (mg/L)		TI
	EC ₅₀ (pH)	LC ₅₀ (pH)	
24	663 (3.99)	1170 (3.99)	1.76
48	433 (4.76)	764 (4.03)	1.76
72	339 (5.20)	502 (4.87)	1.48
96	255 (5.62)	277 (5.06)	1.10

The LC₅₀ values at 48 and 96 h were 764 and 277 mg/L (1 058 and 384 µM), respectively, indicating that FB₁ has a relatively low acute toxicity towards zebrafish embryos. With TI values of 1.76 and 1.10 at 48 and 96 h, respectively, it also displays a low teratogenicity as values ≥2 predict teratogenicity (Selderslaghs *et al.*, 2012). Developmental abnormalities mainly included oedema, reduced heartbeat, reduced blood flow and blood stasis (Figure 3.13).

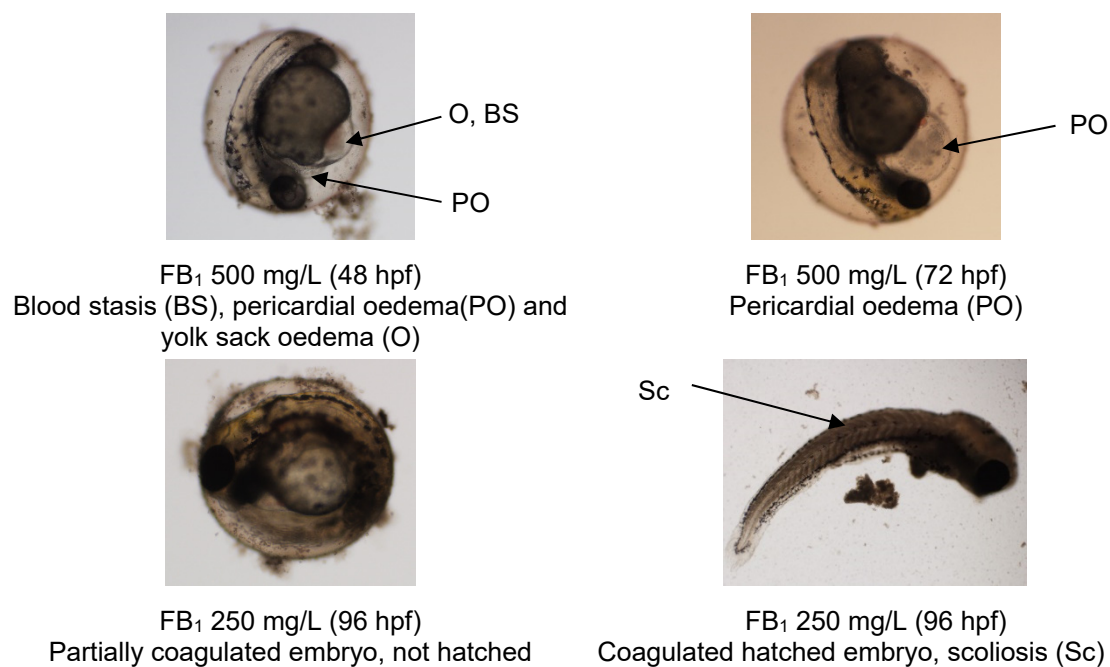


Figure 3.13: Typical developmental abnormalities observed in zebrafish embryos when exposed to FB₁ over a period of 96 hpf

This was the first study on fumonisin toxicity towards zebrafish embryos. No literature for direct comparison was therefore available. However, Zhou *et al.* (2017) reported LC₅₀ values of 0.61 and 16 μ M for aflatoxin B₁ and zearalenone, respectively, which supports the results from the cytotoxicity assays demonstrating that FB₁ is less toxic than these mycotoxins. Figures 3.14 and 3.15 present the dose-response curves to determine the effective and lethal concentrations for HFB₁ towards zebrafish embryos, respectively.

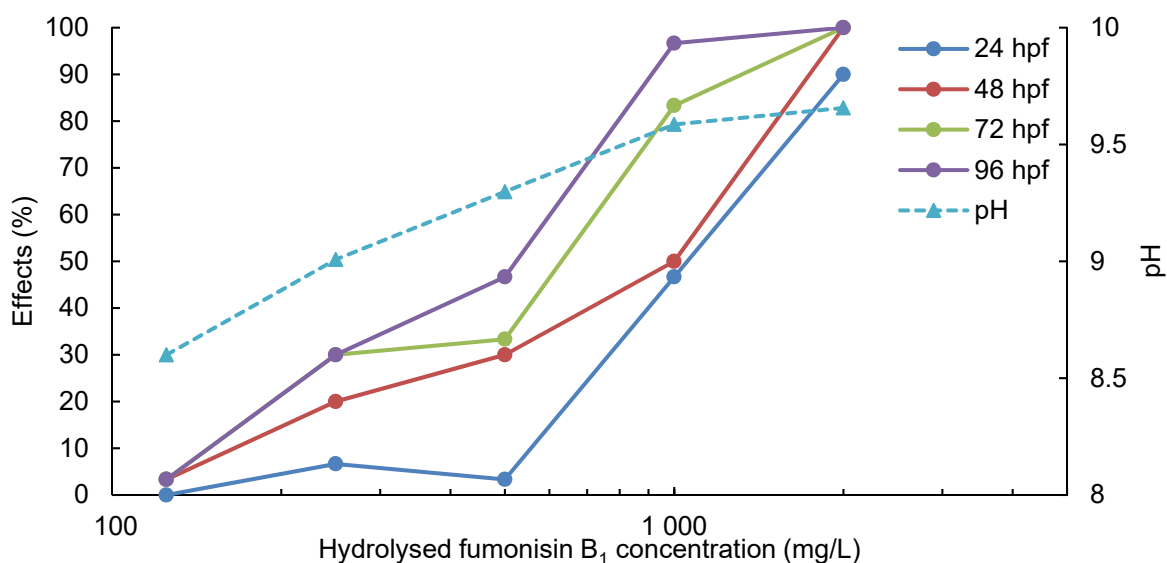


Figure 3.14: Dose response curves to determine the effective concentration for HFB₁ at 24, 48, 72 and 96 hpf

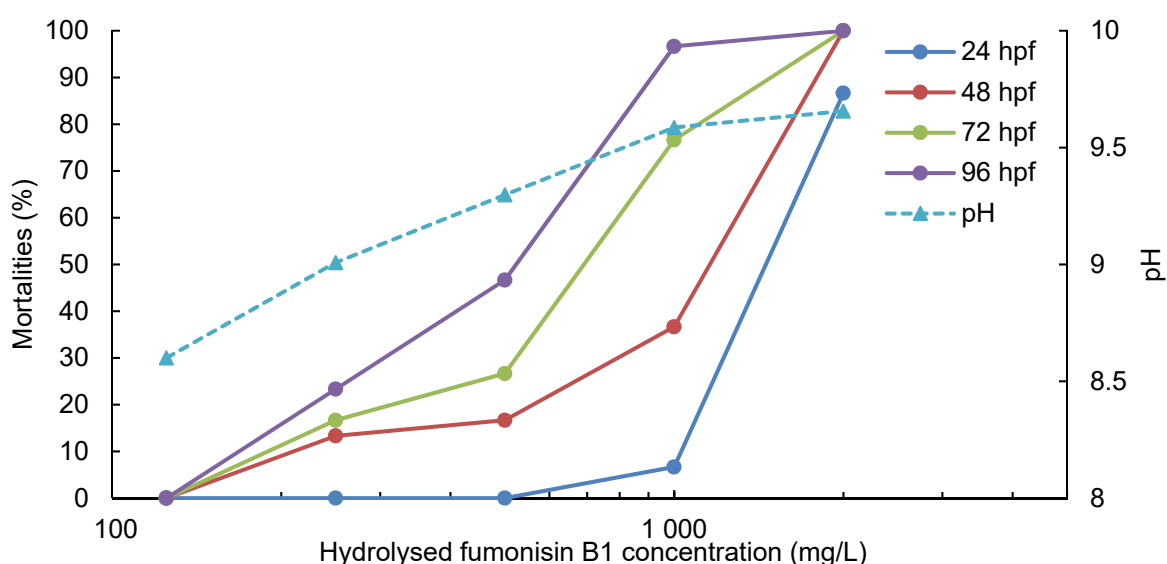


Figure 3.15: Dose response curves to determine the lethal concentration for HFB₁ at 24, 48, 72 and 96 hpf

As the concentration of the HFB₁ solutions increased, the pH increased. The alkalinity is due to the amino group on carbon 2 in the HFB₁ molecule. As with FB₁, it seems like there is a relationship between the pH of the HFB₁ solution and the percentage effects or mortalities in the zebrafish embryos. However, in this case it is a direct relationship and not inverse, as is the case with FB₁. The calculated EC₅₀ and LC₅₀ values (with corresponding calculated pH values), as well as the TI of HFB₁ are summarised in Table 3.7.

Table 3.7: Effective (EC₅₀) and lethal (LC₅₀) concentrations, as well as teratogenicity index (TI) for HFB₁ at 24, 48, 72 and 96 hpf

Time (hpf)	HFB ₁ (mg/L)		TI
	EC ₅₀ (pH)	LC ₅₀ (pH)	
24	1 030 (9.53)	1 500 (10.3)	1.46
48	782 (9.41)	1 090 (9.72)	1.39
72	542 (9.22)	664 (9.42)	1.23
96	441 (9.05)	471 (9.11)	1.07

The LC₅₀ values at 48 and 96 h were 1 090 and 471 mg/L (2 690 and 1 160 µM) respectively, indicating that HFB₁ has a relatively low acute toxicity towards zebrafish embryos. With TI values of 1.39 and 1.07 at 48 and 96 hpf, respectively, it also displays a low teratogenicity. Developmental abnormalities included mainly oedema, reduced heartbeat and reduced blood flow, but occasional cases of malformation of the ears, head and spine were noted (Figure 3.18).

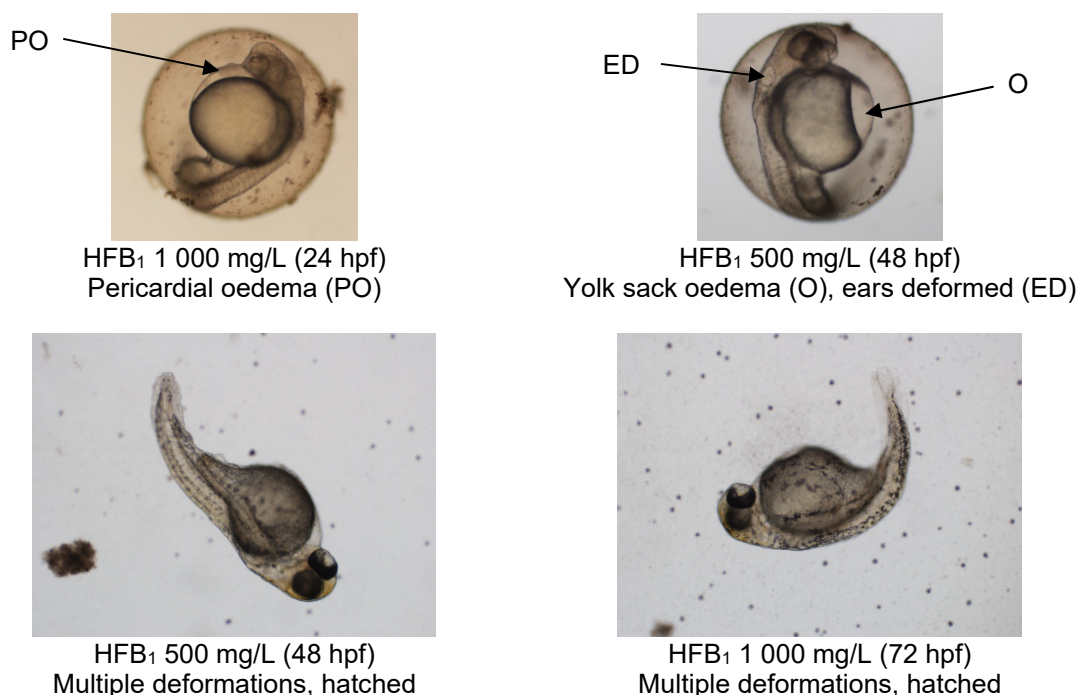


Figure 3.16: Typical developmental abnormalities observed in zebrafish embryos when exposed to HFB₁ over a period of 72 hpf

In the zebrafish FET assay, HFB₁ proved to be less toxic than FB₁. This was apparent from the higher EC₅₀ and LC₅₀ values obtained for HFB₁. Literature on the toxicity of HFB₁ reflects the insufficiencies of the current knowledge to properly

assess its risk to humans. In a study by Gelderblom *et al.* (1993), the short-term liver cancer initiation/promotion model used demonstrated that HFB₁ displayed greater cytotoxicity in primary hepatocytes than FB₁. However, in *in vivo* studies hydrolysed fumonisins are less toxic to laboratory animals and pigs than their parent forms (Voss *et al.*, 1996; Hartl and Humpf, 2000; Grenier *et al.*, 2012). More recently, Hahn *et al.* (2015) reported that hydrolysed and partially hydrolysed fumonisins are non-toxic to rats (Hahn *et al.*, 2015). Conversely, it was demonstrated that HFB is acylated *in vitro* and *in vivo* and that the resulting *N*-acyl-HFB was cytotoxic to the human colonic cell line HT29 (Seiferlein *et al.*, 2007).

3.3.3.4 pH control experiment

According to the OECD guideline, the pH of the test solutions should range between 6.5 and 8.5 (OECD, 2013). At the high concentrations tested, FB₁ and HFB₁ caused pH shifts outside this range, with the lowest pH (4.11) at 2 000 mg/L FB₁ and the highest pH (9.66) at 2 000 mg/L HFB₁. A control experiment using reconstituted water with its pH adjusted covering a range from 2-12 was performed to determine the effect of pH in the absence of fumonisins (Figures 3.17 and 3.18).

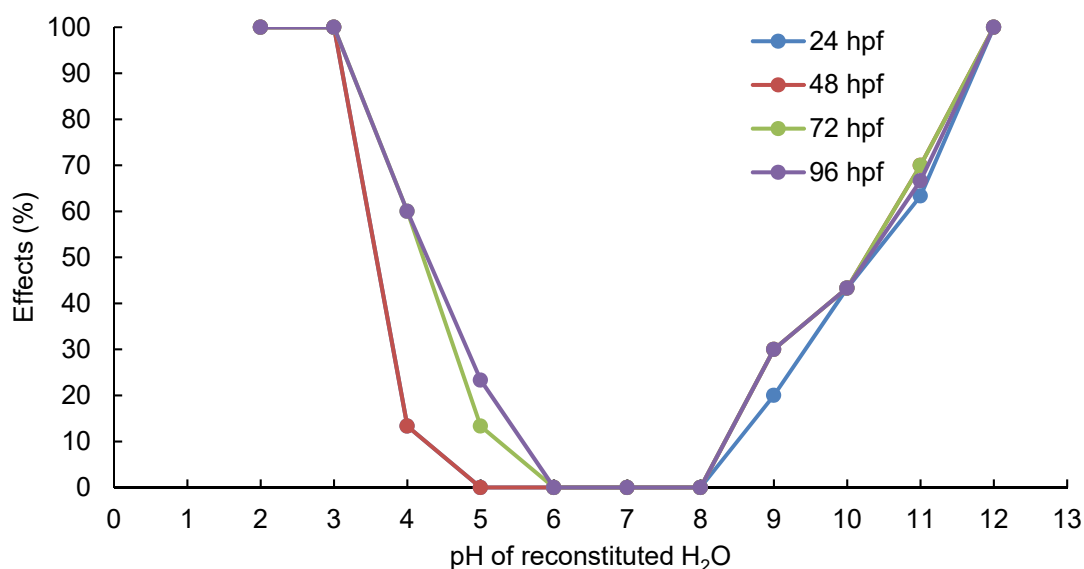


Figure 3.17: The effect of reconstituted water pH on the development of zebrafish embryos at 24, 48, 72 and 96 hpf

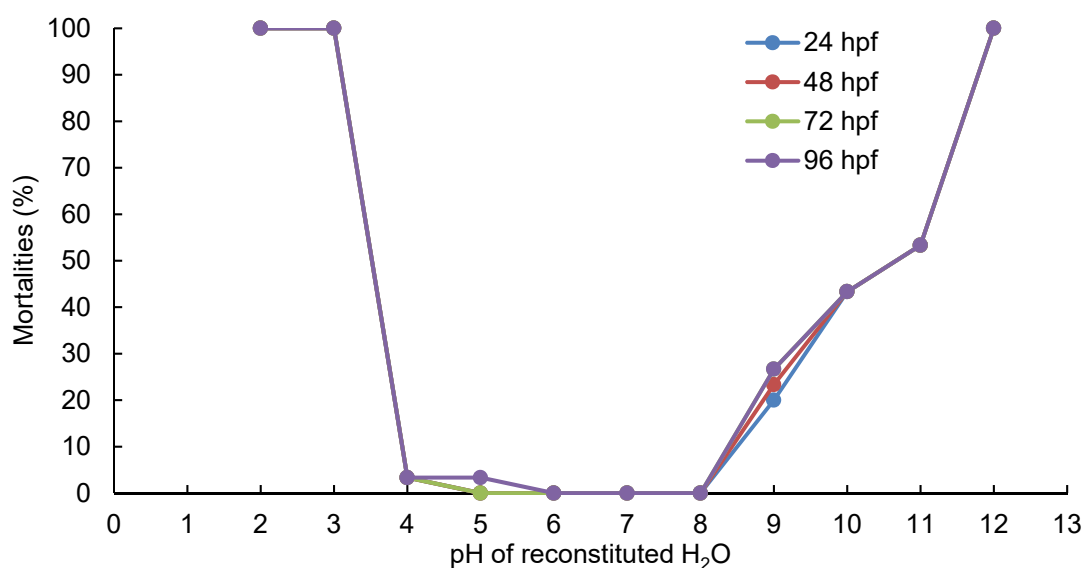


Figure 3.18: The effect of reconstituted water pH on the mortality of zebrafish embryos at 24, 48, 72 and 96 hpf

The data of the pH-response curves were used to calculate effective (EpH₅₀) and lethal (LpH₅₀) pH values in the acidic alkaline ranges (Table 3.8). For the purposes of this study, EpH₅₀ and LpH₅₀ were defined as the pH at which 50% of the embryos exhibit effects or die, respectively.

Table 3.8: Effective (EpH₅₀) and lethal (LpH₅₀) pH values in the acidic and alkaline pH ranges at 24, 48, 72 and 96 hpf

Time (hpf)	EpH ₅₀		LpH ₅₀	
	Acidic	Alkaline	Acidic	Alkaline
24	3.56	10.3	3.48	10.3
48	3.56	10.3	3.48	10.3
72	4.17	10.3	3.48	10.4
96	4.25	10.3	3.49	10.4

For FB₁ the pH at the EC₅₀ and LC₅₀ values ranged from 3.99 to 5.62 and from 3.99 to 5.06, respectively. In the control experiment the EpH₅₀ and LpH₅₀ values (acidic range) varied from pH 3.56 to 4.25 and 3.48 to 3.49, respectively. Although pH played a major role, it did not account for all the effects observed in embryos exposed to FB₁. In the case of HFB₁ the pH at the EC₅₀ and LC₅₀ values ranged from 9.05 to 9.53 and from 9.11 to 10.3, respectively. In the control experiment (alkaline range) the EpH₅₀ pH value was constant at 10.3, while the LpH₅₀ ranged only from 10.3 to 10.4. Therefore, it was speculated that for HFB₁ most of the effects (albeit not all) could be caused by the alkaline pH shift.

3.3.3.5 Toxicity of pH-adjusted FB₁

To determine whether pH-adjusted FB₁ solutions are toxic towards zebrafish embryos, FB₁ solutions (125, 250, 500, 1 000 and 2 000 mg/L) were tested at physiological pH (7.8±0.1). The dose-response curves to determine the effective and lethal concentrations for pH-adjusted FB₁ are presented in Figures 3.19 and 3.20, respectively.

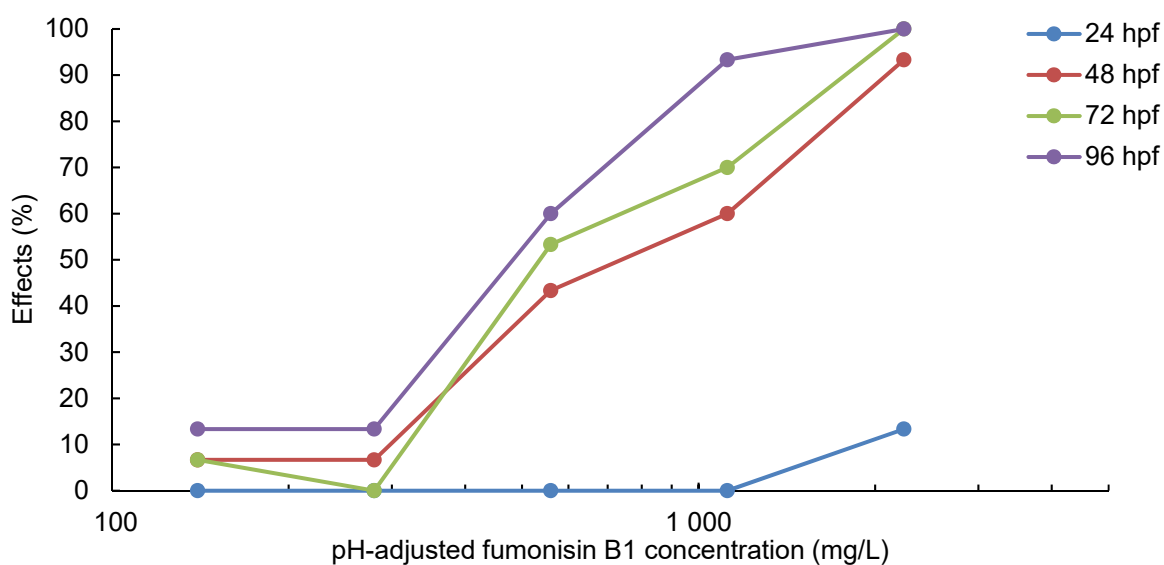


Figure 3.19: Dose response curves to determine the effective concentration for pH-adjusted FB₁ at 24, 48, 72 and 96 hpf

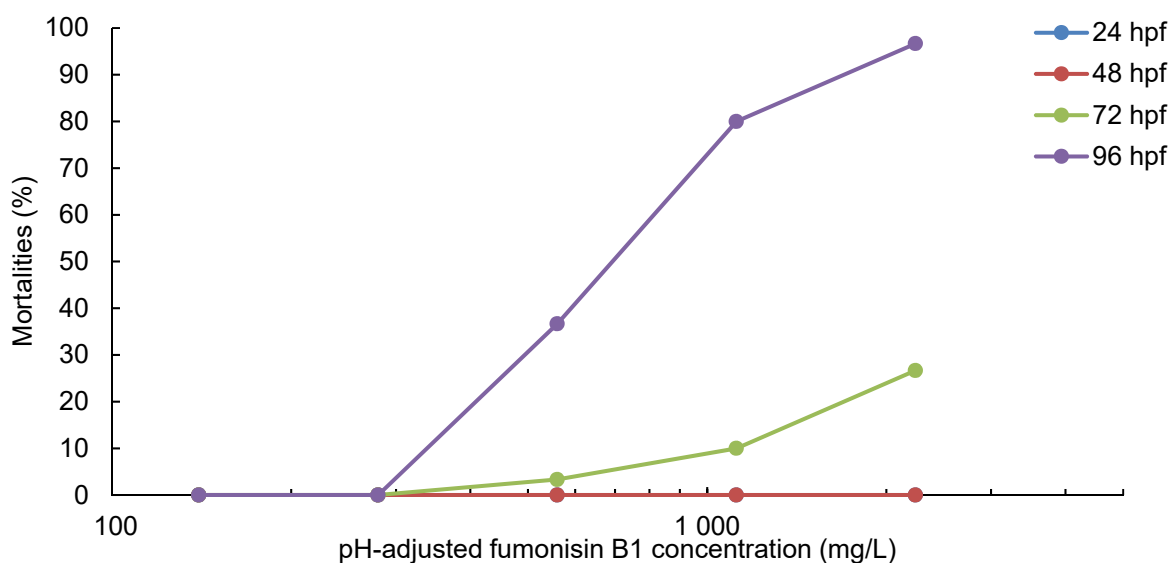


Figure 3.20: Dose response curves to determine the lethal concentration for pH-adjusted FB₁ at 24, 48, 72 and 96 hpf

There were no mortalities in the zebrafish embryos during the first 48 h when exposed to the pH-adjusted FB₁ solutions, therefore the dose response curves for 24 and 48 h coincide on the graph (Figure 3.20). The calculated EC₅₀ and LC₅₀ values, as well as the TI of pH-adjusted FB₁ are provided in Table 3.9.

Table 3.9: Effective (EC₅₀) and lethal (LC₅₀) concentrations, as well as teratogenicity index (TI) for pH-adjusted FB₁ at 24, 48, 72 and 96 hpf

Time (hpf)	pH-adjusted FB ₁ (mg/L)		TI
	EC ₅₀	LC ₅₀	
24	-	-	-
48	749	-	-
72	574	-	-
96	490	682	1.39

The EC₅₀ for pH-adjusted FB₁ was 749 and 490 mg/L (1 040 and 679 µM) at 48 and 96 hpf, respectively. This represents an increase in the EC₅₀ by a factor of 1.7-1.9 when the pH of the FB₁ solutions were adjusted to physiological conditions, compared to when FB₁ was tested without pH adjustment. The LC₅₀ could only be obtained after 96 hpf using the concentrations tested and the value (682 mg/L or 945 µM) was 2.5 times higher than the corresponding concentration without pH adjustment (277 mg/L). This indicates that the acute toxicity towards zebrafish embryos was reduced, but not eliminated when adjusting the pH. The developmental abnormalities observed in zebrafish embryos exposed to pH-adjusted FB₁ solutions (Figure 3.21) were similar to the abnormalities observed in embryos exposed to unadjusted FB₁ solutions (Figure 3.13), but the effects presented at higher concentrations.

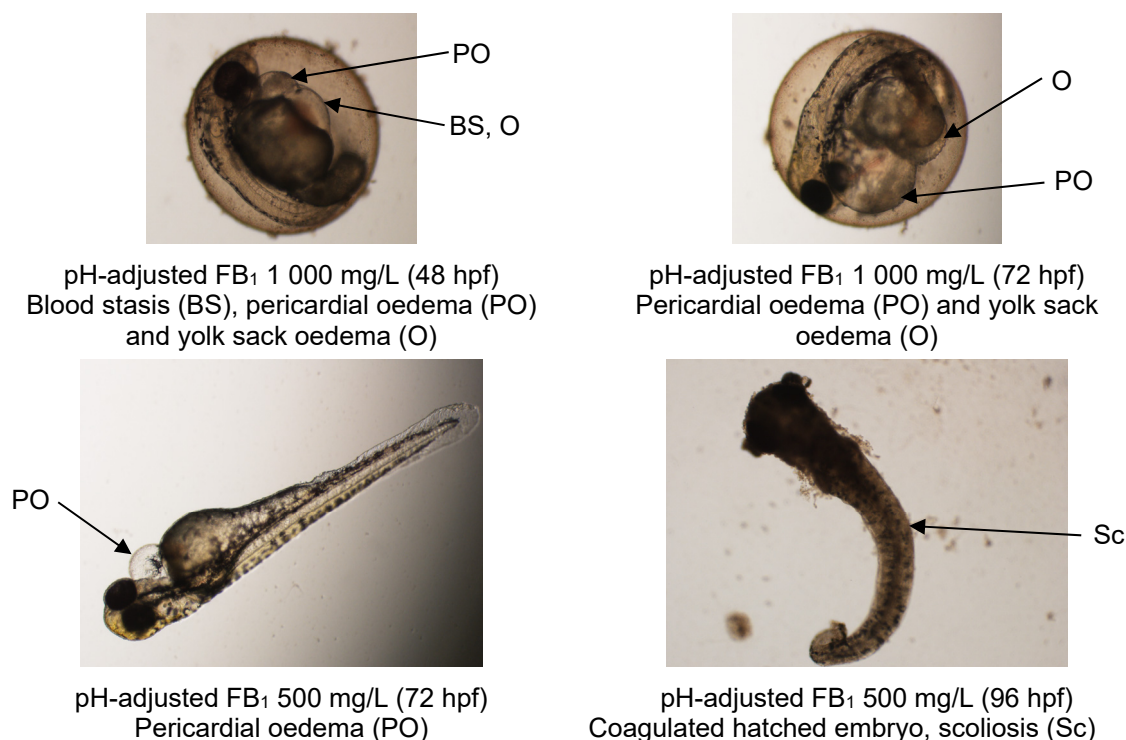


Figure 3.21: Typical developmental abnormalities observed in zebrafish embryos when exposed to pH-adjusted FB₁ over a period of 96 hpf

The toxicity results using the zebrafish FET assay differed from those obtained in the RTL-W1 cell culture cytotoxicity assay. Firstly, the IC₅₀ measured for FB₁ in the cell culture assay (1 260 µg/mL) was higher than the LC₅₀ obtained in the FET (277-1 170 µg/mL). Secondly, the effect of pH was more pronounced in the cell culture assay than in the FET assay. The mode and mechanism whereby FB₁ exerts toxicity is complex, and seems to vary in different cells, tissues and animal species (IARC, 2002). The primary mechanism for the toxic effect of FB₁ is the disruption of sphingolipid metabolism by inhibiting the enzyme ceramide synthase (IARC, 2002; Voss *et al.*, 2007; Stockmann-Juvala and Savolainen, 2008). This leads to an accumulation of sphinganine in cells and tissues, which is correlated with cardiovascular, liver and kidney dysfunction (IARC, 2002).

It is estimated that evolutionary divergence between zebrafish and mammals occurred about 445 million years ago (Peterson *et al.*, 2004). In spite of the long evolutionary separation, there is a high degree of genetic similarity between zebrafish and humans (Harper and Lawrence, 2011). Included in the genetic similarities, is the expression of *ceramide synthase (cers)* genes (Brondolin *et al.*,

2013). The zebrafish genome contains nine *cers* genes with distinct expression patterns during the embryonic development. The expression of these genes is tissue-specific, with elevated transcription in the central nervous system and liver during the development of the embryo. Zebrafish embryos provide a whole animal model and allows for metabolism and systemic interactions, which is an advantage over cell lines (Ducharme *et al.*, 2013).

In the current study both FB₁ and HFB₁ resulted in TI values <2, demonstrating that these substances are not predicted to be teratogenic. There are reports in literature that indicate that the consumption of fumonisin-contaminated maize may be associated with an increased incidence of neural tube defects in humans, a phenomenon which was also observed in several animal model studies (Gelineau-van Waes, 2010). However, in a recent report by the EFSA Panel on Contaminants in the Food Chain, it was deemed that the current evidence is inconclusive (EFSA, 2018). The reason for this viewpoint was that embryotoxicity in rodents were caused only at doses where maternal toxicity was observed, except for Syrian hamsters. Neural tube defects were caused only in sensitive mice strains (inbred LM/c).

Although the correlation between zebrafish and rodent toxicity is high (Ducharme *et al.*, 2013), there are also noteworthy differences between the physiology of mammals and fish; including the fact that fish are ectothermic and lack cardiac septa, lungs and synovial joints (Lieschke and Currie, 2007; Ali *et al.*, 2011). Furthermore, the zebrafish embryos develop outside the female's body and are not affected by her diet during the embryonic development, but are exposed directly to the test substances in the FET. In mammals, some substances ingested by the mother may undergo biotransformation before affecting the embryo. This phenomenon will not necessarily be replicated by the FET. Although there is little evidence that FB₁ undergoes biotransformation, it has been reported to increase biotransformation of aflatoxin B₁ in the hepatoma cell line H4IIE and spleen mononuclear cells of rats (Mary *et al.*, 2015).

3.4 CONCLUSIONS

The results obtained from this part of the study indicate that FB₁ exhibits a very low cytotoxicity towards the RTL-W1 cell line when tested without pH adjustment (IC₅₀ 1 260 µg/mL), and no cytotoxicity when the pH was adjusted to physiological conditions. No genotoxicity was observed for FB₁, FB₂, FB₃ or HFB₁ at concentrations up to 400 µg/mL. The zebrafish FET also yielded high LC₅₀ values for FB₁ (765 and 277 mg/L at 48 and 96 h, respectively), indicating a low acute toxicity. The EC₅₀ for pH-adjusted FB₁ was 749 and 490 mg/L at 48 and 96 hpf, respectively. This represents an increase in the EC₅₀ by a factor of 1.7-1.9 when the pH of the FB₁ solutions were adjusted to physiological conditions, compared to when FB₁ was tested without pH adjustment. The LC₅₀ could only be obtained after 96 hpf using the concentrations tested and the value (682 mg/L) was 2.5 times higher than the corresponding concentration without pH adjustment (277 mg/L). This indicates that the acute toxicity towards zebrafish embryos was reduced, but not eliminated when adjusting the pH. In the zebrafish FET assay, HFB₁ proved to be less toxic than FB₁. The LC₅₀ values for HFB₁ at 48 and 96 h were 1 090 and 471 mg/L (2 690 and 1 160 µM) respectively. The TI values of FB₁ (with and without pH adjustment) and of HFB₁ (without pH adjustment) were <2, indicating a lack of teratogenicity towards zebrafish embryos. None of the FB standards displayed any genotoxic effects towards the trout liver cell line RTL-W1 at concentrations up to 400 µg/mL.

The current study quantified the pH shifts caused by high concentrations of FB₁ and HFB₁ and proved that the pH shifts contribute greatly to their toxicities. It also confirmed that the FET is a useful toxicity screening tool that may account for effects not observable in cell cultures. This is the first study in which the RTL-W1 cell line and zebrafish FET were used to assess fumonisin toxicity. The findings are consistent with most other studies done in other animal models and have broadened the scientific knowledge concerning the toxic effects of FB.

CHAPTER 4

FUMONISIN INTERACTION WITH MAIZE AND ITS MACROCOMPONENTS

4.1 INTRODUCTION

Fumonisin is a common contaminant of South African maize (SAGL, 2016). Free fumonisins can be found in food products as parent fumonisins (e.g. FB₁, FB₂ or FB₃), in a partially hydrolysed form or as a totally hydrolysed form (Falavigna *et al.*, 2012). In partially hydrolysed forms, one of the TCA groups is removed, while both of the TCA groups are absent in fully hydrolysed forms (De Girolamo *et al.*, 2011). Masked fumonisins include covalently and non-covalently bound fumonisins commonly referred to as bound and hidden fumonisins, respectively (Falavigna *et al.*, 2012). These forms of fumonisins, which are not detected by conventional analytical methods, may contribute to the total fumonisin content and associated health risk. According to Shier *et al.* (2003), a significant portion of fumonisins can be modified during typical thermal food processing, without complete loss of *in vitro* toxicity (Shier *et al.*, 2003).

Although maize porridge is consumed by a broad spectrum of South Africans (Du Plessis, 2003; Louw *et al.*, 2010), knowledge on the formation of masked and/or modified fumonisins during its preparation is limited. In general, high heat treatments such as baking, frying and extrusion reduce fumonisin concentrations in food products, whereas mild treatments have little effect (Humpf and Voss, 2004). Shephard *et al.* (2002) reported that the preparation of traditional South African maize porridge (at 100 °C for 20 min) resulted in a 23% reduction of the FB levels. However, a subsequent study by Shephard *et al.* (2012) indicated only a small decrease (11%) in the total amount of fumonisins (Shephard *et al.*, 2012). It is not known whether these reductions could be ascribed to thermal decomposition of the FB, or to binding with food matrix components (e.g. starch and protein). Hence, there is a need for further investigation to determine whether the fumonisins in maize porridge are simply masked and not detoxified.

The study was designed to systematically obtain a better understanding of the binding of FB to maize and to its major macrocomponents. Different temperature and pH conditions were evaluated for their role in FB binding interactions.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and reagents

Fumonisin standards [FB₁, batches A/10-2014 and A/13; FB₂ batch A/14-2015; FB₃ batch A/07+16-2015 and hydrolysed fumonisin B₁ (HFB₁) batch A09-2014] with purities of >96% were obtained from the Institute of Biomedical and Microbial Biotechnology (IBMB), Cape Peninsula University of Technology (CPUT). Stock solutions (1 mg/mL) were prepared in deionised water. Maize starch was obtained from Tongaat-Hulett Starch (Johannesburg, South Africa) and amino acids (glutamic acid, proline, alanine and leucine with purities of >98%) were sourced from Chempure (Pretoria, South Africa). Zein and all other reagents (HPLC grade) were obtained from Sigma Aldrich/Merck (Darmstadt, Germany).

4.2.2 Maize samples

Uncontaminated (fumonisin free) and naturally contaminated maize kernels with high levels of fumonisins (FB₁ 74 500±200 µg/kg, FB₂ 15 900±200 µg/kg and FB₃ 7 210±740 µg/kg) were provided by the Southern African Grain Laboratory (SAGL). The maize samples were previously analysed at SAGL using an exhaustive method optimised for maximal extraction of fumonisins from raw maize (Skhosana, 2016). The samples were milled using setting number 2 (particle size <1 mm) on a Mazzer Major disk attrition mill (Mazzer Luigi S.p.a., Italy) and stored in sealed plastic bags at 4 °C.

4.2.3 Processing of fumonisins with maize and its macrocomponents

The reactions of fumonisins with maize meal and its macrocomponents (zein, maize starch, glucose and the amino acids glutamic acid, proline, alanine and leucine)

were studied under different pH and temperature conditions. A Rapid Visco Analyser (RVA 4500, Perten Instruments, Australia) was used as a reactor, since it allows for fine control of temperature and stirring (quantified shear energy applied) over time. Chemically resistant, lined RVA reaction canisters were used. Samples were processed for 20 min with stirring (160 rpm). The pH of deionised water was adjusted to 4, 7 and 10, respectively, using HPLC grade formic acid or ammonia. The sample weights were as follows: for starch and glucose, 1.00 g; for the amino acid mixture, 250 mg each of glutamic acid, proline, alanine and leucine, for the individual amino acids, 250 mg and for zein, 250 mg. For the amino acids, lower sample weights were used, due to their limited solubility in water (which ranged from 8.6 mg/mL for glutamic acid to 1 625 mg/mL for proline) (Haynes *et al.*, 2017). For zein, the reduced sample weight of 250 mg was also used, because preliminary analyses indicated that zein tended to cause matrix effects during the analysis. Due to difficulty with the determination of FB in the presence of the amino acid mixture, the amounts of the individual amino acids were decreased further to 25 mg in an attempt to limit the interference of the matrix with the determination of the fumonisins.

All samples were spiked with 1.00 mL of a standard solution containing 2.50 µg/mL each of FB₁, FB₂ and FB₃ and made up to a volume of 25.00 mL using pH-adjusted deionised water. The spiked samples contained a final concentration of 100 ng/mL each of FB₁, FB₂ and FB₃. The samples were analysed at the SAGL using a previously validated ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) method used for the routine analysis of maize.

4.2.4 Reaction between fumonisins and glutamic acid at room temperature

Due to difficulty in the construction of matrix-matched standard curves with solutions containing glutamic acid, the reaction between glutamic acid and FB was investigated further. For this purpose, FB₁, HFB₁ and glutamic acid (Glu) solutions (2.00 mg/mL each) were prepared in deionised water (pH 7) and filtered using 0.2 µm GHP syringe filters (Separations, Randburg, South Africa). The FB₁ was mixed with Glu in 1:1 and 1:2 ratios (v/v), without any heating or pH adjustment.

Hydrolysed fumonisin B₁ and Glu mixtures were prepared in the same way. The samples were analysed at the University of Johannesburg using an ultra-performance liquid chromatography–quadrupole time-of-flight–high-definition mass spectrometry (UPLC-QToF-HDMS) method.

4.2.5 Fumonisin determination using UPLC-MS/MS

From the 25.00 mL prepared (Section 4.2.3), a sample of 1.00 mL was diluted to a final volume of 10.0 mL with deionised water. It was then filtered through a 0.2 µm GHP syringe filter into the HPLC vials and analysed against matrix-matched standards (unless indicated otherwise) using the UPLC-MS/MS. Matrix-matched standards were prepared by diluting 1.00 mL of a blank sample to 10.0 mL with deionised water and using stock standards to prepare a set of matrix-matched standards of FB₁, FB₂ and FB₃ for the construction of a standard curve.

The UPLC-MS/MS analyses were conducted using a Waters Acquity system equipped with a binary pump and hyphenated with a triple quadrupole detector (TQD) fitted with an electrospray ionization (ESI) source (Waters, Milford, MA, USA), The system was controlled using MassLynx® software. The sample was introduced as a 5 µL injection volume. Separation was performed on an Acquity BEH C₁₈ (2.1 x 50 mm, 1.7 µm) column at 30 °C. A mobile phase flow rate of 0.4 mL/min was applied and gradient elution was carried out as indicated in Table 4.1. The standard conditions for analysis were used for all samples, unless stated otherwise.

Table 4.1: Standard and slow ramp conditions used for UPLC-MS/MS analysis of samples to determine fumonisins

Standard method			Slow ramp method		
Time (min)	% Eluent A	% Eluent B	Time (min)	% Eluent A	% Eluent B
0.00	90	10	0.00	90	10
3.00	90	10	3.00	90	10
10.00	30	70	15.00	30	70
10.10	10	90	15.50	30	70
12.00	10	90	15.60	10	90
12.10	90	10	17.00	10	90
15.00	90	10	18.00	90	10
			21.00	90	10

Eluent A = 0.5 mM ammonium acetate and 0.1% formic acid in water

Eluent B = 0.1% formic acid in acetonitrile

Nitrogen (20 L/h, 3 kV) and argon were used as the nebulising and collision gases, respectively, and the ESI was conducted in the positive-ion mode. For each compound, one precursor and two product ions were detected, with the one product ion used for quantification and the second for confirmation. The parameters used for the MS quantification of the fumonisins are provided in Table 4.2.

Table 4.2: Parameters applied for the quantification of fumonisins using UPLC-MS/MS

Parameter	FB ₁	FB ₂	FB ₃
Precursor ion (<i>m/z</i>)	722.4	706.4	706.4
Quantifier product ion (<i>m/z</i>)	352.3	336.4	336.5
Confirmation product ion (<i>m/z</i>)	334.3	318.3	318.3
Cone voltage (V)	42	40	40
Collision energy (eV)	40	40	40
Retention time (min)			
Standard method	6.33	7.08	6.79
Slow ramp method	8.02	9.24	8.76

The calibration data for fumonisins in different matrices are summarised in Appendix 1. The method performance was evaluated according to the SANCO/12571 guidelines and spiked blank samples (at the expected fumonisin concentration) were included with each set of samples, ensuring acceptable accuracy (SANCO, 2013). The limit of detection (LOD) and limit of quantification (LOQ) for FB₁, FB₂ and FB₃ in the different matrices are provided in Table 4.3.

Table 4.3: Limits of detection (LOD) and quantification (LOQ) for fumonisins in the different matrices using the UPLC-MS/MS method

Matrix	FB ₁ (ng/mL)		FB ₂ (ng/mL)		FB ₃ (ng/mL)	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Neat	0.625	1.25	0.696	1.39	0.641	1.28
Starch	2.50	5.00	2.67	5.33	2.59	5.18
Glucose	0.625	1.25	0.696	1.39	0.641	1.28
Zein	2.50	5.00	2.67	5.33	2.59	5.18
Amino acid mix	5.00	10.0	250	533	259	518
Alanine	2.50	5.00	2.67	5.33	2.59	5.18
Glutamic acid	50.0	100	250	533	259	518
Leucine	2.50	5.00	2.67	5.33	2.59	5.18
Proline	2.50	5.00	2.67	5.33	2.59	5.18
Maize meal	2.50	5.00	2.67	5.33	2.59	5.18

Amino acid mix contained contained 1 mg/mL each of glutamic acid, proline, alanine and leucine

Acceptable LOQ's for FB₁, FB₂ and FB₃ were achieved in all the matrices, except for the amino acid mixture and the glutamic acid solution.

4.2.6 Fumonisin determination using UPLC-QToF-HDMS

An Acquity UPLC (Waters, Milford, MA, USA) coupled in tandem to a Waters Synapt G1 QToF HDMS mass spectrometer was used to conduct the analyses. The injection volume was 1 µL. Chromatographic separations were carried out on a Waters HSS T3 C₁₈ column (150 mm x 2.1 mm, 1.8 µm) at 60 °C. A binary solvent mixture was used at a flow rate of 0.4 mL/min and gradient elution was applied as summarised in Table 4.4. Samples were kept in the auto sampler at 6 °C until injection.

Table 4.4: Gradient elution applied for UPLC-QToF-HDMS determination of fumonisins

Time (min)	% Eluent A	% Eluent B
0.00	100	0
2.00	100	0
15.00	2	98
17.00	2	98
20.00	100	0

Eluent A = water containing 10 mM formic acid (natural pH of 2.3)

Eluent B = acetonitrile containing 10 mM formic acid

The Synapt G1 mass spectrometer was used in V-optics and operated in ESI mode to enable detection of ESI-compatible compounds. Leucine enkephalin (50 pg/mL) was used as a reference for mass calibration to obtain typical mass accuracies between 1 and 5 mDalton (mDa). The mass spectrometer was operated in both positive and negative ionisation modes. The capillary voltage was maintained at 2.0 kV, and the sampling cone and extraction cone set at 30 V and at 4.0 V, respectively. The scan time was 0.1 sec, covering the 50 to 1500 Dalton mass range. The source and desolvation temperatures were 120 °C and 450 °C, respectively. Nitrogen gas was used as the nebulisation and cone gas at flow rates of 550 L/h and 50 L/h, respectively. Waters MassLynx® 4.1 (SCN 872) software was used to control the hyphenated system and perform all data acquisition and

analysis. The transitions used for the MS quantification of fumonisins in ESI positive mode are provided in Table 4.5.

Table 4.5: Transitions used for the quantification of FB₁ and HFB₁ in the QToF-HDMS method in ESI positive mode

Mycotoxin	Precursor ion (<i>m/z</i>)	Fragmentation ions observed (<i>m/z</i>)		Retention time (min)
FB ₁	722.39	352.32	334.31	7.69
HFB ₁	406.35	388.33	352.32	7.41

Compound identification was further confirmed by analysing all standards and samples with low and high collision energy settings of the collision cell. To minimise compound fragmentation, a low energy setting of 3 eV was used, while a collision energy ramp of 10 – 50 eV was selected to enhance fragmentation of molecules. The calibration data for FB₁ and HFB₁ are provided in Appendix 2. The method performance was evaluated according to the SANCO/12571 guidelines and spiked blank samples (at the expected fumonisin concentration) were included to determine recovery rates and ensure acceptable accuracy (SANCO, 2013). The LOD and LOQ for FB₁ and HFB₁ using the UPLC-QToF-HDMS method are given in Table 4.6.

Table 4.6: Limits of detection (LOD) and quantification (LOQ) for FB₁ and HFB₁ using the UPLC-QToF-HDMS method

Mycotoxin	LOD (µg/mL)	LOQ (µg/mL)
FB ₁	0.004	0.030
HFB ₁	0.005	0.040

4.2.7 Statistical analysis

Statgraphics® Centurion XVI version 16.1.17 software (Statpoint Technologies Inc., The Plains, Virginia, USA) was used to analyse the data. Analysis of Variance (ANOVA) was performed and a *p*-value of <0.05 for the F-test was considered to indicate statistically significant differences between samples. Means were compared with the aid of Fisher's LSD (Least Significant Difference) intervals. All analyses were performed in triplicate.

4.3 RESULTS AND DISCUSSION

In this section, the reaction of FB with maize and its macrocomponents is discussed. Stiff maize porridge contains 24.5% starch, 2.7% protein and 0.6% lipids (Wolmarans *et al.*, 2010). This study focussed starch and protein, since these are the major maize macrocomponents and the mechanisms by which FB interact with starch and proteins are less well understood than the interaction with lipids (Section 2.6.3).

4.3.1 Reaction of fumonisins with starch

Starch, a glucose polymer, is the main carbohydrate in maize. Whole maize kernel and maize endosperm contain about 62% and 87% starch on a dry weight basis, respectively (Shukla and Cheryan, 2001). Maize starch occurs in the form of polyhedral and rounded granules with an average size of 30 μm (Buléon *et al.*, 1998). Native starch is insoluble in cold water, but as the temperature is increased to the gelatinisation temperature, water is absorbed and the granules swell irreversibly (Coultate, 2016a). The crystalline order of the granule is also disrupted, and the viscosity of the suspension increases dramatically. In Figure 4.1, the effect of processing starch for 20 min at 30 and 90 °C on the recoveries of FB₁, FB₂ and FB₃, is illustrated. These temperatures were selected to represent conditions below and above the gelatinisation temperature (70-75 °C) of starch in maize flour (Coral *et al.*, 2009). The 20 min processing time is typical for the preparation of traditional stiff maize porridge (Van der Merwe, 1999). The pH values were selected to cover a range from acidic to alkaline conditions. Traditional stiff maize porridge is prepared with tap water (or another potable water source) and the pH is expected to be neutral. Porridge made from fermented maize (e.g. akamu) has an acidic pH of around 4, due to lactic acid fermentation (Ogbonnaya and Chidinma, 2012). Alkaline pH is not expected during the preparation of maize porridge but was included to complete the range of conditions. Other maize-based products, for example tortillas, include processing steps that take place under alkaline pH conditions (Brooker, 2015).

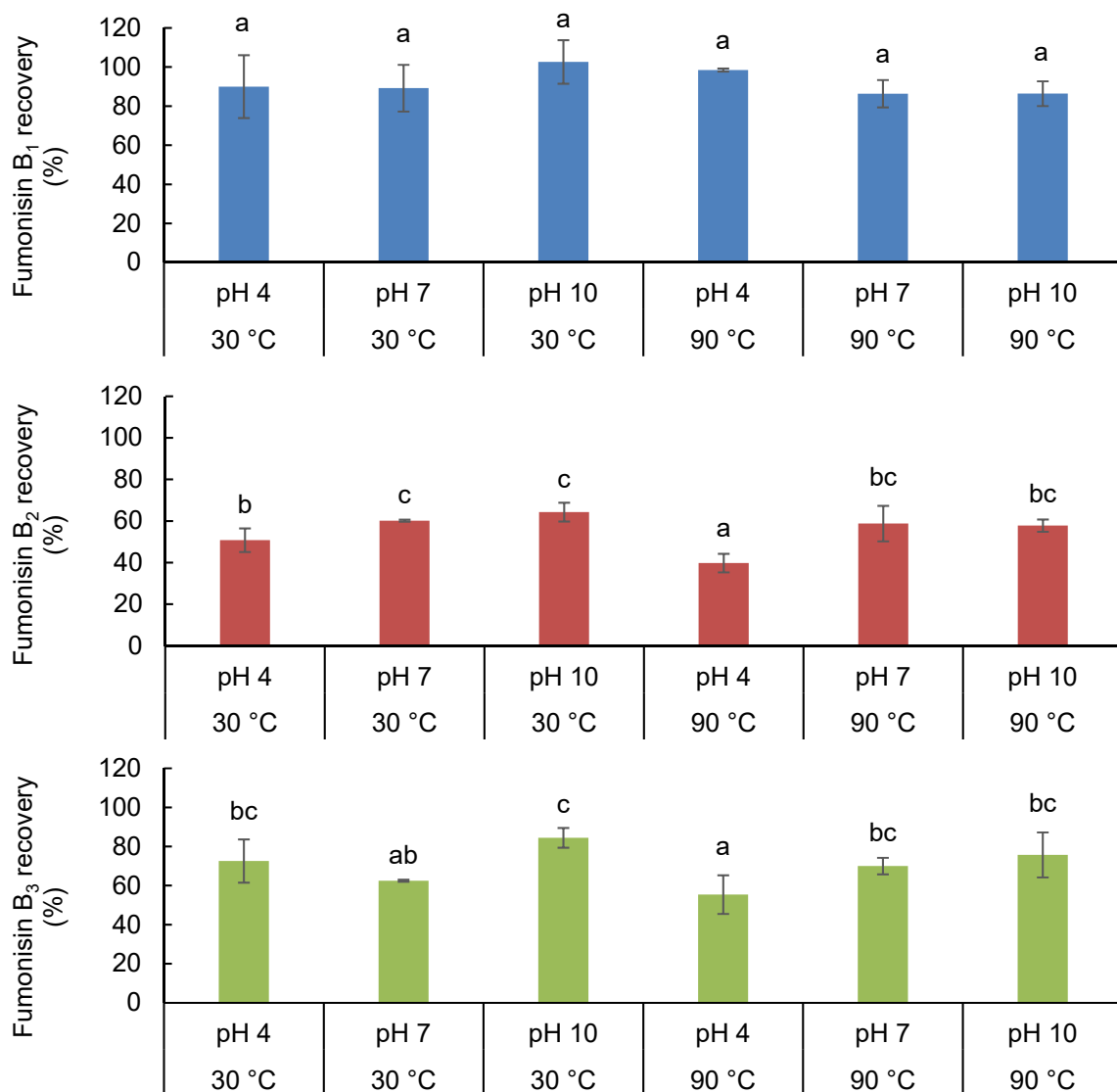


Figure 4.1: Effect of processing starch for 20 min at 30 or 90 °C on the recovery of fumonisins B₁, B₂ and B₃ at pH 4, 7 and 10
Means with any same letter are not significantly different ($p>0.05$)

The differences in the recovery of FB₁ for the treatments were not statistically significant ($p>0.05$), indicating that there was no reaction between FB₁ and starch under these conditions, and that no masking took place. For FB₂ there were significant differences ($p=0.0010$) between the treatments. The recovery of FB₂ at 90 °C and pH 4 was only 39.7%, which was significantly lower than those of the other treatments. For FB₃ there were significant differences ($p=0.0131$) between the treatments. The lowest recovery (55.3%) was for FB₃ at 90 °C and pH 4, which was significantly lower than for most of the other treatments.

To confirm the trends observed, the effects of heating at 90 °C for extended times (20, 40 and 60 min) on the recovery of FB₁, FB₂ and FB₃ from starch were also determined (Figure 4.2).

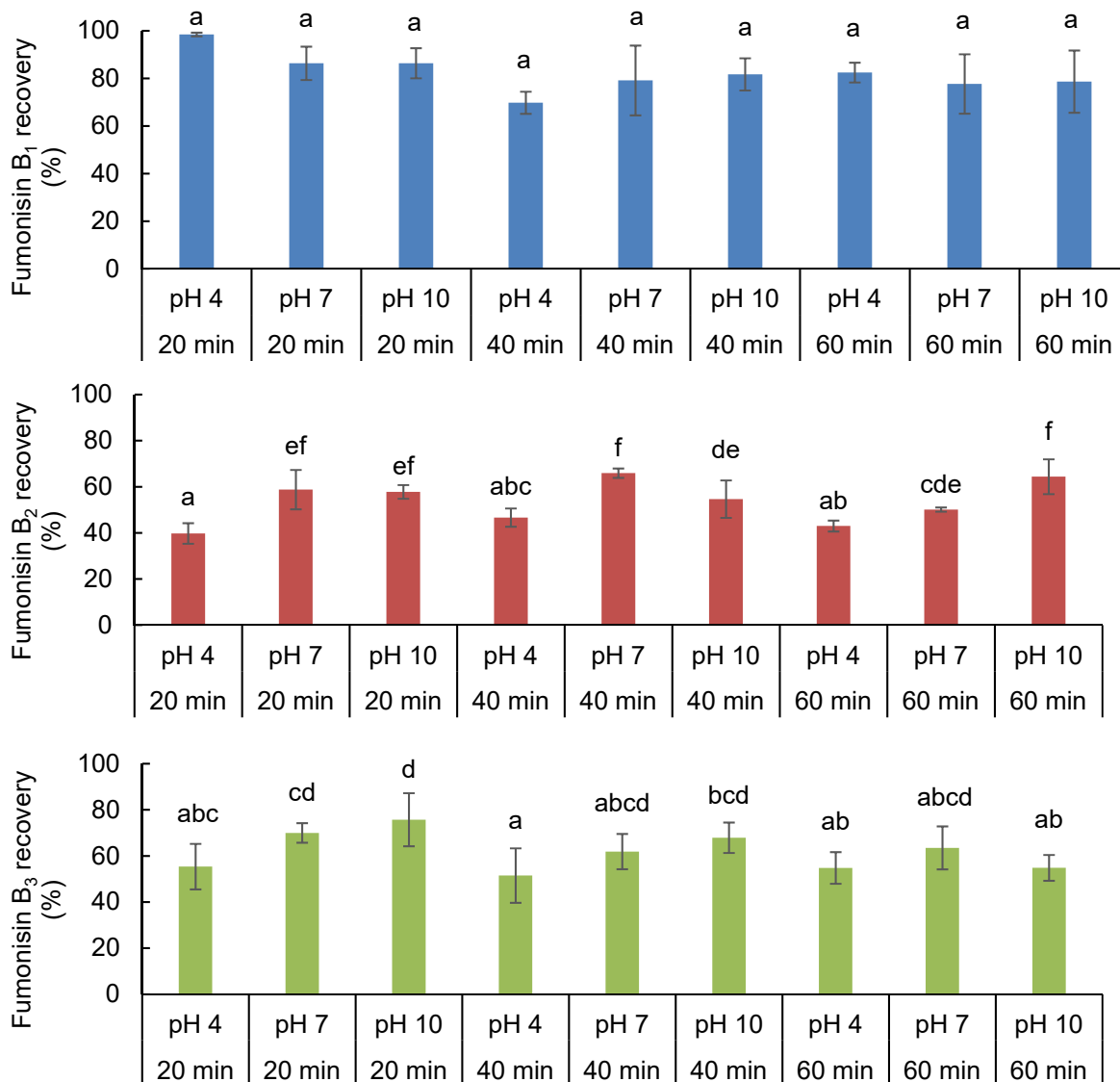


Figure 4.2: Effect of processing starch at 90 °C for 20, 40 or 60 min on the recovery of fumonisins B₁, B₂ and B₃ at pH 4, 7 and 10
Means with any same letter are not significantly different ($p>0.05$)

The differences in the recoveries of FB₁ for the extended heating treatments were not statistically significant ($p>0.05$), indicating that there was no reaction between FB₁ and starch, or any masking of FB₁, even with prolonged heating above the gelatinisation temperature. For FB₂, there were significant differences ($p=0.0001$) between the treatments and the recovery of FB₂ at pH 4 ranged from 39.7 to 46.6%

and was significantly lower than most of the other treatments. For FB₃, there were also significant differences ($p=0.0327$) between the treatments, but the trends were not as clear. The recovery of FB₃ was significantly higher at pH 10 than at pH 4 for the 20 min and 40 min treatments. However, there were no significant differences in the recoveries of FB₃ at different pH values when processed for 60 min.

Although starch appears to play a key role in the associative interaction occurring between fumonisin and maize macrocomponents, there are still gaps in the existing knowledge regarding the nature of the interactions (Dall'Asta and Battilani, 2016). Using a model system, Seefelder *et al.* (2003) demonstrated that FB₁ can react with methyl α -D-glucopyranoside (representing starch) *via* the TCA side chains. However, it is not known to what extent to this reaction takes place in food products during processing (Humpf and Voss, 2004). Kim *et al.* (2002) reported that FB₁ and FB₂ seem to be unstable in corn starch, with fumonisins apparently disappearing after 5.5 months storage at room temperature, since they were not recovered from the spiked samples. In addition, the recoveries of fumonisins directly after spiking was poor, with only 50 - 60% being recovered from corn starch.

More recent publications highlighted the importance of starch gelatinisation in the recovery of fumonisins from maize samples, as well as the formation of modified fumonisins (Giorni *et al.*, 2015; Dall'Asta and Battilani, 2016). These authors, on the one hand, hypothesised that starch gelatinisation renders the maize matrix more accessible to solvent extraction, thereby increasing the recovery of FB. Conversely, they hypothesised that the amylose fraction of starch may be involved in the formation of modified fumonisins, which are not detected and quantified by the standard analytical methods. It is well known that amylose is able to form inclusion complexes with small non-polar molecules and the hydrophobic domains of amphiphilic compounds (Putseys *et al.*, 2010). With this knowledge, Giorni *et al.* (2015) argued that it could be possible that fumonisins, having a 20-carbon aminopolyhydroxy-alkyl chain backbone, could interact with amylose in a similar way, thereby forming a modified fumonisin that could be released upon digestion. However, when examining the three-dimensional (3D) structures of FB₁, FB₂ and

FB₃ using the ChemIDplus databasis⁷ and comparing it to that of eicosanoic acid (a 20-carbon saturated fatty acid), it is evident that unlike the fatty acid, the 3D structures of fumonisins are not linear (Appendix 3). In addition, the fumonisins have an amino group and two or more hydroxyl groups attached to the carbon backbone, resulting in more electrostatic charges and a more polar molecule than the fatty acid, therefore the hypothesis proposed by Giorni *et al.* (2015) seems questionable and should be verified.

Inclusion complexes in starch may be detected and characterised using techniques such as X-ray diffraction, differential scanning calorimetry, Fourier-transform infrared spectroscopy and pasting properties (Wang *et al.*, 2016). Nevertheless, to synthesise detectable amounts of an amylose inclusion complex, one would require 0.3-10% of the ligand (Putseys *et al.*, 2010), which would be the fumonisin molecule in this case. Due to the high cost and limited availability of FB standards, such experiments were not feasible in the current study.

The current study indicates that the recoveries of FB₁, FB₂ and FB₃ processed in the presence of starch differed. This may possibly suggest that the position of the hydroxyl groups on the carbon backbone could play a role in the interaction of FB with starch. Another potential explanation is that the UPLC-MS/MS method used was better optimised for accurate quantification of FB₁ rather than for FB₂ and FB₃.

4.3.2 Reaction of fumonisins with glucose

Whole maize contains only a small amount of free sugars (1.61 to 2.22% on a dry weight basis) (Kent and Evers, 1994). The reaction of fumonisin B₁ with reducing sugars such as glucose, occurs *via* the C2-amino group and results in the formation of *N*-(carboxymethyl) fumonisin B₁ (Howard *et al.*, 1998) and a number of other Maillard reaction products, including the fumonisin B₁-glucose Schiff's base *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₁ (Lu *et al.*, 2002). The rate and complexity of the

⁷ <https://chem.nlm.nih.gov/chemidplus/> [Accessed 28/02/2018]

reaction increase with a corresponding increase in temperature. The Maillard reaction is known to be influenced by pH (Martins *et al.*, 2001; Van Boekel, 2001). In addition, the loss of FB₁ in an aqueous medium (in the absence of sugars) is more rapid and extensive under alkaline (pH 10) and acidic conditions (pH 4), than at neutral pH (Jackson *et al.*, 1996c). For these reasons, pH 4 and 10 were selected for this study, and the reaction of fumonisins with glucose was investigated at 90 °C, to represent a temperature that would be applied during the preparation of porridge.

The results indicate that glucose does not have a significant effect on the recovery of FB₁, FB₂ or FB₃ when processed for 20 min at pH 4 and 10 at 90 °C (Figure 4.3). However, the recovery of FB₂ was significantly lower at pH 4 than pH 10 (irrespective of whether glucose was added or not). This was not the case for FB₁ and FB₃.

The current study confirms that fumonisins do not react with glucose under temperature and time parameters typically encountered during cooking of maize porridge. However, it should be noted that glucose conjugates of fumonisins have been synthesised by applying higher temperatures over prolonged time (Lu *et al.*, 2002; Seefelder *et al.*, 2003; Matsuo *et al.*, 2015). Glucose derivatives of mycotoxins may also be formed through plant metabolism as a natural mechanism to reduce mycotoxin accumulation (Lattanzio *et al.*, 2012). While the chemical reaction between fumonisins and glucose is well described in literature, there is currently limited information on naturally occurring fumonisin glucoside derivatives in unprocessed and processed maize. Only one study reported the presence of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₂ and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B₃ in naturally contaminated corn (maize) flour (Matsuo *et al.*, 2015). Low levels of *N*-(carboxymethyl) fumonisin B₁ have been detected in some samples of commercially processed food products (tortilla chips, nachos chips and corn flakes) (Seefelder *et al.*, 2001).

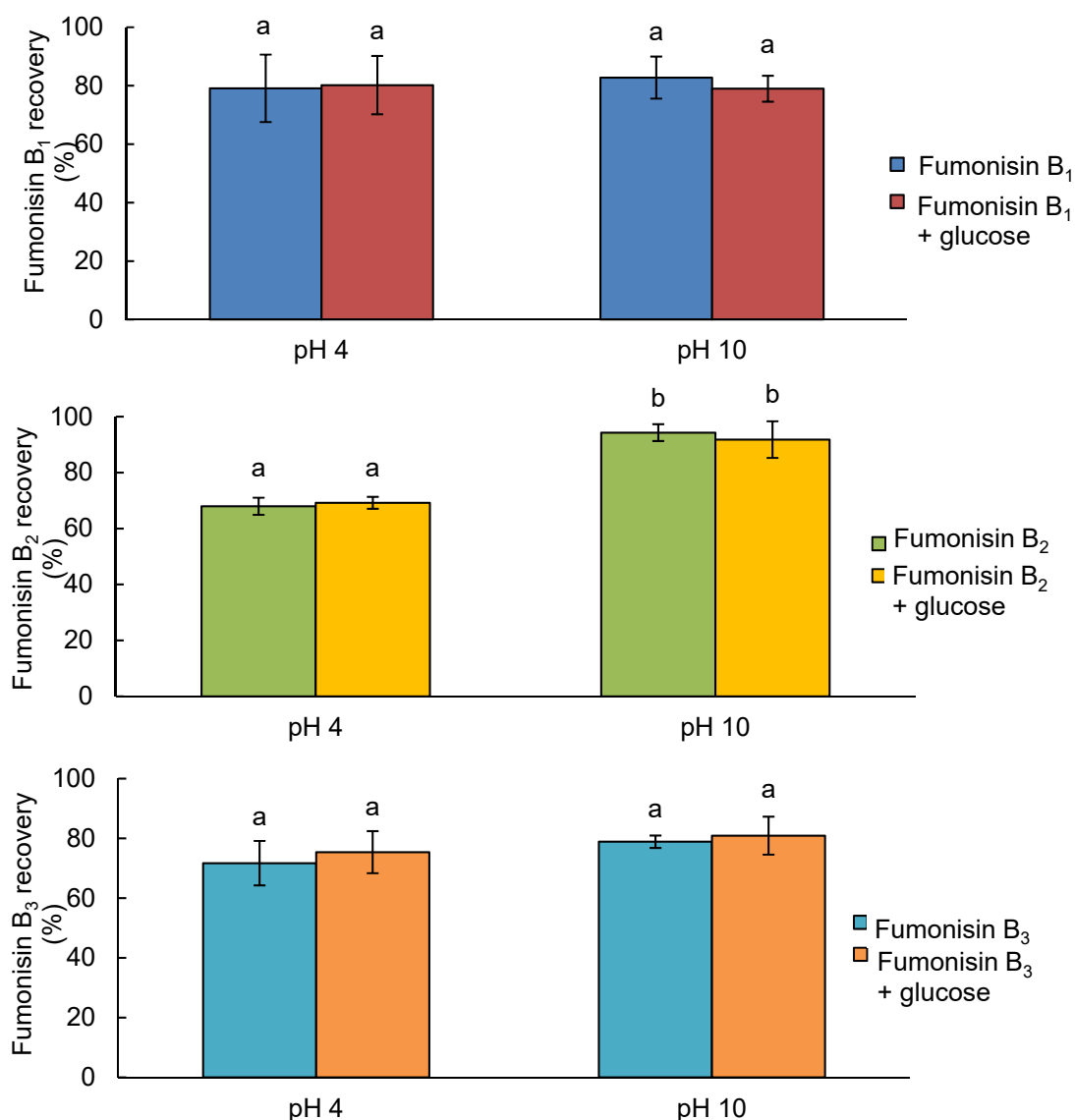


Figure 4.3: Recoveries of fumonisins B₁, B₂ and B₃ after processing in the presence of glucose for 20 min at 90 °C and pH 4 and 10
Means with any same letter are not significantly different ($p>0.05$)

4.3.3 Reaction of fumonisins with zein

Like most cereal grains, maize kernel and maize endosperm contain about 7.8% and 8% protein, respectively (Shukla and Cheryan, 2001). The main protein in maize endosperm is the prolamin protein, zein, which occurs in the form of protein bodies. Zein consists of four fractions, namely α -, β -, γ and δ -zein, with α -zein being the major fraction (Luo and Hu, 2017). In this study, processing under different pH and temperature conditions did not have a significant effect on the recovery of FB₁ in a matrix containing zein (Figure 4.4). On the other hand, recovery of FB₂ was

significantly ($p=0.0001$) higher at pH 10 than at pH 4 and 7. The recovery of FB₃ was also significantly increased ($p=0.0002$) at pH 10, although these values were comparable to the recovery at pH 4 processed at 90 °C.

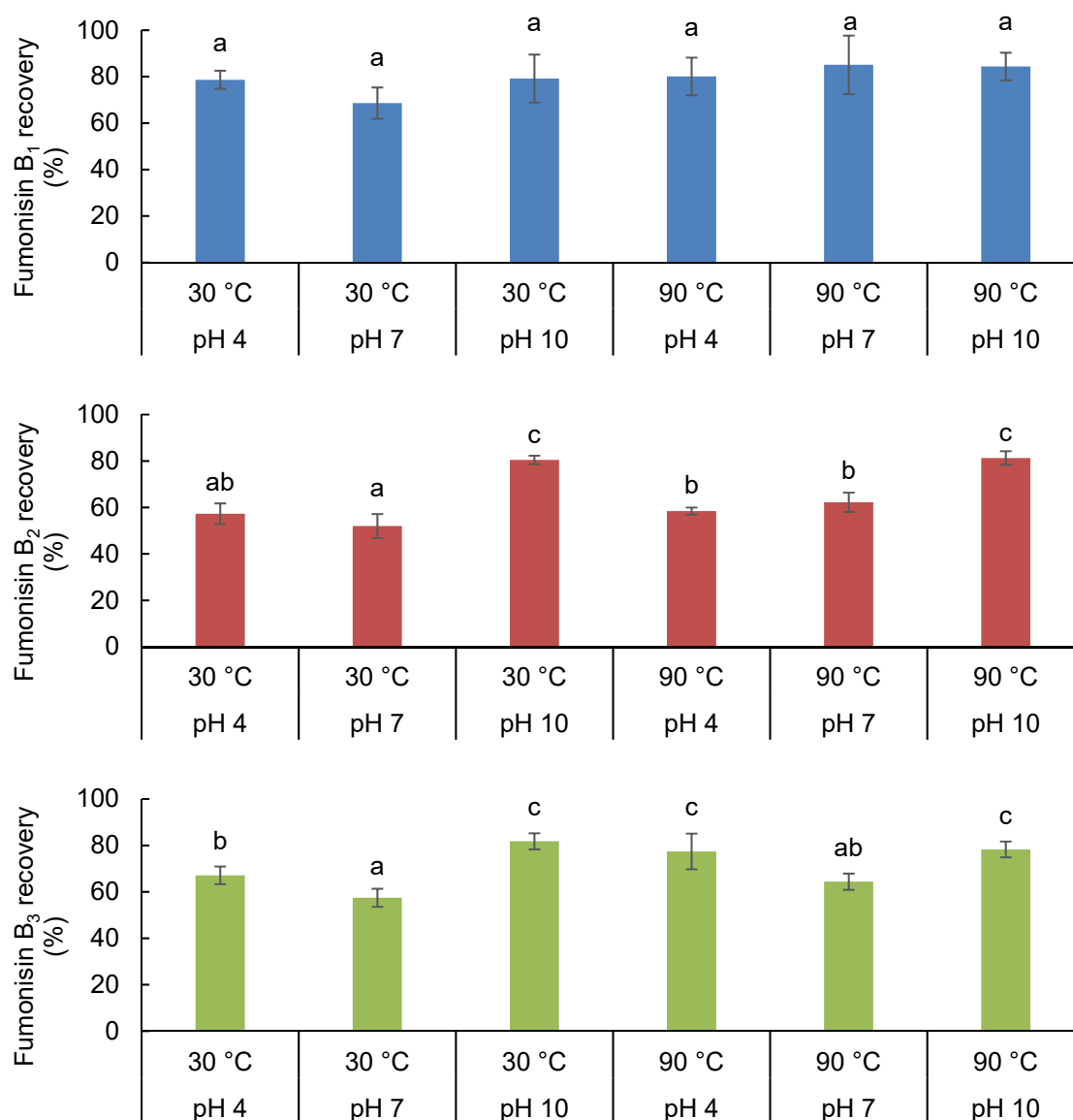


Figure 4.4: Effect of processing zein for 20 min at 30 or 90 °C on the recovery of fumonisins B₁, B₂ and B₃ at pH 4, 7 and 10
Means with any same letter are not significantly different ($p>0.05$)

As is the case with starch, current knowledge on the interaction of fumonisins with proteins is insufficient to fully understand the mechanisms involved. According to the available literature, in model systems, the TCA side chains of FB₁ react with amino acids and proteins *via* functional groups, including the ϵ -amino moiety of

lysine and the thiol function of cysteine, to form covalently bound products (Seefelder *et al.*, 2003; Shier *et al.*, 2003). However, as maize zein is deficient in lysine and cysteine (Shukla and Cheryan, 2001), it is unlikely that this reaction could explain the lower recoveries observed for FB₂ and FB₃ at pH 4 and 7.

Another possible explanation is that the fumonisins may be physically entrapped by the protein matrix (Mangia, 2009; Dall'Asta *et al.*, 2010). As previously stated by Shukla and Cheryan (2001), due to its amino acid composition (consisting mainly of hydrophobic (75%) amino acid residues and containing less hydrophilic amino acid residues (25%)), zein is insoluble in water (Shukla and Cheryan, 2001). The secondary structure of zein consists of 50 to 60% α -helix and 15% β -sheet, whereas the tertiary structure has been proposed as an elongated rectangular prism-like shape, with the upper and lower surfaces containing glutamine loops that are hydrophilic, while the other four surfaces are highly hydrophobic (Luo and Hu, 2017). This arrangement allows zein to form aggregates, which might trap fumonisins and result in lower recovery. The data obtained in our study did not provide a clear answer explaining why the zein matrix would trap FB₂ and FB₃, but not FB₁. A possible explanation for the slightly better recovery of FB₂ and FB₃ observed at pH 10 compared to pH 4 and 7, may be due to the increased solubility of zein under alkaline conditions (Shukla and Cheryan, 2001).

4.3.4 Reaction of fumonisins with amino acids

The main amino acids in maize protein are glutamic acid, proline, alanine and leucine (Sodek and Wilson, 1971; Shukla and Cheryan, 2001). In the current study, the determination of fumonisins in the presence of a mixture of amino acids proved to be problematic. It was not possible to construct a reliable matrix-matched standard curve using a mixture of amino acids (glutamic acid, proline, alanine and leucine) as a matrix (Appendix 1). Hence, the recovery of FB from the amino acid mixture was quantified directly against neat standards (Figure 4.5). Although processing did not have a significant effect on the recovery of FB₁, it is noticeable that the recoveries of FBs from the mixture were low. This could indicate a chemical reaction of FBs with amino acids, or an analytical interference.

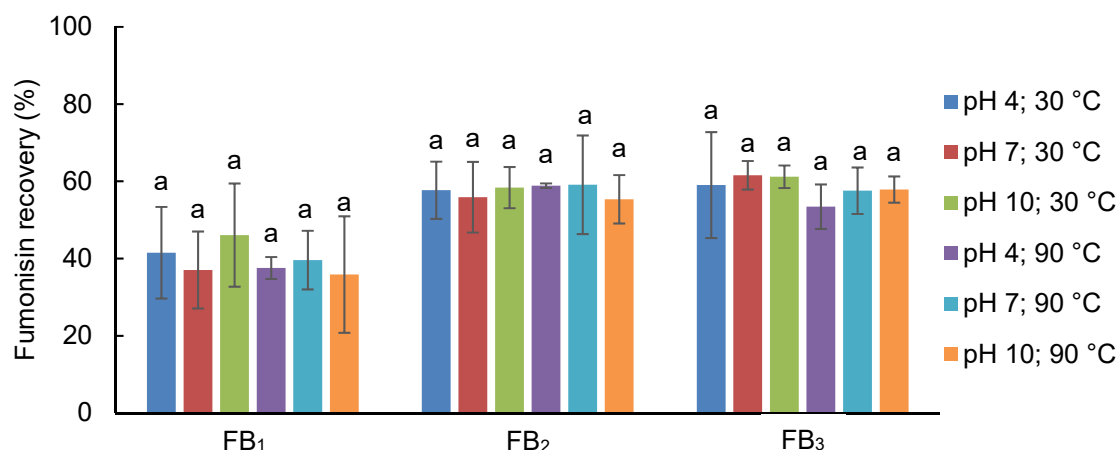


Figure 4.5: Recoveries* of fumonisins from a mixture of amino acids** processed for 20 min under different pH and temperature conditions

*Analysed against neat standards

**Mixture contained 10 mg/mL each of glutamic acid, proline, alanine and leucine

Means with any same letter are not significantly different ($p > 0.05$)

In an attempt to decrease the matrix effect of the amino acids, the concentration of the individual amino acids was decreased from 10.0 mg/mL to 1.00 mg/mL and in addition to the amino acid mixture, individual solutions were also prepared. Fumonisins were spiked into the solutions (without any pH adjustment or processing) and the UPLC parameters were adjusted to a slower ramp in an attempt to improve the analytical results. The recoveries were determined by analysing *versus* both neat and matrix matched standards (Table 4.7).

Table 4.7: Recoveries of FB spiked into solutions containing amino acids quantified *versus* neat and matrix matched standards

Amino acid	FB recovery (ng/mL) vs neat standards			FB recovery (ng/mL) vs matrix matched standards		
	FB ₁	FB ₂	FB ₃	FB ₁	FB ₂	FB ₃
Glutamic acid	-*	-	-	-	-	-
Proline	12.1	16.3	17.5	11.4	14.0	13.0
Alanine	11.0	13.8	14.7	9.70	12.0	11.8
Leucine	10.4	15.2	14.6	10.4	15.6	12.5
Amino acid mix	-	-	-	-	-	-

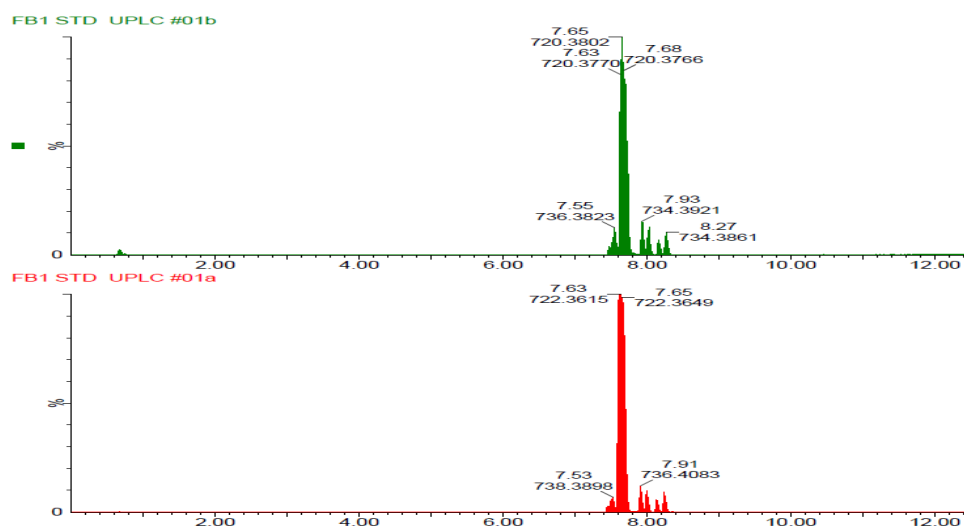
Amino acid concentration was 1 mg/mL, the mixture contained 1 mg/mL each of glutamic acid, proline, alanine and leucine, FB was spiked at a concentration of 10.0 ng/mL, values are the means of duplicate analyses, *UPLC-MS/MS response too low for quantification

Fumonisins could be quantified in solutions containing proline, alanine or leucine, but not in the glutamic acid solution or the mixture of amino acids. This indicates that glutamic acid is somehow involved in the poor recovery obtained. All amino

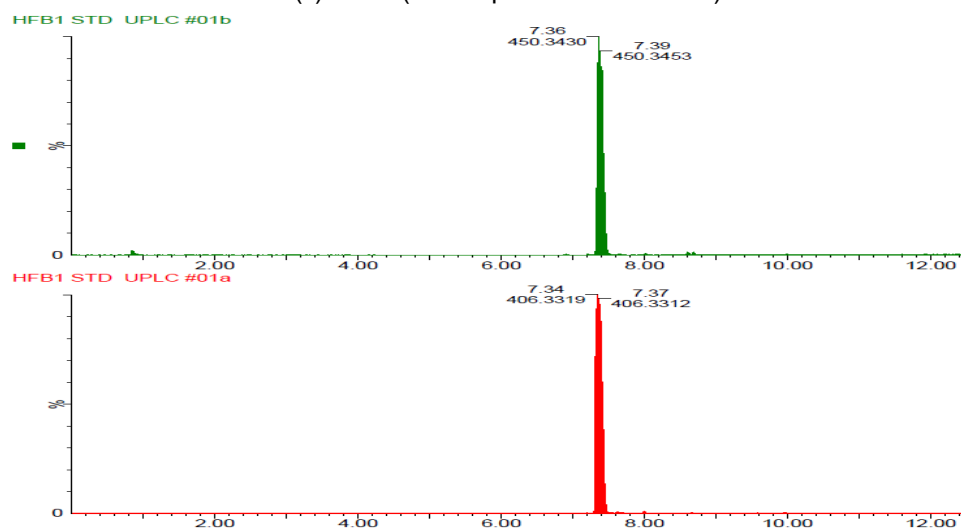
acids contain a central carbon atom, which carries amino and carboxyl groups, as well as a side chain (Coultate, 2016b). Glutamic acid's side chain imparts acidic properties and is electrically charged, whereas the side chains of proline, alanine and leucine are non-polar and hydrophobic in nature. The interaction of glutamic acid with FB was investigated further by UPLC-QToF-HDMS analysis. The chromatograms of FB₁, HFB₁ and glutamic acid are presented in Figure 4.6.

Sharp peaks were observed on the chromatograms of the FB₁ and HFB₁ standards. For glutamic acid, the ESI negative mode generated a peak with a molecular mass of 146 g/mol, which corresponds to the molecular weight of glutamic acid. However, in ESI positive mode a peak with a molecular mass of 130 g/mol was observed, which corresponds to pyroglutamic acid. The free amino group of glutamic acid can cyclise to form the lactam, pyroglutamic acid (Gayte-Sorbier *et al.*, 1985). Although this reaction is favoured at pH values ranging from 2 to 3, and in the presence of oxygen and high temperatures; it even takes place under refrigerated conditions. The natural pH of the 2 mg/mL glutamic acid solution was 3.45.

(i) FB₁ (ESI- top and ESI+ bottom)



(ii) HFB₁ (ESI- top and ESI+ bottom)



(iii) Glutamic acid (ESI- top and ESI+ bottom)

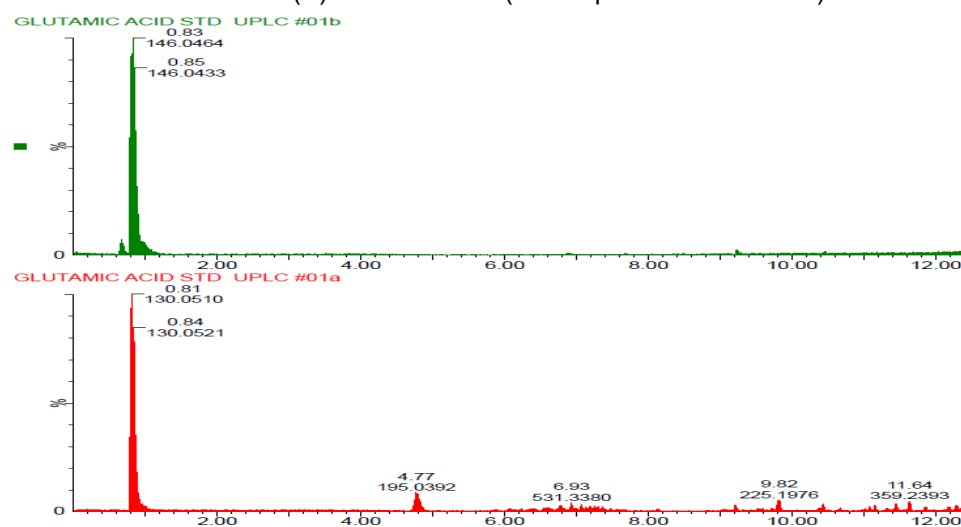
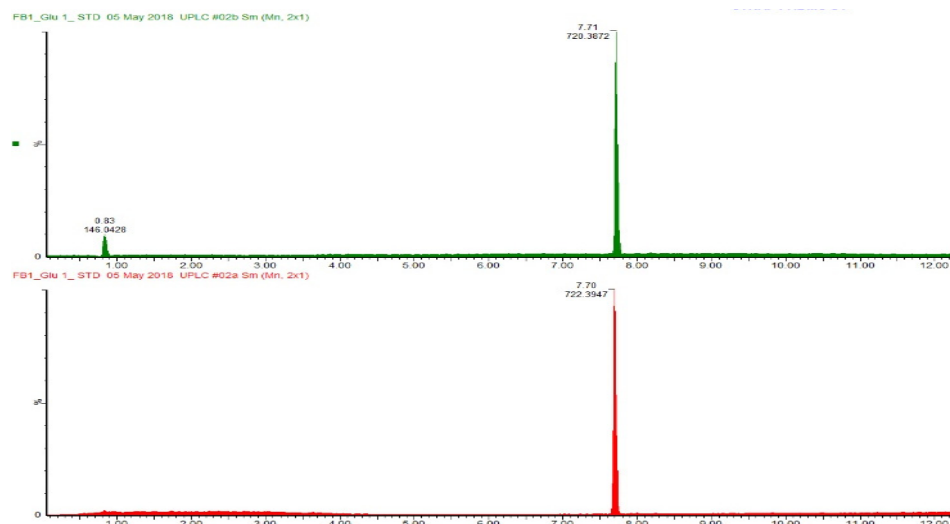


Figure 4.6: Chromatograms of fumonisin B₁ (i), hydrolysed fumonisin B₁ (ii) and glutamic acid (iii) in ESI negative and positive modes

The chromatograms obtained after combining glutamic acid with FB₁ and HFB₁, at room temperature are presented in Figure 4.7.

(i) Glu and FB₁ (ESI- top and ESI+ bottom)



(ii) Glu and HFB₁ (ESI- top and ESI+ bottom)

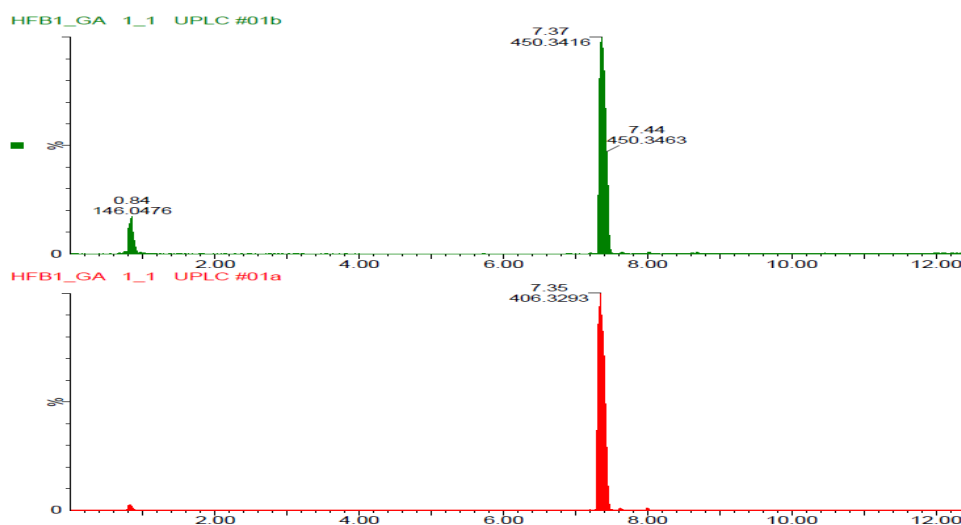


Figure 4.7: Chromatograms of mixtures (1:1) of glutamic acid with FB₁ (i) and HFB₁ (ii) in ESI negative and positive modes

The results demonstrate that combining FB₁ with glutamic acid does not result in any new chemical components forming, even when increasing the ratio of FB₁:glutamic acid to 1:2 (results not shown). In addition, HFB₁ was not affected by glutamic acid and remained unchanged, regardless of whether it was mixed with glutamic acid in 1:1 or 1:2 ratios. Using the UPLC-QToF-HDMS, FB₁ could be

detected in the presence of glutamic acid ($94\pm 11\%$ recovery of FB₁) and there was no evidence that modified fumonisins were formed in the presence of glutamic acid at ambient temperature.

4.3.5 The reaction of fumonisins in maize porridge

Fumonisin B₁, B₂ and B₃ recoveries from uncontaminated maize meal spiked with fumonisins, as well as naturally contaminated maize meal, were measured after processing under different pH (4, 7 and 10) and temperature (30 and 90 °C) conditions.

4.3.5.1 Uncontaminated maize meal spiked with fumonisins

From Figure 4.8 it is evident that, in the uncontaminated spiked maize meal, the variation in the recovery of FB₁ between the treatments was not statistically significant ($p>0.05$). This confirms that cooking under different pH and temperature conditions did not influence the spiked FB₁ in the maize meal. The recovery of FB₁ from the uncontaminated spiked maize meal was also high, ranging 88.8 to 110%.

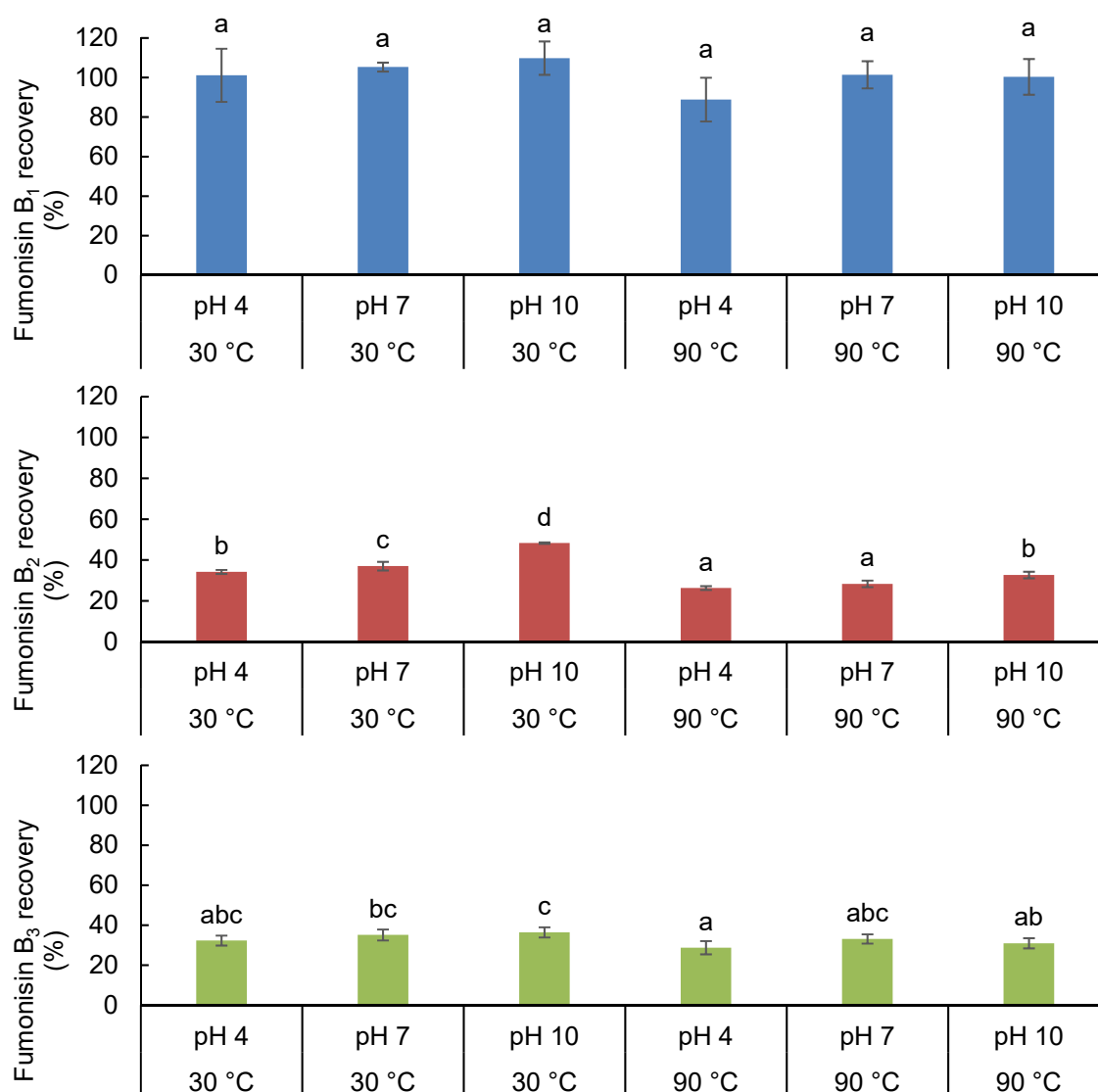


Figure 4.8: The effect of processing for 20 min under different pH and temperature conditions on the recovery of fumonisins B₁, B₂ and B₃ from uncontaminated maize meal spiked with fumonisins
Means with any same letter are not significantly different ($p > 0.05$)

There were significant differences in the recoveries of FB₂ ($p=0.0001$) between the treatments and the recoveries of FB₂ from uncontaminated spiked maize meal were low, ranging from 26.2 to 48.3%. A similar tendency was observed for FB₃, where significant differences ($p=0.0448$) between the treatments were measured and the recoveries ranged from 28.7 to 36.4%. For both FB₂ and FB₃, the lowest recovery was for the pH 4, 90 °C treatment.

4.3.5.2 Naturally contaminated maize meal

In the naturally contaminated maize meal (Figure 4.9), there were significant differences in the recovery of FB₁ ($p=0.0017$), FB₂ ($p=0.0001$) as well as FB₃ ($p=0.0001$).

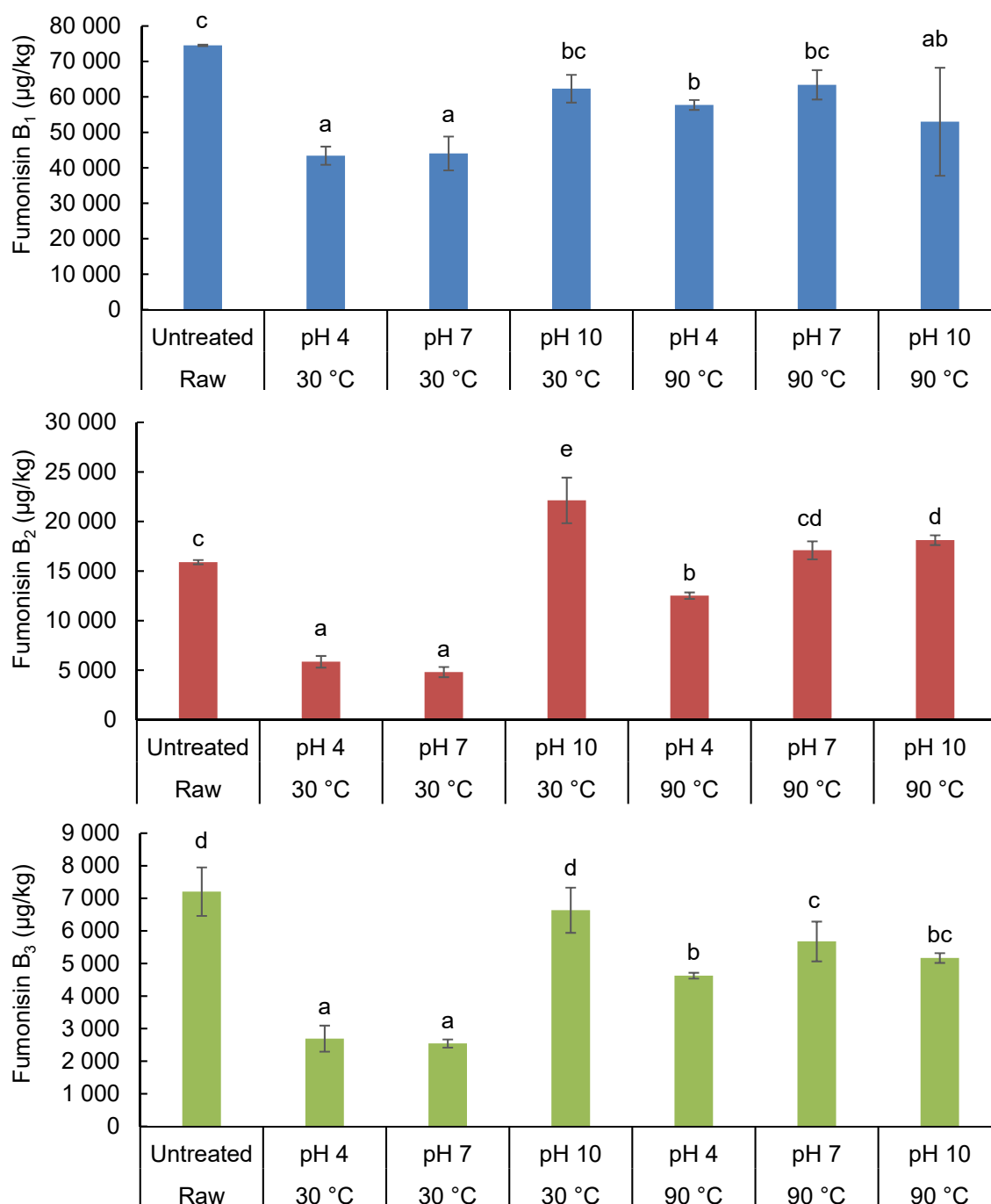


Figure 4.9: The effect of processing for 20 min under different pH and temperature conditions on the measured FB₁, FB₂ and FB₃ in naturally contaminated maize

Means with any same letter are not significantly different ($p>0.05$)

Overall, lower recoveries were obtained at pH 4 and 7, when processing at 30 °C. In naturally contaminated maize meal the fumonisins may be trapped in the matrix. The exhaustive method, which was used at SAGL to analyse the untreated raw sample, involves a 2 h extraction step using a solvent mixture containing water:methanol:acetonitrile (50:25:25 v/v/v) (Skhosana, 2016). In the current study, the experimental procedure to determine the fumonisins did not involve solvent extraction but instead used the “dilute and shoot” method. For this reason, it is possible that some of the fumonisins in the samples at pH 4 and 7, processed at 30 °C, were still trapped in the matrix. Processing at 90 °C gelatinized the starch and disrupted the matrix, which probably resulted in better extraction of fumonisins from the matrix. Although alkaline conditions are known to cause hydrolysis of FB to HFB (De Girolamo *et al.*, 2016), this reaction did not seem to take place in the current experiment, because the recovery of FB was high in all the pH 10 samples. This pH, although alkaline, was milder than the conditions used by De Girolamo *et al.* (2016) during nixtamalization [1-5% Ca(OH)₂] and the 2 M NaOH often used to hydrolyse masked fumonisins in maize samples before analysis (Dall'Asta *et al.*, 2008).

Fumonisin stability under different pH and temperature conditions (in the absence of a food matrix) was studied extensively by Jackson and co-workers more than two decades ago (Jackson *et al.*, 1996a; Jackson *et al.*, 1996b; Jackson *et al.*, 1996c). Only recently, a study was published in which the effects of pH and temperature on the stability of fumonisins in a maize matrix (baked dough) was investigated (Bryla *et al.*, 2017). These authors obtained higher free fumonisin levels in maize dough prepared using a pH 7.5 buffer compared to those obtained when using pH 5.5 and 3.5. The difference was ascribed to a higher extraction efficiency of the fumonisins at pH 7.5. It was speculated that, at pH 7.5, a proton from one of the carboxylic acid groups would be donated to the amino group, resulting in a zwitterion and increased solubility.

The properties, as well as intra- and intermolecular interactions of ampholytes are influenced by pH (Pagliara *et al.*, 1997). Based on the chemical structure of FB₁ and

using the ChemAxon Chemicalize online platform⁸, the following pK_a values are predicted for FB₁: 3.16, 3.75, 4.77 and 5.35 (for the carboxylic acid groups) and 9.53 (for the amino group). Fumonisin B₁ may exist in solution in four different electrical states: cationic, charged with a net charge of zero (zwitterion), uncharged neutral and anionic. Due to the number of ionisable groups on the molecule, there are 20 different possible microspecies (Appendix 4). At the isoelectric point of FB₁, which is calculated to be 3.43, the zwitterionic forms are the most abundant and the solubility of the molecule is at its lowest. It is well known that the solubility of amphoteric compounds (e.g. proteins) are reduced at the isoelectric point, because at that pH the negative and positive charges on the molecule are balanced, reducing repulsive electro-static forces (Novák and Havlíček, 2016).

According to the results of the ChemAxon Chemicalize properties calculation⁸, all four carboxylic acid groups, as well as the amino group, are expected to be ionised at pH 7, resulting in a net negative charge on the molecule. At pH 10, the carboxylic acid groups are ionised, but not the amino group, resulting in an even larger negative charge on the molecule. In addition, it is estimated that the solubility of FB₁ will increase as the pH is increased above the isoelectric point and reach a maximum at pH 5.2. Further increases in pH are not expected to affect the solubility. Considering these facts, the reasoning by Bryla *et al.* (2017) regarding the solubility and existence of FB as a zwitterion around pH 7 seems incorrect. Nonetheless, in the current study, the pH does appear to play a role in the recovery of fumonisins from maize and its macrocomponents. Lower fumonisin recoveries were often obtained at pH 4, although the data was not conclusive.

4.4 CONCLUSIONS

This is the first study in which the effects of pH and temperature were studied in maize under conditions simulating the preparation of traditional maize porridge. None of the treatments (pH 4, 7 and 10) or temperatures (30 and 90 °C) resulted in a significant decrease in the recovery of FB₁ from starch, glucose, zein or maize

⁸ <https://chemicalize.com> [Accessed 06/03/2018]

meal spiked with FB. These results confirm the stability of FB₁ in maize porridge and thereby support the findings of Shephard and co-workers (Shephard *et al.*, 2002; Shephard *et al.*, 2012). Although the recovery of FB₁ from the mixture of amino acids was low, the UPLC-QToF-HDMS analysis demonstrated that no modified fumonisins were formed in the presence of glutamic acid at ambient temperature. The results of this study did not provide a clear understanding as to why the recoveries of FB₂ and FB₃ were overall consistently lower than those of FB₁ in spiked samples. This highlights the challenges associated with the determination of fumonisins in different food matrixes and the importance of validating the methods for each matrix. More research is required to determine the nature of the interaction of FB₂ and FB₃ with maize and its macrocomponents.

CHAPTER 5

BIOACCESSIBILITY OF FUMONISINS FROM MAIZE PORRIDGE

5.1 INTRODUCTION

Maize is a major staple worldwide (Nuss and Tanumihardjo, 2010) and is consumed in the form of maize porridge by a broad spectrum of the community in southern Africa (Du Plessis, 2003; Louw *et al.*, 2010). According to newly introduced South African regulations for fumonisin levels in foodstuffs, raw maize and maize meal may not contain more than 4 000 and 2 000 µg/kg of fumonisins (sum of B₁ and B₂), respectively (SA, 2016). However, there is not sufficient information available to establish to what extent fumonisins in maize porridge are released in the human gut. According to Gonzalez-Arias *et al.* (2013), the amount of fumonisins ingested, as well as the effect of food preparation and the bioavailability of the toxin should be measured to assess dietary fumonisin. The release of a food component or contaminant from its matrix (bioaccessibility) can be used as an indicator for its uptake and metabolic utilisation (bioavailability), because release in the GIT is a prerequisite for absorption in the body (Versantvoort *et al.*, 2005). Furthermore, bound mycotoxins may be regarded as detoxified if they are not released from the food matrix during food processing or in the human digestive system (Berthiller *et al.*, 2013).

In this study, the bioaccessibility of fumonisins from maize porridge was investigated by using a dynamic, computer-controlled model of the stomach and small intestine (tiny-TIM). This validated model predicts human digestive conditions better than static digestion models (Schaafsma, 2005; Verwei *et al.*, 2016), thereby providing a more accurate method for the evaluation of the risk associated with the consumption of fumonisin-contaminated maize porridge.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of maize porridge

Uncontaminated (fumonisin-free) and naturally contaminated maize kernels with high levels of fumonisins (FB₁ 74 500±200 µg/kg, FB₂ 15 900±200 µg/kg and FB₃ 7 210±740 µg/kg) were provided by the Southern African Grain Laboratory (SAGL). The maize samples were previously analysed at SAGL using an exhaustive method optimised for maximal extraction of fumonisins from raw maize (Skhosana, 2016). The samples were milled using setting number 2 (particle size <1 mm) on a Mazzer Major disk attrition mill (Mazzer Luigi S.p.a., Italy) and stored in sealed plastic bags at 4 °C. Stiff maize porridge was prepared according to a method adapted for microwave cooking (Van der Merwe, 1999). Briefly, tap water (94.00 g) and sodium chloride (0.71 g) were placed in 250 mL plastic beakers, covered with Glad® cling wrap (Clorox Africa (PTY) Limited, South Africa) and heated for 4 min 10 s on full power in a 1400 W microwave oven (Moulinex, France) to bring the solutions to boiling point. Maize meal (38.39 g) (two blank maize and three contaminated maize) was added to each beaker and stirred well to combine, without the formation of lumps. The beakers were again covered with cling wrap and cooked for 2 min 20 s on full power, after which another 31.00 g of tap water was added. The samples were mixed for 30 s, covered and cooked for another 2 min 40 s on full power. After the final cooking step, the porridge was stirred thoroughly, covered again and allowed to cool for 10 min at room temperature. The porridge was freshly prepared for the dynamic gastrointestinal trial.

5.2.2 Dynamic gastrointestinal model (tiny-TIM)

The bioaccessibility of fumonisins from maize porridge was determined in the TNO Gastrointestinal Model of the stomach and small intestine (tiny-TIM) at Triskelion (Zeist, Netherlands). For the blank, two independent replicates were performed using uncontaminated porridge, and for the fumonisin-contaminated maize porridge, three independent replicates were run.

5.2.2.1 Description of the tiny-TIM model

The tiny-TIM is a dynamic, multi-compartmental *in vitro* system that makes use of computer simulation to mimic the conditions in the lumen of the GIT during transit and digestion of a meal (Havenaar *et al.*, 2013; Minekus, 2015; Verwei *et al.*, 2016). The system contains a gastric compartment and a compartment for the small intestine (Figure 5.2). Each compartment consists of two glass jackets with a flexible silicone inner tube. The space between the glass and the silicone tube is filled with water, which controls the temperature (37 ± 0.5 °C) and simulates muscular peristaltic contractions by modulating the pressure on the flexible walls of the inner tube. The gastric and intestinal compartments are connected by a peristaltic valve pump, which simulates the pyloric sphincter and allows the chyme to transit through the system over time. Digested and dissolved molecules are dialysed from the intestinal lumen through a semipermeable membrane unit connected to the small-intestinal compartment. This represents the bioaccessible fraction that is potentially available for absorption in the small intestine.

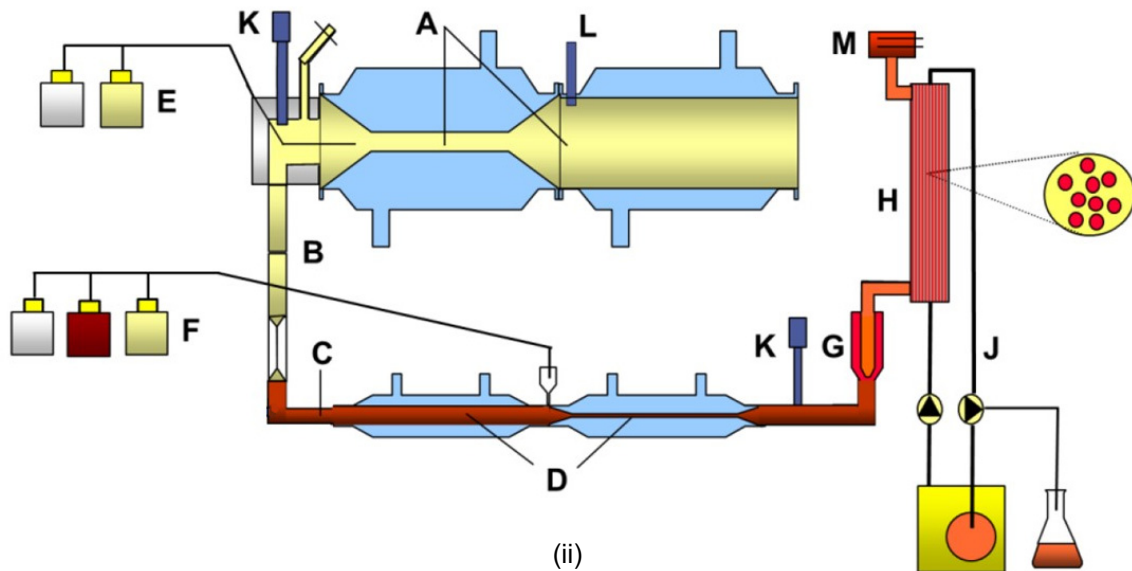
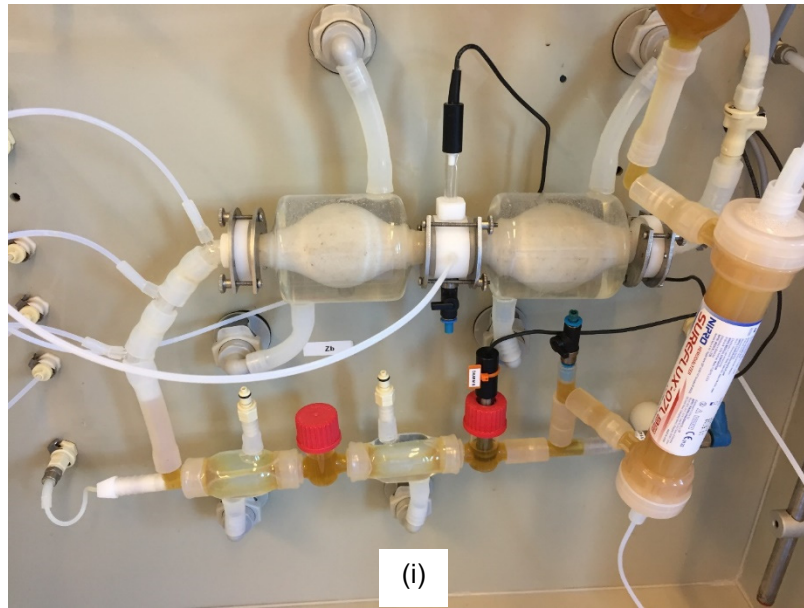


Figure 5.1: Photograph (i) and schematic (ii) of the tiny-TIM system

(A: gastric compartment, B: pyloric valve, C: intestinal content (chime), D: small-intestinal compartment, E: gastric secretion, F: intestinal secretion, G: pre-filter, H: dialysis membrane unit, J: dialysate (bioaccessible fraction), K: pH electrodes, L: pressure sensor, M: level sensor) Photo: B du Plessis, Schematic (Minekus, 2015)

5.2.2.2 Simulated gastrointestinal conditions

The tiny-TIM experiment was performed using settings simulating the average physiological conditions in the GIT of a healthy adult human taking a light meal (Havenaar *et al.*, 2016). The intestinal transit times, pH values and secretion and composition of the bile, pancreatic juice and enzymes were controlled as summarised in Table 5.1.

Table 5.1: Stages in gastrointestinal digestion and tiny-TIM parameters for healthy adult simulation (Minekus *et al.*, 1995; Havenaar *et al.*, 2013; Bellmann *et al.*, 2016; Havenaar *et al.*, 2016; Verwei *et al.*, 2016)

Digestion phase	tiny-TIM parameters for healthy adult	
	Enzymes	Description
Oral	α -amylase 1800 units (A6380, Sigma-Aldrich)	Mix prepared porridge (100 g) manually with water and artificial saliva (45 g) Artificial saliva contains α -amylase and physiological concentrations of electrolytes (NaCl, KCl and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) Add gastric start fluid (5 g) containing lipase, pepsin and amylase, as described below
Gastric	gastric lipase (65 U/mL, 80612, Sigma-Aldrich) pepsin (1 mg/mL, P7012, Sigma-Aldrich) α -amylase (150 U/mL, A6380, Sigma-Aldrich)	Secretion gastric fluid 0.5 mL/min pH adjusted from 5.5 to 1.7 in 120 min with HCl (1 M) Peristaltic mixing 3 times per min Gastric half-emptying time 70 min
Small-intestinal	pancreatin from porcine pancreas 4xUSP P1750, (Sigma-Aldrich), 7% (w/v) containing trypsin, amylase, lipase and protease	Bile (porcine bile extract, P8631, Sigma-Aldrich), 4% (w/v) Physiological concentrations of electrolytes (NaCl, KCl and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) Adjustment of pH to 7 with 1 M NaHCO_3 Peristaltic mixing 6 times per min Intestinal fluid secretion 1 mL/min Dialysis through a cartridge with hollow-fiber semi-permeable membranes (Sureflux 07L, Nipro Europe, Belgium; cut-off of 5-7 kDa, surface area for absorption of 0.7m^2) at a rate of 10 mL/min
Total duration 6 h		

The enzyme concentrations in the compartments changed over time, as it is a dynamic system. The dynamics of the gastric emptying and pH decline for this simulation are illustrated in Figure 5.2 and the set-points were computer controlled.

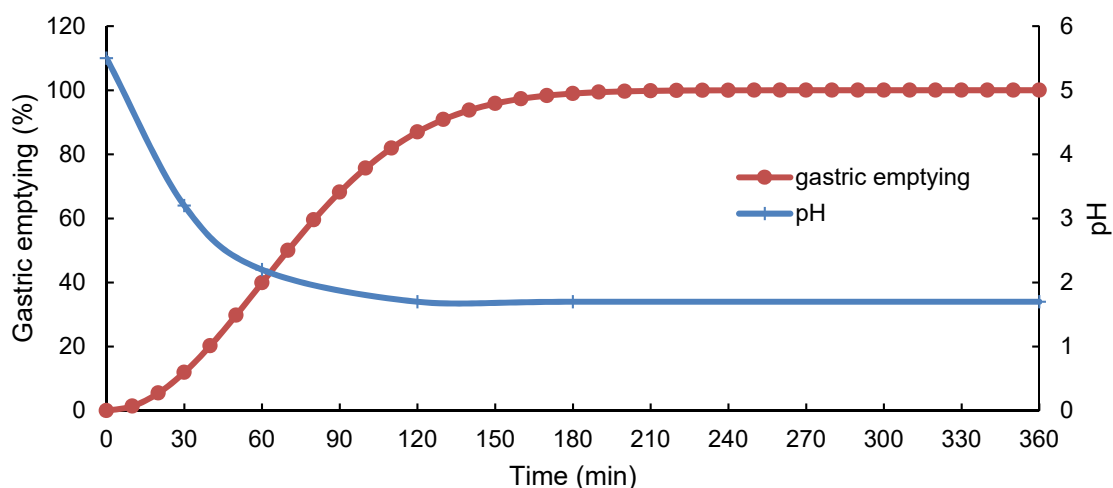


Figure 5.2: Gastric emptying and pH decline in the tiny-TIM protocol simulating physiological conditions in human adults after the intake of a light meal

All secretion fluids (e.g. gastric and pancreatic juice, bile and electrolytes) were freshly prepared, the pH electrodes calibrated, and new semipermeable membranes installed prior to the experiment. The secretion fluids contained physiologically relevant concentrations of electrolytes, enzyme activities, and bile salts at site-specific pH levels.

5.2.2.3 Sampling

The dialysate from the intestinal compartment (representing the bioaccessible fraction) was collected every hour for 6 h and the volume recorded. The residues in the gastric and intestinal compartments, respectively, were collected at the end of the experiment. Each compartment was rinsed with water and the rinse was pooled with the residue sample. Immediately after collection, porridge, dialysate and residue samples were frozen on dry ice and stored in a freezer at -20 °C. The samples were freeze-dried in a Virtis AdVantage Plus Laboratory Benchtop Freeze Dryer (SP Scientific, Warminster, PA) for eight days and stored at -80 °C until analysis.

5.2.3 Alkaline hydrolysis of small intestinal residue

Alkaline hydrolysis was performed according to a method previously optimised for maize samples (Skhosana, 2016). The freeze-dried small intestinal residue samples were dispersed in 15 mL deionised water, after which 15 mL of 4 M KOH was added. The samples were mixed well and then agitated on a mechanical shaker (220 rpm) for 24 h. After hydrolysis of the fumonisins, 15 mL ethyl acetate was added and the samples were mixed manually for one min and placed on a shaker for 10 min. The samples were centrifuged for 10 min at 1 438 rcf and the supernatant (ethyl acetate phase) transferred to a 50 mL centrifuge tube. The remaining pellet was re-suspended in 15 mL ethyl acetate, then agitated and centrifuged as described above. The first and second extracts were combined and dried under vacuum at room temperature in a centrifugal concentrator (Genevac™ EZ-2plus, Genevac Ltd., Ipswich, UK).

5.2.4 Fumonisin determination using by UPLC-QToF-HDMS

5.2.4.1 Chemicals and reagents

Fumonisin standards (FB₁, batches A/13 and A/22, FB₂ batch B/05, FB₃ batch A/16 and hydrolysed fumonisin B₁ (HFB₁) batch A09-2014) with purities of >96% were obtained from the Institute of Biomedical and Microbial Biotechnology (IBMB), Cape Peninsula University of Technology (CPUT). Stock solutions were prepared in deionised water containing 1% acetonitrile. Unless otherwise stated, all other reagents were HPLC grade and obtained from Sigma Aldrich/Merck, Darmstadt, Germany.

5.2.4.2 Sample preparation and analysis

Dried samples were extracted with 3 mL of deionised water containing 1% (v/v) acetonitrile, centrifuged for 10 min at 1 438 rcf and the supernatants were filtered through 0.45 µm and 0.2 µm GHP syringe filters (Separations, South Africa).

Fumonisin were determined using the UPLC-QToF-HDMS method which was described for FB₁ and HFB₁ in Section 4.2.6. The transitions used for the MS quantification of FB₂ and FB₃ in ESI positive mode are provided in Table 5.2. The transitions for FB₁ and HFB₁ were provided in Table 4.5 (Section 4.2.6).

Table 5.2: Transitions used for the quantification of FB₂ and FB₃ using the QToF-HDMS method in ESI positive mode

Mycotoxin	Precursor ion (<i>m/z</i>)	Fragmentation ions observed (<i>m/z</i>)		Retention time (min)
FB ₂	706.40	336.32	318.31	8.59
FB ₃	706.39	336.32	318.31	8.25

The calibration data for FB₁, FB₂, FB₃ and HFB₁ is provided in Appendix 2. The method performance was evaluated according to the SANCO/12571 guidelines. The LOD and LOQ for FB₂ and FB₃ using the UPLC-QToF-HDMS method are provided in Table 5.3 (the data for FB₁ and HFB₁ was reported in Table 4.6, Section 4.2.6).

Table 5.3: Limits of detection (LOD) and quantification (LOQ) for FB₂ and FB₃ using the UPLC-QToF-HDMS method

Mycotoxin	LOD (µg/mL)	LOQ (µg/mL)
FB ₂	0.050	0.980
FB ₃	0.015	0.045

5.2.5 Statistical analysis

Statgraphics® Centurion XVI version 16.1.17 software (Statpoint Technologies Inc., The Plains, Virginia, USA) was used to analyse the data. Analysis of variance (ANOVA) was performed and a *p*-value of <0.05 for the F-test was considered to indicate statistically significant differences between samples. Means were compared with the aid of Fisher's LSD (Least Significant Difference) intervals. All analyses were performed in triplicate.

5.3 RESULTS AND DISCUSSION

The chromatogram of FB₁, FB₂ and FB₃ in a dialysate sample obtained after *in vitro* digestion of fumonisin-contaminated maize porridge (Figure 5.3) show that the UPLC-QToF-HDMS method separated the fumonisins efficiently. Fumonisin B₁ was eluted first (7.69 min), followed by FB₃ (8.25 min) and FB₂ (8.59 min).

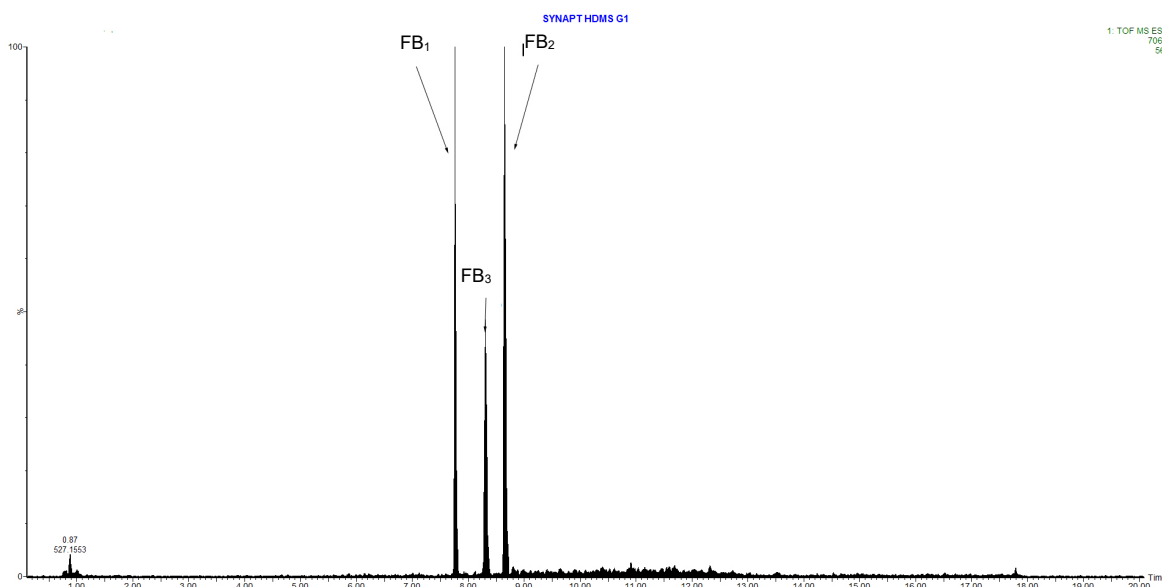


Figure 5.3: Chromatogram a dialysate sample obtained after *in vitro* digestion of fumonisin-contaminated maize porridge, indicating the presence of FB₁, FB₂ and FB₃

The fumonisin content in the dialysate and residue samples collected after simulated human digestion of maize porridge is presented in Table 5.4.

Table 5.4: Fumonisin B₁, B₂ and B₃ in dialysate and residue samples collected after simulated human digestion of stiff maize porridge in a dynamic gastrointestinal model (tiny-TIM)

Sample	Fumonisin B ₁		
	Blank porridge (µg/100 g porridge)	Contaminated porridge (µg/100 g porridge)	Recovery from spiked blank (%)
Dialysate fraction			
0-60 min	ND	307±30 ^b	77.7±7.5 ^a
60-120 min	ND	500±96 ^c	86.2±1.5 ^a
120-180 min	ND	282±30	83.0±21.1 ^a
180-240 min	ND	56.4±15.7 ^a	83.0±24.1 ^a
240-300 min	ND	9.15±3.93 ^a	81.9±28.6 ^a
300-360 min	ND	ND	73.4±46.6 ^a
Gastric residue	ND	3.15±0.99 ^a	79.8±34.6 ^a
Intestinal residue	ND	ND	-
Fumonisin B ₂			
	Blank porridge (µg/100 g porridge)	Contaminated porridge (µg/100 g porridge)	Recovery from spiked blank (%)
Dialysate fraction			
0-60 min	ND	164±7 ^c	78.4±2.6 ^d
60-120 min	ND	311±36 ^e	89.0±3.2 ^{de}
120-180 min	ND	210±32 ^d	97.7±1.0 ^e
180-240 min	ND	50.6±1.2 ^b	77.3±4.6 ^d
240-300 min	ND	9.96±2.18 ^a	62.0±2.9 ^c
300-360 min	ND	1.96±0.64 ^a	48.6±1.2 ^b
Gastric residue	ND	ND	14.0±0.7 ^a
Intestinal residue	ND	ND	-
Fumonisin B ₃			
	Blank porridge (µg/100 g porridge)	Contaminated porridge (µg/100 g porridge)	Recovery from spiked blank (%)
Dialysate fraction			
0-60 min	ND	21.1±1.6 ^b	75.8±3.4 ^{bc}
60-120 min	ND	44.2±4.7 ^c	79.3±0.7 ^{cd}
120-180 min	ND	25.6±4.3 ^b	90.0±0.1 ^e
180-240 min	ND	5.03±1.14 ^a	82.7±0.7 ^d
240-300 min	ND	ND	77.3±1.0 ^{bcd}
300-360 min	ND	ND	72.4±0.6 ^b
Gastric residue	ND	ND	45±0.1 ^a
Intestinal residue	ND	ND	-
Hydrolysed fumonisin B ₁			
	Blank porridge (µg/100 g porridge)	Contaminated porridge (µg/100 g porridge)	Recovery from spiked blank (%)
Dialysate fraction			
0-60 min	ND	ND	98.2±4.7 ^b
60-120 min	ND	ND	91.3±2.9 ^b
120-180 min	ND	ND	81.4±25.2 ^b
180-240 min	ND	ND	46.5±16.4 ^a
240-300 min	ND	ND	27.2±2.0 ^a
300-360 min	ND	ND	21.6±1.2 ^a
Gastric residue	ND	ND	92.9±0.1 ^b
Intestinal residue	ND	ND	-
Intestinal residue, hydrolysed	-	14.6±1.4	74.1±7.8 ^b

ND not detected, - not analysed

For each toxin, means with any same letter in a column are not significantly different ($p>0.05$)

The values after (±) are standard deviations

No fumonisins were detected in the uncontaminated (blank) maize porridge or any of its dialysate or residue fractions. Fumonisins (B₁, B₂ and B₃) were detected in the dialysate samples from contaminated maize. The 60-120 min dialysate fraction contained the highest fumonisin concentrations (500, 311 and 44.2 µg/100 g porridge of FB₁, FB₂ and FB₃, respectively). No HFB₁ was detected in any of the samples. This result is in agreement with De Girolamo *et al.* (2016) who reported that HFB₁ was not formed during cooking of maize in the absence of lime. The gastric residue samples from the contaminated porridge contained only traces of FB₁ and no fumonisins were detected in the intestinal residue samples. Because the intestinal residue was viscous and turbid, these samples were hydrolysed with alkali to release any trapped fumonisins which might be present. Upon hydrolysis, a small amount of HFB₁ (14.6 µg/100 g porridge) was detected in the intestinal residue of the contaminated porridge sample. This corresponds to 26.0 µg FB₁/100 g porridge and is only about 2% of the FB₁ present in the contaminated porridge.

There were significant differences in the recovery of fumonisins from the various samples collected during the *in vitro* digestion procedure. This could be expected, because the composition of each fraction is unique and essentially represent a different matrix. Due to the cost of the analysis and the number of different matrices, it was not practical to analyse each sample against its matrix-matched standards. For most of the samples, the recoveries (measured against neat standards) were acceptable and in line with the SANCO guideline (SANCO, 2013). However, for a number of samples (FB₂ 300-360 min dialysate and gastric residue, FB₃ gastric residue, HFB₁ 180-240 min, 240-300 min and 300-360 min dialysates) the recoveries were unacceptably low, indicating possible matrix interference.

The cumulative recovery (%) of FB₁, FB₂ and FB₃ from stiff maize porridge prepared from contaminated maize meal, after simulated human digestion, is presented in Figure 5.4.

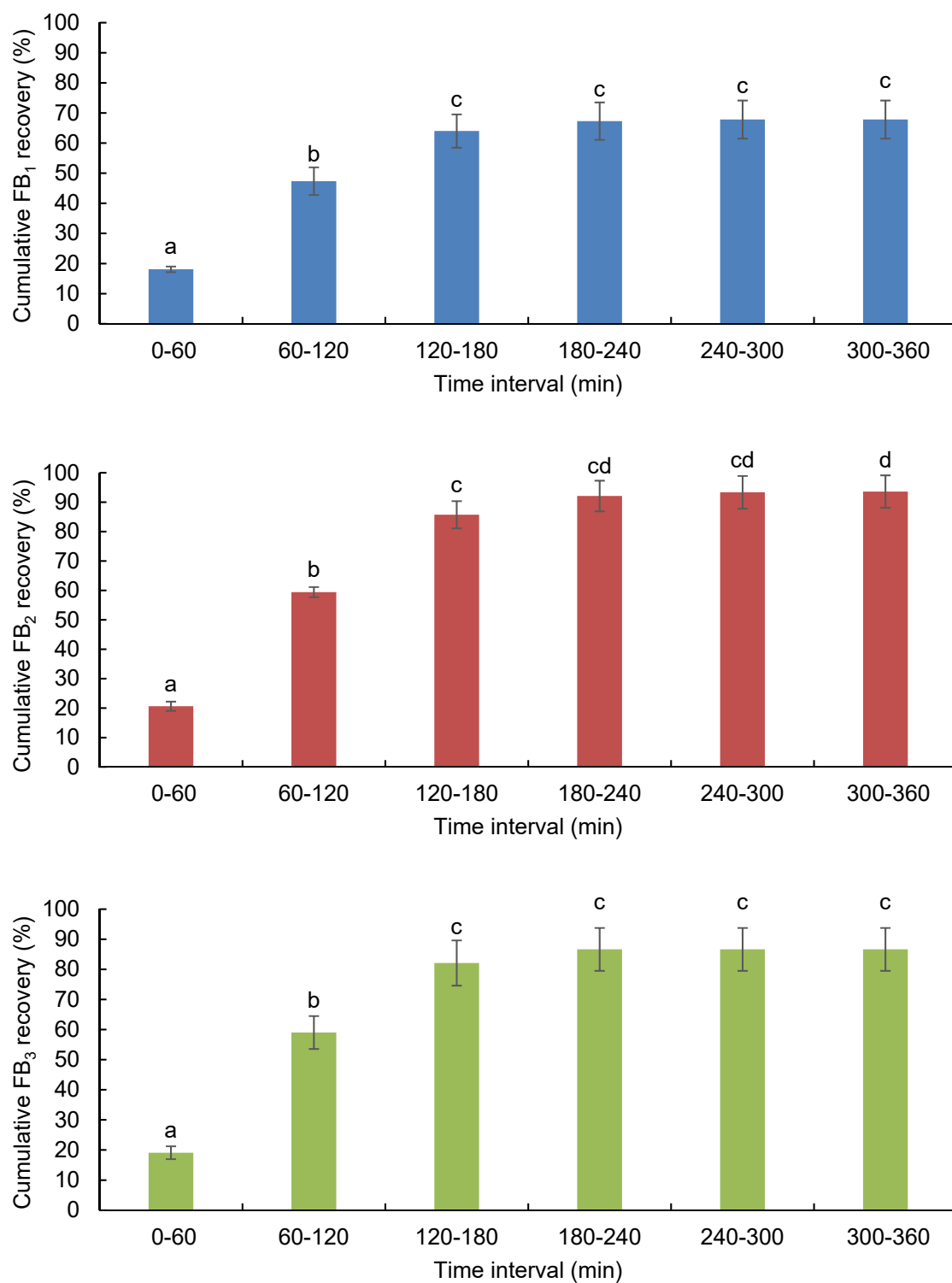


Figure 5.4: Cumulative recovery of fumonisins B₁, B₂ and B₃ in dialysate during simulated human digestion of maize porridge in a dynamic gastrointestinal model (tiny-TIM)

The results demonstrate that fumonisin FB₁, FB₂ and FB₃ are released rapidly from stiff maize porridge made from naturally contaminated maize meal. In the first 120 min, 47% of the FB₁ and 59% FB₂ and FB₃ are released from the maize porridge and detected in the dialysate. After 360 min, 68%, 94% and 87% of FB₁, FB₂ and FB₃, respectively, are bioaccessible. The results of the current study are in agreement with those of Avantaggiato *et al.* (2007), who used a dynamic *in vitro* digestion procedure simulating the digestive tract of pigs to analyse mycotoxin-contaminated feed containing maize and wheat. They reported that the majority of the fumonisins were absorbed within the first 2 h of digestion and that 105% and 89% of FB₁ and FB₂, respectively, were released from the feed matrix during the 6 h period.

Although the tiny-TIM model system provides a good simulation of the physiological conditions during digestion, it has limitations in terms of lipophilic compounds. Digested and dissolved molecules are separated through a semipermeable membrane with a 5-7 kDa molecular weight limit (Verwei *et al.*, 2016). However, lipids are not solubilised during digestion. Instead they are incorporated into nanoscale structures containing bile salts, namely micelles (8-20 nm) and vesicles (40-200 nm) (Meynier and Genot, 2017). Lipids are absorbed by the intestinal epithelial cells where lipoproteins structures known as chylomicrons are formed that are transported by the lymphatic system. The tiny-TIM system contains the appropriate enzymes for fat digestion (gastric and pancreatic lipase), as well as adequate quantities of bile extract for the formation of micelles. Nevertheless, the micelles are too large to pass through the semipermeable membrane and are therefore not removed with the dialysate (Minekus, 2015).

Fumonisin are water soluble (EC, 2000), therefore the shortcomings of the tiny-TIM system in terms of separating digested lipid compounds from the lumen is not expected to have a major effect on the results of the experiment. Indeed, the results demonstrated that the majority of the FB present in the porridge could be recovered in the dialysate over the 6 hour period. However, since FB is structurally related to the cellular sphingolipids sphinganine and sphingosine (Figure 2.3) the possibility of FB being taken up by the transport systems of lipids should be considered. There is evidence that FB₁ is able to transfer from an aqueous environment into a lipid phase

containing cholesterol and/or bile salts (Mahfoud *et al.*, 2002). This would make it possible for FB to be incorporated into micelles and transported by the lymphatic system, as well as enable enterohepatic circulation.

The fact that the fumonisins in contaminated maize porridge become available for absorption rapidly during digestion, imply that it is likely that the intestine could be exposed to high concentrations of FB after consuming porridge made from contaminated maize meal. Literature provides evidence that FB is poorly absorbed by the intestinal epithelium (Shier, 2000), however, the exposure of the intestine to high concentrations of FB remains a concern, since the intestine is a possible target for fumonisin toxicity (Bouhet and Oswald, 2007). In addition, there are indications that exposure to FB₁ could increase the trans-cellular and para-cellular permeability of pig intestines (Lallès *et al.*, 2009), which reduces the ability of the intestinal epithelium to act as a barrier to protect the organism from harmful chemicals and microbial pathogens (Akbari *et al.*, 2017). A damaged intestinal barrier is associated with the development of chronic intestinal inflammatory diseases and malabsorption of nutrients, which can cause growth impairment in children and animals (Chen *et al.*, 2018).

5.4 CONCLUSIONS

This is the first time that the bioaccessibility of FB in maize porridge has been determined using a dynamic model simulating human digestion. The study presents new information and insight into the kinetics of FB bioaccessibility and confirms that the low bioavailability is not due to strong association of the toxin with the intestinal content, but that it is in fact rapidly released from maize porridge. More research is required to determine the effect of digestion on the release of cryptic fumonisins from food matrices, particularly from staples such as maize porridge.

CHAPTER 6

CONTROL OF *FUSARIUM* SPP. ISOLATED FROM SOIL AND MAIZE BY MEANS OF SELECTED ESSENTIAL OILS

6.1 INTRODUCTION

The genus *Fusarium* is a complex polyphyletic grouping that occurs worldwide, in many different environments (Summerell *et al.*, 2010). It encompasses a variety of fungal plant pathogenic species, which cause enormous production losses in agriculture and horticulture, including food crops such as wheat and maize. There are two key diseases affecting maize ears that are caused by *Fusarium* spp., namely Fusarium ear rot (primarily due to *F. verticillioides*, *F. subglutinans* and *F. proliferatum*) and Gibberella ear rot (primarily due to *F. graminearum*) (Munkvold, 2003). In addition to being plant pathogens of agricultural importance, these fungi produce mycotoxins, which are toxigenic secondary metabolites that are of major concern for the human food and animal feed industries (Ncube *et al.*, 2011).

Fusarium verticillioides is the most common maize pathogen. It is ubiquitous and infects the plant at all stages of development, typically by airborne conidia *via* the silk channel or wounds and systemically, due to infected seeds (Munkvold and White, 2016). The disease cycle of *Fusarium* in maize is characterised by a saprophytic, as well as a pathogenic stage, and the fungus can overwinter in maize debris and soil, infecting germinating seeds and roots in the next season, and moving up the plant through systemic growth (Battilani *et al.*, 2003; Munkvold and White, 2016).

Public concern over the health and environmental hazards associated with synthetic pesticides is on the increase. Essential oils are natural volatile organic compounds that are isolated from aromatic plants using steam distillation and hydrodistillation (Saad *et al.*, 2013). These volatile secondary metabolites are produced as a defence mechanism by plants to function as antibacterial, antiviral, antifungal and insecticidal agents (Bakkali *et al.*, 2008). Since EOs are biodegradable and relatively non-toxic

to mammals, birds and fish, they are considered to be eco-friendly (Dwivedy *et al.*, 2016) and are gaining popularity as green pesticides (Koul *et al.*, 2008).

The aim of this study was to evaluate the potential of selected EOs for the biocontrol *Fusarium* spp. present in soil and maize. Furthermore, for the treatment with the best potential, the effect of the EO on *FUM* gene expression and fumonisin production was assessed using two fumonisin-producing *F. verticillioides* strains.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals, reagents and microbiological media

Essential oils [lemongrass (*Cymbopogon citratus*), spearmint (*Mentha spicata*) and thyme (*Thymus vulgaris*)] were purchased from Holistic Emporium (Johannesburg, South Africa). The selected EOs from this supplier were previously analysed and their chemical compositions are provided in Table 2.6. All other chemicals, reagents and microbiological media were obtained from Sigma Aldrich/Merck (Darmstadt, Germany).

6.2.2 *Fusarium* cultures

A *F. verticillioides* strain (MRC 826), which is known to produce fumonisins, was purchased from the Institute of Biomedical and Microbial Biotechnology, Cape Peninsula University of Technology, Cape Town, South Africa. In addition, the Southern African Grain Laboratory (SAGL) supplied a sample of naturally contaminated maize meal with confirmed high levels of fumonisins (FB₁ 74 500±200 µg/kg, FB₂ 15 900±200 µg/kg and FB₃ 7 210±740 µg/kg), from which *F. verticillioides* was isolated. All other *Fusarium* cultures used in this study were isolated from soil or maize as described in Section 6.2.5.

6.2.3 Soil and maize sampling

Sampling took place during July, shortly after the 2016 harvesting season and was limited to the Mpumalanga and Free State provinces (Figure 6.1), since these provinces contribute 24% and 40%, respectively, of the national maize production in South Africa (SAGL, 2016).

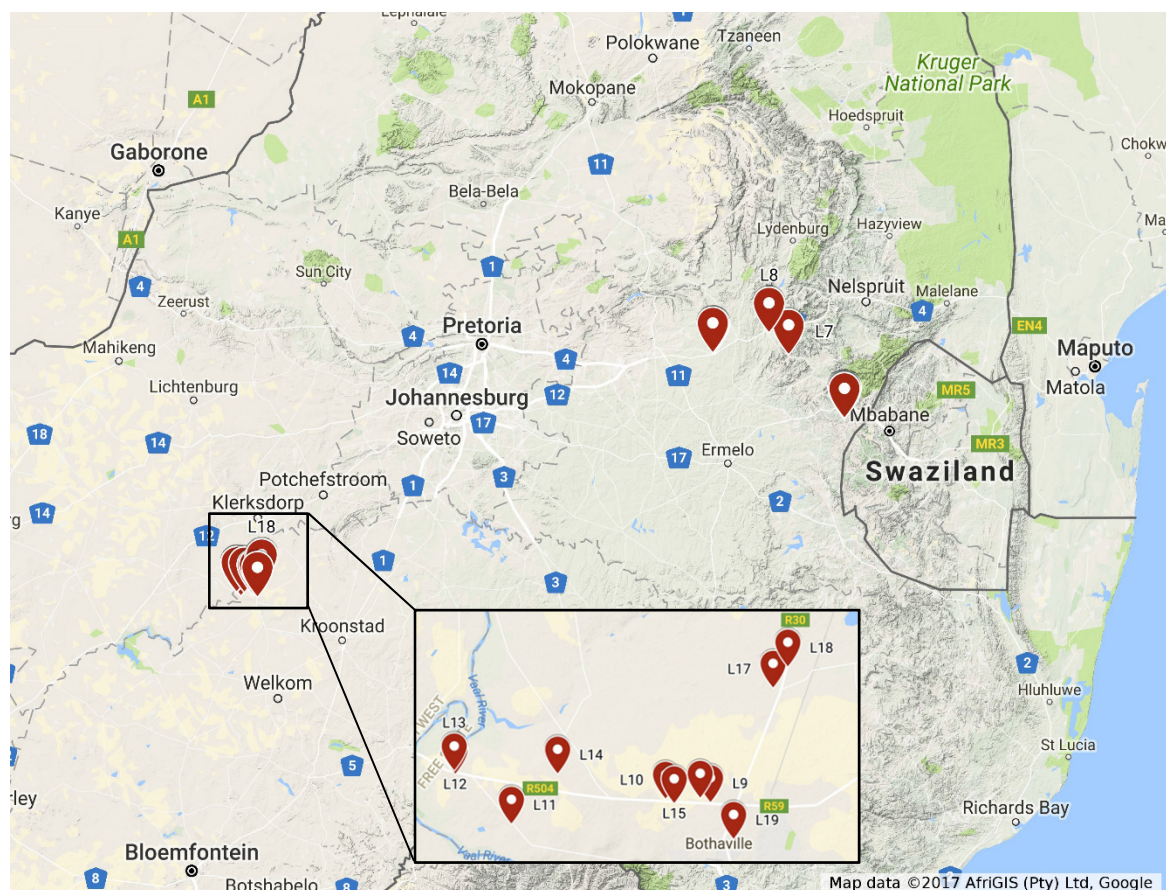


Figure 6.1: Map indicating sites in the Mpumalanga and Free State provinces where soil and maize samples were collected

The prevailing weather conditions and coordinates of each location were recorded (Tables 6.1 and 6.2).

Table 6.1: Coordinates and description of samples collected in Mpumalanga Province, South Africa

Location	Coordinates	Sample ID	Sample description	Date*
R104, 26 km from Belfast Nkangala District Municipality, Mpumalanga province	S25°48'52.8" E29°53'06.6"	L1	Surface soil Rhizosphere soil	10/07/2016
R104, 26 km from Belfast Nkangala District Municipality, Mpumalanga province	S25°48'43.3" E29°52'40.1"	L2	Surface soil Rhizosphere soil	10/07/2016
Mayibuye maize mill, Dundonald Gert Sibande District Municipality, Mpumalanga province	S26°13'31.5" E30°49'28.5"	L3	Maize residue in silos and mill	11/07/2016
Dundonald Gert Sibande District Municipality, Mpumalanga province	S26°13'24.4" E30°48'35.5"	L4	Surface soil Rhizosphere soil Maize ears Maize kernels on field	11/07/2016
Dundonald Gert Sibande District Municipality, Mpumalanga province	S26°13'59.6" E30°48'52.0"	L5	Surface soil Rhizosphere soil	11/07/2016
Dundonald Gert Sibande District Municipality, Mpumalanga province	S26°13'57.7" E30°48'56.9"	L6	Surface soil Rhizosphere soil Cobs with a few kernels	11/07/2016
R541, 28 km from eNtokozweni Nkangala District Municipality, Mpumalanga province	S25°49'24.9" E30°25'09.4"	L7	Surface soil Rhizosphere soil Cobs with a few kernels	11/07/2016
R541, 6 km from eNtokozweni Nkangala District Municipality, Mpumalanga province	S25°41'00.1" E30°16'42.8"	L8	Surface soil Rhizosphere soil	11/07/2016

*Weather conditions on 10-11 July 2016 were sunny and cold with temperatures ranging from 8-13 °C

Table 6.2: Coordinates and description of samples collected in Free State Province, South Africa

Location	Coordinates	Sample ID	Sample description	Date*
Bothaville Lejweleputswa District, Free State	S27°21'19.3" E26°36'56.2"	L9	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°21'13.1" E26°35'29.7"	L10	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°21'55.8" E26°30'32.8"	L11	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°20'30.7" E26°28'45.0"	L12	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°20'24.6" E26°28'43.0"	L13	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°20'30.3" E26°32'01.7"	L14	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°21'20.9" E26°35'46.4"	L15	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°21'11.3" E26°36'36.2"	L16	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°18'03.9" E26°38'56.5"	L17	Surface soil Rhizosphere soil Maize ears	25/07/2016
Bothaville Lejweleputswa District, Free State	S27°17'27.6" E26°39'25.2"	L18	Surface soil Rhizosphere soil Maize ears	26/07/2016
Thuso mill, Bothaville Lejweleputswa District, Free State	S27°22'21.7" E26°37'40.3"	L19	Maize kernels Maize meal	26/07/2016

*Weather conditions on 25-26 July 2016 were rainy with thunderstorms, hail and temperatures ranging from 6-11 °C

Soil sampling was performed according to the guidelines of the ISO 10381-6 method (ISO, 2009). Surface soil samples (2 cm depth) were taken after removal of surface vegetation cover (if any present), using a shovel sterilised with 70% ethanol. For rhizosphere soil, plants were removed and the soil from the roots were collected. At least 15-20 equal-sized, random samples of surface and rhizosphere soil were collected from each field by moving across the field in a Z-pattern. These samples were pooled to create a composite sample of surface and rhizosphere soil, respectively, for each field, which was placed in loosely-tied polyethylene bags. In addition, where available, maize ears were collected from the field and stored in cloth bags. Moist samples were air dried at room temperature with forced air circulation before storage. All soil samples were passed through a 2 mm sieve to remove plant material and stones (if present) and ensure aerobic conditions. All samples were subsequently stored in the dark at 4 ± 2 °C with free access of air.

6.2.4 Soil characterisation

6.2.4.1 Soil texture

Soil texture was assessed using a combination of the feel method (Ali and Narayan, 2013) and the field method (Foth, 1990). A sample of soil was moistened with water and kneaded until it was homogenous and had the consistency of putty. It was formed into a ball and squeezed between the thumb and forefinger to attempt to form a ribbon. The mud was evaluated for stickiness, stiffness, the feel between the fingers, ball formation and ribbon formation. The soil texture was then classified into one of the major classes, as illustrated in Figure 6.2.

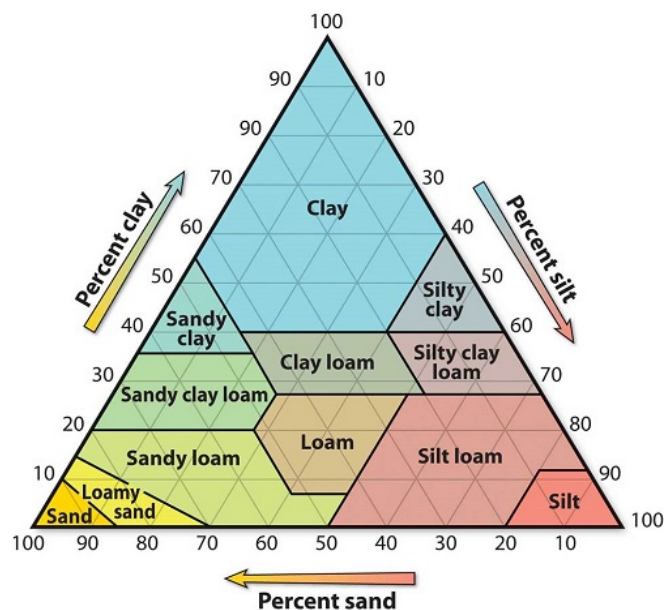


Figure 6.2: Soil texture triangle, illustrating the composition of the major textural classes⁹

6.2.4.2 Moisture determination

Moisture content was determined by measuring the weight loss on drying 3.00 g of sieved soil at 105 °C in a Kern Moisture balance (model MLB_N, Kern & Sohn, Balingen, Germany) to constant weight (automatic shutoff for change to weighing value ≤ 1 mg within 120 s). Analyses were performed in triplicate and the moisture content was expressed in percentage (m/m).

6.2.4.3 pH and conductivity

The pH of the soil was measured in 0.01 M CaCl_2 (Henderson *et al.*, 2008) using an Orion Star A211 pH meter (Thermo Scientific™, Waltham, USA). A 20.00 mL volume of 0.01 M CaCl_2 was added to 10.00 g of air-dried, sieved soil. The soil suspension was stirred intermittently for 30 min and allowed to stand for 1 h, after which the pH of the clear supernatant was measured. The conductivities of the same samples were measured using a Hach HQ40d multi-conductivity meter (Hach Company, Loveland, USA) and the conductivity of the 0.01 M CaCl_2 solution was

⁹ <http://soils4teachers.org/files/images/s4t/texture-triangle.jpg> [Accessed 06/03/2018]

subtracted from the measured values. Analyses for pH and conductivity were performed in triplicate.

6.2.4.4 Trace elements

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was employed to detect trace elements in the soil. The soil samples were digested using microwave acid digestion (MARS Xpress microwave system; CEM Corporation, USA) in a two-stage procedure. In the first stage, soil samples (0.500 g) were digested with 65% HNO₃ (9 mL), 32% HCl (3 mL) and 40% HF (1 mL) in closed vessels using the following protocol: ramping to 175 °C (8.75 °C/min), holding at 175 °C for 20 min, and cooling for 15 min. In the second stage, saturated H₃BO₃ (1 mL) was added and the same microwave heating protocol was followed. All reagents used were Suprapur grade (Sigma-Aldrich, Johannesburg, South Africa). After digestion, samples were filtered through MN 617 filter paper (Macherey-Nagel, Düren, Germany) and made up to 50.00 mL with deionised water. Reagent blanks were prepared using the same procedure.

A SPECTRO ARCOS ICP-OES analyser (SPECTRO Analytical Instruments GmbH, Kleve, Germany) with the torch position in a fixed radial view, equipped with a Scott Double Pass spray chamber, a cross-flow nebuliser and a charge coupled device detector, was used for elemental analysis. The instrument operating conditions were as follows: plasma power 1 400 W, peristaltic pump speed 30 rpm, coolant flow (12 L/min), auxiliary flow 1 L/min and nebuliser flow 1 L/min. Calibration curves were constructed by preparing mixed standard solutions containing nitrite salts of Al, Ba, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Sr, Ti, V, Zn and Zr at concentrations of 1.00, 3.00, 5.00, 7.00, 10.00, 15.0, 20.0, 30.0, 40.0 and 45.0 mg/L from Spectrascan standard solutions (Kungsbacka, Sweden). Linear curves were fitted to the calibration data (Appendix 5). Limits of detection (LOD) and quantification (LOQ) were calculated from the calibration data using the Equations 2 and 3 below.

$$\text{LOD} = 3 \frac{S_{y/x}}{b} \quad \text{.....Equation 2}$$

where $S_{y/x}$ is the standard error of the calibration regression line and b is the slope of the calibration regression line

$$LOQ = 10 \frac{S_{y/x}}{b} \quad \text{.....Equation 3}$$

where $S_{y/x}$ is the standard error of the calibration regression line and b is the slope of the calibration regression line

The wavelength, LOD and LOQ for each element is provided in Table 6.3.

Table 6.3: Wavelengths, detection and quantification limits for elemental analysis

Element	Wavelength (nm)	Limit of detection (mg/L)	Limit of quantification (mg/L)
Al	394	4.62	15.4
Ba	455	4.63	15.4
Ca	393	4.80	16.0
Co	239	4.52	15.1
Cr	284	4.62	15.4
Cu	325	5.13	17.1
Fe	238	4.73	15.8
K	767	5.44	18.2
Mg	280	4.94	16.5
Mn	258	5.03	16.8
Na	589	4.89	16.3
Sr	408	4.33	14.4
Ti	334	4.79	16.0
V	293	4.57	15.2
Zn	206	4.59	15.3
Zr	344	4.80	16.0

Additional dilutions were performed for samples for which the results were outside the calibration range. All analyses were performed in triplicate and results were expressed in mg/kg soil, on a dry weight basis.

6.2.5 Isolation, characterisation and identification of fungi

6.2.5.1 Isolation of fungi from soil and maize

Soil (1 g) was suspended in sterile Ringer solution, serial dilutions made (10^{-1} to 10^{-5}) and plated on Potato Dextrose Agar (PDA) containing 50 mg/L chloramphenicol to inhibit bacterial growth. For isolation of fungi from maize kernels, two different approaches were followed. For the direct plating method, whole maize kernels were surface disinfected with 1% NaOCl for 5 min, rinsed three times with sterile distilled water and air dried on a paper towel in a laminar flow cabinet (Thompson *et al.*, 2013). Thereafter single kernels were cut aseptically in

longitudinal halves and placed directly on the surface of the PDA medium in Petri dishes. Kernels were sampled from the top, middle and bottom of three randomly selected cobs from each location. In the second method, maize kernels (surface disinfected and not disinfected, respectively) were crushed aseptically in a mortar and pestle, serial dilutions (10^{-1} to 10^{-5}) were prepared using sterile distilled water and plated on PDA. All plates were incubated at 25 °C for 7 days, after which fungi were isolated by sub-culturing on PDA.

6.2.5.2 *Morphological characterisation of fungal isolates*

Morphologically the genus *Fusarium* is characterised by the production of septate hyphae that range in colour from white to pink, red, purple or brown (Montville *et al.*, 2012). Isolates with morphological properties and pigmentation consistent with *Fusarium* spp. were transferred to Carnation Leaf Agar (CLA) to promote sporulation and enable presumptive identification. This medium is low in carbohydrates and rich in complex natural substances that promote abundant production of typical and uniform conidia (Leslie and Summerell, 2008). After 7-10 days of incubation at 25 °C, water mounts of the cultures were prepared on glass slides, and examined using an Olympus® BX43 light microscope to determine the presence, shape and size of macroconidia, microconidia and chlamydospores. The guidelines for morphological identification, described by Leslie and Summerell (2008), were used to select cultures presumed to be *Fusarium* spp. The identities were subsequently confirmed through genetic analysis.

6.2.5.3 *Genetic identification of Fusarium spp.*

Genetic analysis was performed at Inqaba Biotech® (Pretoria, South Africa). Genomic DNA was extracted from pure fungal cultures using a ZR Fungal/Bacterial DNA Kit™ (catalogue no D6005, Zymo Research Corp., Irvine, USA). Internal transcribed spacer (ITS) sequencing was performed for preliminary identification of fungi presumed to be *Fusarium* spp. (based on morphological properties, refer to previous section) and identities were confirmed by calcium modulated protein (calmodulin) gene targeted sequencing, which is more specific than ITS. The ITS target region was amplified using EconoTaq® PLUS GREEN 2X Master Mix

(Lucigen, Middleton, USA) with ITS1 and ITS4 primer sequences (5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3', respectively) (White *et al.*, 1990). For the calmodulin sequencing, amplification of the 16S target region was performed using DreamTaq™ DNA polymerase (Thermo Scientific™, Waltham, USA), with two 16S rDNA primer sequences, namely 16S-27F and 16S-1492R (5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-CGGTTACCTTGTTACGACTT-3', respectively).

The PCR products were analysed and extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit, Catalogue No. D4001), followed by sequencing in the forward and reverse directions. Clean-up was performed with a Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™ (Catalogue No. D4050) and the purified fragments were analysed on an ABI 3500xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, US). The CLC Bio Main Workbench v7.6 was used to analyse the data generated by the genetic analyser, and results were obtained by a Basic Local Alignment Search Tool (BLAST)¹⁰.

6.2.6 *In vitro* antifungal activity of selected essential oils

Three EOs, namely lemongrass, spearmint and thyme were selected based on price, availability in South Africa and antifungal potential towards *Fusarium spp.* (Seseni *et al.*, 2015; Morcia *et al.*, 2016). The agar dilution method was selected to assess the antifungal activity of the EOs. Unlike the broth method, this technique does not present turbidity problems. In addition, it is not hampered by limited diffusion of non-polar components as in the agar diffusion method (Saad *et al.*, 2013). Lemongrass, spearmint and thyme EOs were tested at concentrations of 100, 500, 750, 1 000 and 2 000 µL/L in PDA. Tween® 20 (a non-ionic surfactant) was added at 0.05% to ensure solubility and even distribution of the EO in the agar, without chemically interacting with the EO or affecting the organisms tested at the level that it was applied. The negative controls contained Tween® 20, but no EOs.

¹⁰ https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch [Accessed 19/04/2018]

Conidia from selected *Fusarium* spp. isolated from soil and maize were harvested from 7-day-old cultures grown on PDA. A sterile aqueous solution containing 0.1% each of Tween® 20 and PDA was poured onto the culture plates and the surface was superficially scraped with a bent glass rod to facilitate the release of the conidia. The suspension was filtered through a sterile muslin cloth and the conidia were counted using a Neubauer Chamber (Celeromics, Cambridge, UK). Thereafter the concentration of the conidia in the suspension was adjusted to $1 \times 10^6/\text{mL}$.

Plates were inoculated, in the centre, with 10 μL of the conidial suspension, sealed with Parafilm™ and incubated at 25 °C. After 7 days, the diameter of the mycelial growth was measured as the average of two perpendicular readings using a digital Vernier calliper (KTV, Major Tech Pty Ltd, Johannesburg, South Africa). The percentage inhibition was calculated according to Equation 4.

$$\% \text{ Inhibition} = \left[\frac{(\text{average diameter negative control} - \text{average diameter treatment})}{\text{average diameter negative control}} \right] \times 100$$

.....Equation 4

Based on the first set of results, the best performing EO was selected and tested against a wider range of *Fusarium* isolates and at smaller concentration intervals, to determine the minimum inhibitory concentration (MIC).

6.2.7 *FUM* gene expression

The experimental design for the preliminary and follow-up experiments to determine the effect of lemongrass EO on the expression of *FUM1* and *FUM21* genes in two *F. verticillioides* cultures is illustrated in Figure 6.3. The *FUM1* and *FUM21* genes were selected for this study, because the expression of these genes was reported to mirror fumonisin production by *F. verticillioides* (Fanelli *et al.*, 2013). Conidial suspensions were prepared as described in Section 6.2.6, inoculated into a PDA broth medium containing lemongrass EO, and incubated at 25 °C. Samples taken at specific time intervals were stored at -80 °C until completion of the experiment,

after which they were analysed to determine *FUM* gene expression. In the preliminary experiment, the concentration of the spore suspension used for inoculation was 1×10^6 spores/mL and lemongrass EO concentrations of 400 $\mu\text{L/L}$ (the MIC value) and 100 $\mu\text{L/L}$ (25% of the MIC) were applied. The samples were incubated for 7 days. In the follow-up experiment, the concentration of the spore suspension was increased to 1×10^7 spores/mL and the incubation period was increased to 28 days. Instead of 400 $\mu\text{L/L}$ lemongrass EO (the MIC value), 200 $\mu\text{L/L}$ (50% of the MIC) was selected as the highest concentration. For each experiment, three independent replicates were performed.

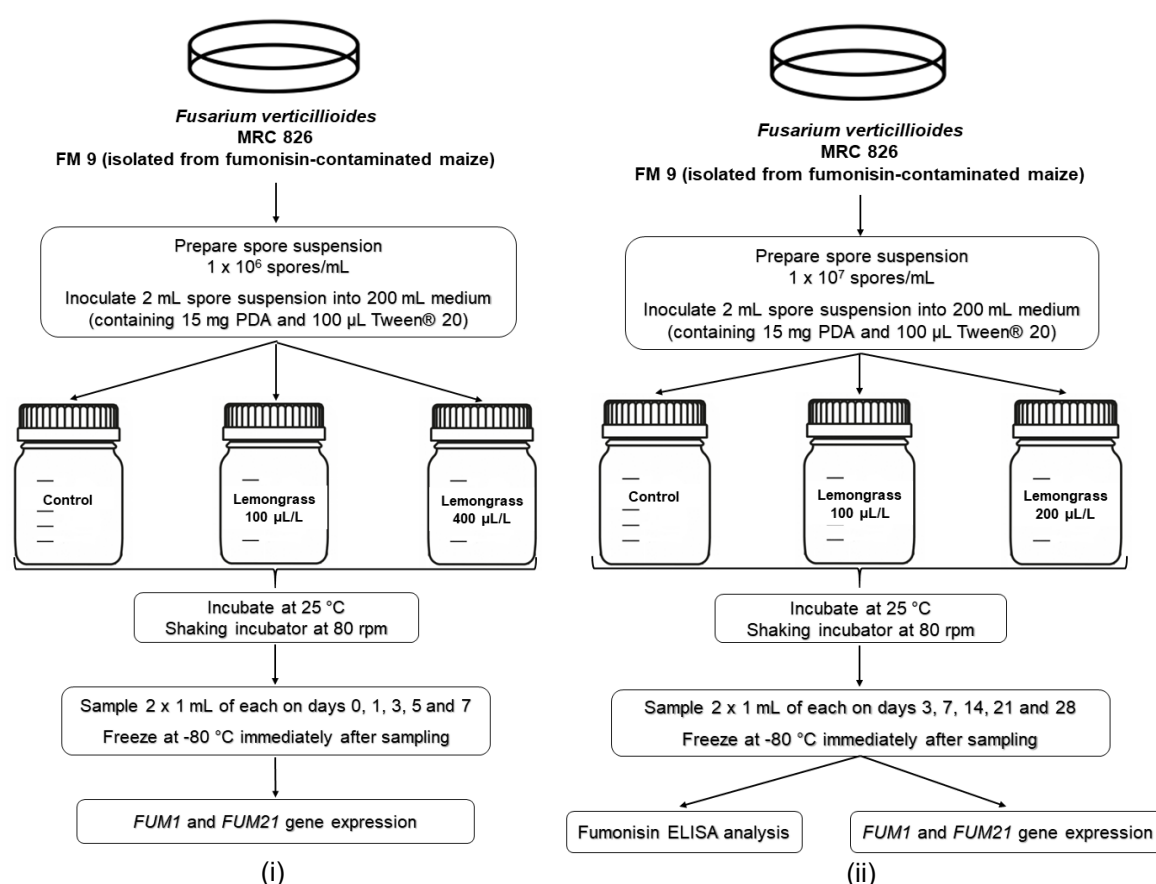


Figure 6.3: Experimental design for the preliminary (i) and follow-up (ii) experiments to determine *FUM* gene expression

Fungal ribonucleic acid (RNA) was isolated using a Quick-RNA™ fungal/bacterial miniprep kit (catalogue no R2014, Zymo Research Corp., Irvine, USA) according to the manufacturer's instructions. Briefly, samples were homogenised in a ZR BashingBead™ system and DNA was eliminated using a Zymo-Spin™ IIICG Column. The RNA was subsequently transferred to a Zymo-Spin™ IIC Column

where it was digested with DNase I, purified with RNA buffer and eluted with DNase/RNase-free water.

The complementary DNA (cDNA) synthesis and Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) were performed using a Luna[®] Universal one-step RT-qPCR kit (New England BioLabs[®] Inc., Ipswich, USA). This method involves first the conversion of RNA to cDNA by a reverse transcriptase, followed by amplification of the cDNA by a DNA-dependent DNA polymerase, enabling quantitation *via* qPCR. The following primer sequences were used: 5'-GTCAGAACTATTGGAACGCA-3' and 5'-GGACTCCCTTCTGCATGTATCG-3' for the *FUM1* transcripts FUM1_F2 and FUM1_R3; 5'-ATGCAGATCCGGAAGGTGTTC-3' and 5'-TGTAATCTCGTCTGCAATCAAATCC-3' for the *FUM21* transcripts FUM21_F3 and FUM21_R3; and lastly 5'-ACATCCAGACAGCCCTTTGT-3' and 5'-GTTTCCGATGAAGGTCTGAAG-3' for the *TUB2* beta-tubulin transcripts PQ-TUB-F2 and PQ-TUB-R2 (Fanelli *et al.*, 2013). After each PCR cycle, DNA amplification was measured by real-time fluorescence of a double-stranded DNA binding dye, determining the point where the fluorescence signal was confidently detected over the background fluorescence (defined as the quantification cycle or C_q). The threshold cycle (C_t) was inversely proportional to the amount of the target gene. Values ≤ 29 was indicative of a strong positive reaction, while 30-37 was regarded as a positive reaction with moderate amounts of the target gene. The gene expression analysis was performed in triplicate for each independent replicate of the experiment.

For the standard curve, RNA serial dilutions (1000, 100, 10.0, 1.00 and 0.100 ng) were prepared and run in triplicate for all the target genes (*TUB2*, *FUM1* and *FUM21*). The C_q was plotted against the log of the input concentration and the NEB online qPCR tool¹¹ was used to evaluate the efficiency of the qPCR, verifying that the linear fit of the data had a slope of -3.6 to -3.1 (corresponding to a reaction efficiency of 90-110%) and a correlation coefficient (R^2) of ≥ 0.98 (Appendix 6).

¹¹ <http://nebiocalculator.neb.com/#!/qPCRGen> [Accessed 09/04/2018]

The comparative cycle threshold method ($\Delta\Delta C_t$) was used to quantify gene expression, normalising the target genes (*FUM1* and *FUM21*) to an endogenous reference (*TUB2*) and to the treatment controls (Equations 5, 6, 7 and 8). Normalisation of gene expression data is required to compensate for variation in tissue mass between samples.

$$\Delta C_t = FUM \text{ gene } C_t - TUB \text{ gene } C_t \quad \text{.....Equation 5}$$

$$\text{Relative gene expression} = 2^{-\Delta C_t} \quad \text{.....Equation 6}$$

$$\Delta\Delta C_t = FUM \text{ gene } \Delta C_t (\text{treatment}) - FUM \text{ gene } \Delta C_t (\text{control}) \quad \text{.....Equation 7}$$

where the treatment and control samples are those containing, and not containing lemongrass EO, respectively, taken on the same day

$$\text{Fold difference in expression relative to control} = 2^{-\Delta\Delta C_t} \quad \text{.....Equation 8}$$

Using the fold difference calculation, down-regulated genes are identified by values ranging between 0 and 1, while up-regulated genes are reflected by values from 1 to infinity. The standard deviation of the ΔC_t value was calculated from the standard deviations of the target and reference C_t values, using the Equation 9:

$$s = \sqrt{s_1^2 + s_2^2} \quad \text{.....Equation 9}$$

where s is the standard deviation of the ΔC_t values, s_1 is the standard deviation of the reference C_t values and s_2 is the standard deviation of the target C_t values

6.2.8 Fumonisin determination using the ELISA method

Fumonisin production in the PDA broth by *F. verticillioides* in the presence and absence of lemongrass EO was determined using a competitive colorimetric ELISA test kit (catalogue no 1038, Bioo Scientific, Austin, USA). The instructions were followed according to the standard testing protocol. Briefly, fumonisin standards (0, 1.00, 5.00, 10.0, 25.0 and 50.0 ng/mL), as well as a blank sample and spiked blank sample, were added in duplicate to different wells. The PDA broth sample was diluted 1:9 with 35% methanol and 50 μ L of each sample was added in duplicate

wells. After adding the fumonisin antibody mix, the contents were mixed gently and incubated for 30 min at room temperature, and then decanted and washed three times with wash solution. The plate was inverted and dried on paper towels prior to the addition of the TMB chromogenic substrate. The antibody mix was then carefully mixed and incubated for 1 min, after which the stop buffer was added and the colour intensity was measured at a wavelength of 450 nm in a Multiskan™ microplate spectrophotometer (Thermo Scientific™, Waltham, USA). The results were calculated with the Bioo Scientific MaxSignal® ELISA Analysis Program in Excel. Because there is cross-reactivity with the fumonisins when using the ELISA kit (FB₁ 100%, FB₂ 94% and FB₃ 145%), the obtained results may be considered as an indication of the total FB content.

6.2.9 Statistical analysis

Statgraphics® Centurion XVI version 16.1.17 software (Statpoint Technologies Inc., The Plains, Virginia, USA) was used to analyse the data. Analysis of Variance (ANOVA) was performed and a *p*-value of <0.05 for the F-test was considered to indicate statistically significant differences between samples. Means were compared with the aid of Fisher's LSD (Least Significant Difference) intervals. All analyses were performed in triplicate, unless stated otherwise.

6.3 RESULTS AND DISCUSSION

6.3.1 Soil characteristics

The characteristics of the soil samples collected at different locations in the Mpumalanga and Free State provinces are summarised in Table 6.4.

Table 6.4: Texture, moisture content, pH and conductivity of soil samples collected at different locations

Sample code	Soil texture	Sampling position	Moisture content (% m/m)	pH	Conductivity (mS/cm)
L1	silty clay loam	surface	3.98±0.12 ⁿ	6.69±0.02 ^o	76.4±16.7 ^k
		rhisosphere	4.70±0.19 ^o	6.65±0.03 ^o	191±3 ^r
L2	silty clay loam	surface	3.25±0.11 ^k	5.77±0.04 ^g	49.0±3.5 ^{fg} ^{hi}
		rhisosphere	4.58±0.03 ^o	5.92±0.02 ⁱ	159±89 ^p
L4	loam	surface	3.79±0.19 ^m	5.03±0.03 ^b	61.4±8.9 ^j
		rhisosphere	4.90±0.09 ^p	5.58±0.03 ^f	97.0±9.7 ^l
L5	loam	surface	3.65±0.34 ^m	5.31±0.05 ^c	177±21 ^q
		rhisosphere	5.16±0.20 ^q	5.31±0.02 ^c	119±14 ⁿ
L6	loam	surface	3.46±0.09 ^l	5.07±0.02 ^b	148±13 ^p
		rhisosphere	4.90±0.11 ^p	4.57±0.03 ^a	133±9 ^o
L7	loam	surface	2.89±0.05 ^j	5.54±0.06 ^f	291±5 ^s
		rhisosphere	4.08±0.02 ⁿ	5.43±0.01 ^e	114±4 ^{mn}
L8	clay loam	surface	6.08±0.06 ^r	5.66±0.01 ^f	70.4±13.0 ^{jk}
		rhisosphere	6.57±0.12 ^s	5.67±0.03 ^f	90.0±7.5 ^l
L9	loamy sand	surface	0.667±0.036 ^{ab}	5.57±0.04 ^f	15.7±10.1 ^a
		rhisosphere	0.947±0.018 ^{cde}	5.53±0.02 ^f	41.7±6.2 ^{cdef}
L10	loamy sand	surface	0.596±0.018 ^a	6.04±0.04 ^j	17.0±0.6 ^a
		rhisosphere	0.546±0.125 ^a	5.38±0.02 ^d	31.4±2.9 ^{bc}
L11	loamy sand	surface	0.542±0.048 ^a	6.11±0.06 ^k	28.0±2.5 ^{ab}
		rhisosphere	0.836±0.049 ^{bc}	5.30±0.07 ^c	47.4±0.6 ^{efgh}
L12	sandy loam	surface	1.08±0.05 ^e	6.45±0.04 ^m	17.0±5.9 ^a
		rhisosphere	0.944±0.041 ^{cde}	6.83±0.05 ^p	46.0±8.3 ^{defg}
L13	loamy sand	surface	0.827±0.027 ^{bc}	6.32±0.01 ^l	33.4±11.6 ^{bcd}
		rhisosphere	0.862±0.073 ^{cd}	5.34±0.04 ^{cd}	35.0±0.6 ^{bcd}
L14	clay loam	surface	1.68±0.08 ⁱ	5.45±0.02 ^e	26.7±0.0 ^{ab}
		rhisosphere	1.73±0.05 ⁱ	5.44±0.01 ^e	45.7±12.1 ^d ^{efg}
L15	loamy sand	surface	1.07±0.03 ^e	6.70±0.03 ^o	34.4±1.2 ^{bcd}
		rhisosphere	1.02±0.08 ^{de}	6.53±0.02 ⁿ	60.7±2.0 ^{hij}
L16	loamy sand	surface	0.924±0.037 ^{cde}	6.49±0.01 ^{mn}	27.4±1.5 ^{ab}
		rhisosphere	1.26±0.13 ^f	6.47±0.03 ^m	91.7±3.0 ^l
L17	sandy loam	surface	1.29±0.06 ^{fg}	6.06±0.01 ^{jk}	52.4±3.1 ^{fg} ^{hi}
		rhisosphere	1.61±0.05 ^{hi}	5.86±0.02 ^h	103±6 ^{lm}
L18	loam	surface	1.44±0.06 ^{gh}	5.68±0.07 ^f	57.0±1.5 ^{ghi}
		rhisosphere	1.27±0.08 ^{fg}	5.59±0.03 ^f	70.0±0.6 ^{jk}

Means with any same letter in a column are not significantly different ($p>0.05$)

The values after (±) are standard deviations

The soil samples represent a variety of textural classes, ranging from loamy sand to silty clay loam. The moisture content of the soil samples was low, as can be expected since they were dried before storage. The soil pH was moderately acidic, ranged between 4.6 and 6.8 and did not differ much between surface and rhizosphere samples from the same location. The pH of the soil is known to affect plant growth and the activity of microorganisms (Miller and Donahue, 1990). In general fungi tolerate acidity better than bacteria. Strong acidity inhibits nitrogen-fixing bacteria and the bacteria that decompose soil organic material. Alkaline soil pH can also have a negative effect, because it reduces the solubility and availability of many micronutrients, including iron, zinc, copper and manganese. Most agricultural crops grow well in soil with a pH around 6.5. Conductivity provides an index of the salinity of the soil (Ali and Narayan, 2013). Higher conductivities were measured in the rhizosphere soil samples than in the surface soil samples, except for L5, L6 and L7, where the conductivity was significantly higher in the surface soil and L13 where there was not significant difference between the conductivity of the surface and rhizosphere soil.

Tables 6.5 provides a summary of the average, lowest and highest concentrations of the different trace elements detected in the soil samples. Additional data is provided in Appendices 7 and 8. The micronutrients K, Ca, Mg, B, Zn, Cu, Mn, Fe, and Mo are required for plant growth (Horneck *et al.*, 2015), whereas elevated levels of potentially toxic metals such as V, Cr, Mn, Co, Zn, Pb and U are detrimental and may indicate environmental pollution (Voica *et al.*, 2012). The major trace elements were Fe (6 880-135 000 µg/kg), Al (4 930-15 000 µg/kg), K (887-8 840 µg/kg) and Ti (2 020-9 130 µg/kg), whereas Ba, Ca, Co, Cr, Mg, Mn, V and Zr occurred in minor quantities (ranging from 0-3 090 µg/kg). Low amounts of Cu (7.41-130 µg/kg) were detected in samples from Locations 1 and 8 and Zn (57.8-96 µg/kg) in Location 8.

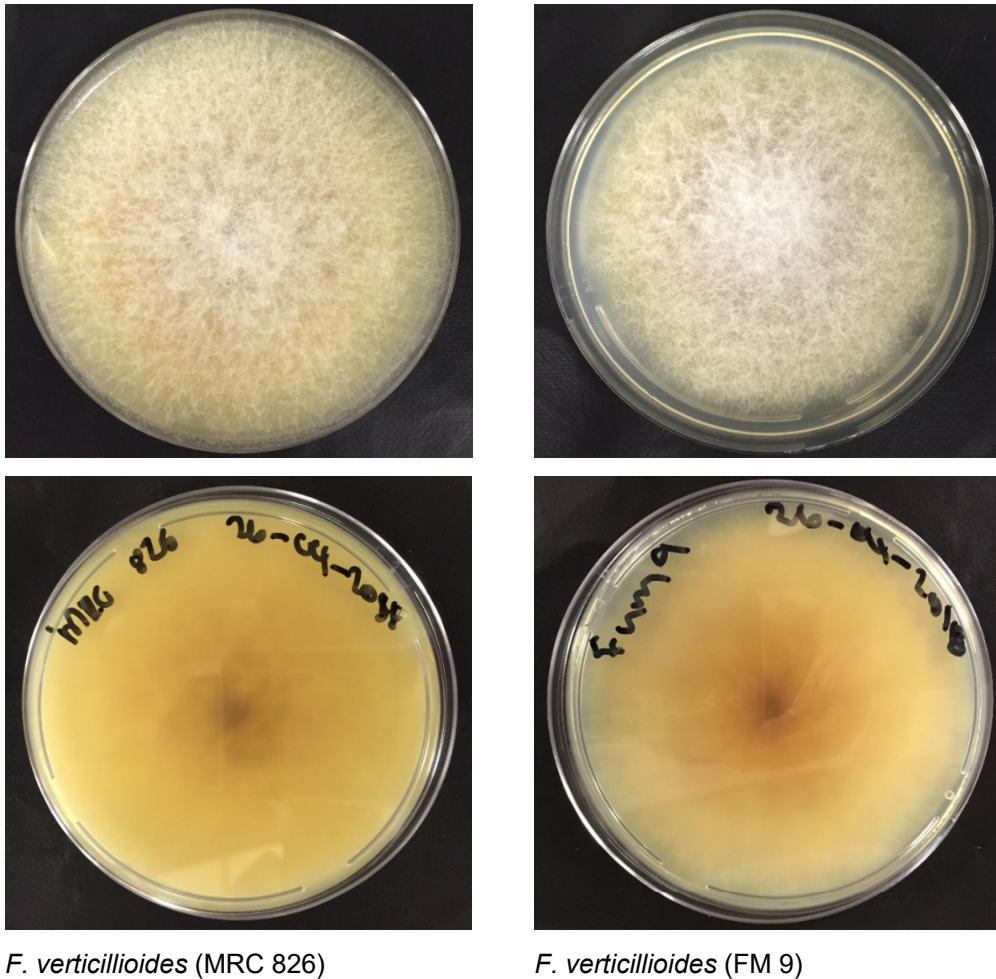
Table 6.5: Average, lowest and highest concentrations of trace elements detected in soil samples collected from locations in the Mpumalanga and Free State provinces

Element	Trace element concentration (µg/kg)					
	Mpumalanga province			Free state province		
	Low	Average	High	Low	Average	High
Al	4 930	12 000	15 000	5 340	9 010	11 600
Ba	11.2	107	232	107	133	311
Ca	185	1 150	2 770	486	970	1 610
Co	69.9	253	513	0	65.2	60.4
Cr	76.4	256	644	16.1	93.0	114
Cu	0.00	37.1	130	0	0	0
Fe	27 100	68 900	135 000	6 880	23 900	25 200
K	887	2 260	4 190	2 940	3 920	8 840
Mg	46.4	905	3 090	263	349	653
Mn	98.3	763	1 550	61.9	235	315
Ti	3 110	6 170	9 130	2 020	3 670	4 570
V	22.4	133	272	2.44	37.4	44.5
Zn	0.00	22.0	96.0	0	0	0
Zr	50.6	79.8	139	0	48.4	19.3

The trace element composition and levels varied between the different locations and could be related to the soil textural classes and use of fertilisers in the soil. Overall the soil samples from Mpumalanga had higher trace element concentrations than the samples from the Free State.

6.3.2 *Fusarium* spp. isolated from soil and maize

Three criteria are used for the identification of *Fusarium*, namely morphological, biological (sexual cross-fertility) and phylogenetic (DNA sequencing) (Summerell *et al.*, 2010). Since *Fusarium* is a large, complex genus and there are relatively few readily distinguishable morphological characteristics (e.g. spore size and shape, presence of micro- and macroconidia), this study made use of morphological characterisation for preliminary identification, which was confirmed by DNA sequencing to ensure accurate identification of the fungal isolates. Figure 6.4 illustrates the appearance of *F. verticillioides* on PDA after 10 days, comparing the colony morphology of the standard culture (MRC 826) to one of the cultures isolated from fumonisin-contaminated maize (FM 9).

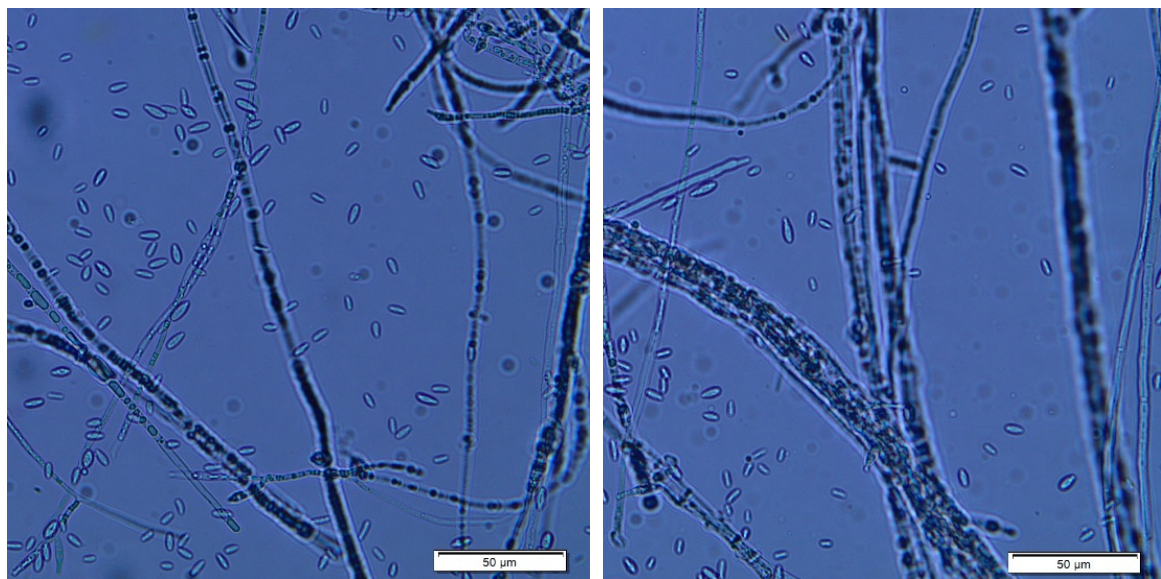


F. verticillioides (MRC 826)

F. verticillioides (FM 9)

Figure 6.4: Appearance of *Fusarium verticillioides* (MRC 826 and FM 9) after 10 days growth on PDA (the top plate in each pair is the upper surface and the lower plate is the under surface)

The appearance of the *F. verticillioides* isolates was typical with white mycelia and the development of greyish orange pigmentation in the agar over time (Leslie and Summerell, 2008). The other presumed *Fusarium* isolates had similar colony morphologies, displaying dense arial mycelium that were initially white or cream in colour, and turned tan, brown, orange or violet with maturation. The microscopic appearance of MRC 826, and an isolate from contaminated maize (FM 9), is illustrated in Figure 6.5.



F. verticillioides (MRC 826)

F. verticillioides (FM 9)

Figure 6.5: Micrographs of *Fusarium verticillioides* indicating fungal mycelia and spores

The hyphae were septate and microconidia were typically oval-shaped without septa. No chlamydospores or macroconidia were observed. Although *F. verticillioides* is able to produce long, slender, thin-walled macroconidia, it is stated in literature that they may be hard to locate, even when the cultures are grown under standardised conditions (Leslie and Summerell, 2008).

Based on morphological properties, 55 isolates from the soil and maize samples were selected for verification, of which genetic analysis confirmed that a total of 25 isolates were indeed *Fusarium* spp. (Table 6.6).

Table 6.6: The identities of *Fusarium* spp. isolated from soil and maize as confirmed through genetic analysis

Location	Sample type	Sample code	Sample description	Identity
L3	Silo maize residue	L3SL1-14	Silo 1, plate 14	<i>F. verticillioides</i>
		L3SL2-29	Silo 2, plate 29	<i>F. verticillioides</i>
	Mill maize residue	L3M-44	Mill, plate 44	<i>F. oxysporum</i>
L9	Soil	S9c70	Surface soil, plate 70	<i>F. solani</i>
		S9c71	Surface soil, plate 71	<i>F. equiseti</i>
		S9c72	Surface soil, plate 72	<i>F. equiseti</i>
	Maize kernels	L9C2M	Cob 2, middle	<i>F. verticillioides</i>
		L9-125	Ground, plate 125	<i>F. verticillioides</i>
L10	Soil	S10c77	Surface soil, plate 77	<i>F. brachygibbosum</i>
	Maize kernels	L10-127	Ground maize, plate 127	<i>F. verticillioides</i>
L12	Maize kernels	L12SS	Surface sterilised, ground	<i>F. verticillioides</i>
L13	Maize kernels	L13-25	Ground, plate 25	<i>F. verticillioides</i>
L14	Maize kernels	L14-61	Ground, plate 61	<i>F. verticillioides</i>
L15	Maize kernels	L15-35	Ground, plate 35	<i>F. verticillioides</i>
		L15SS	Surface, sterilised ground	<i>F. verticillioides</i>
L16	Maize kernels	L16C1B	Location 16 cob 1 bottom	<i>F. oxysporum</i>
		L16C3M	Location 16 cob 2 top	<i>F. oxysporum</i>
		L16C2T	Location 16 cob 3 middle	<i>F. oxysporum</i>
L17	Maize kernels	L17C3T	Location 17 cob 3 top	<i>F. verticillioides</i>
		L17C3M	Location 17 cob 3 middle	<i>F. verticillioides</i>
		L17SS	Surface sterilised ground	<i>F. verticillioides</i>
L18	Maize kernels	L18C1B	Cob 1 bottom	<i>F. verticillioides</i>
		L18SS	Surface sterilised ground	<i>F. verticillioides</i>
SAGL	Maize meal	FM 9	Fumonisin-contaminated	<i>F. verticillioides</i>
		FM 15	Fumonisin-contaminated	<i>F. verticillioides</i>

The only soil samples from which *Fusarium* spp. were isolated, were Locations 9 (*F. equiseti* and *F. solani*) and 10 (*F. brachygibbosum*). No fumonisin-producing fusaria were detected in any of the soil samples, not even from locations where *F. verticillioides* were isolated from the maize kernels. This was attributed to the fact that the soil was sieved before analysis, thereby removing plant debris in which the

fungi survive. Alternatively, it is possible that the maize was infected *via* a different route, e.g. through airborne or waterborne microconidia. Two species of *Fusarium* were isolated from maize, namely *F. oxysporum* (Location 3 mill residue and Location 16 maize) and *F. verticillioides* (Location 3 silo residue, maize from Locations 9, 10, 12, 13, 14, 15, 17 and 18, as well as fumonisin contaminated maize meal). The sampling was limited, since the purpose was to isolate relevant fusaria and not to conduct a comprehensive survey. Nevertheless, it was evident that the predominant *Fusarium* spp. was *F. verticillioides*, which is in agreement with literature (Beukes *et al.*, 2017). The pathogen was also detected in surface-sterilised maize samples, indicating a systemic infection. The presence of *F. verticillioides* was also confirmed in the SAGL maize meal sample with a high FB content.

6.3.3 *In vitro* antifungal activity of lemongrass, spearmint and thyme essential oils against selected *Fusarium* isolates

Five *Fusarium* isolates with different identities [*F. brachygibbosum* (S9c70), *F. equiseti* (S9c71), *F. oxysporum* (L16C3M), *F. solani* (S9c70) and *F. verticillioides* (MRC 826)] were selected to evaluate the antifungal potential of lemongrass, spearmint and thyme EOs (Table 6.7). The percentage inhibition of mycelial growth increased significantly as the concentration of the EOs were increased, indicating that the inhibitory effect of the EOs was proportional to its concentration. At a concentration of 500 $\mu\text{L/L}$, lemongrass EO was able to prevent the growth of all five cultures, whereas spearmint and thyme could only inhibit all five cultures at 1 500 $\mu\text{L/L}$. The efficacy of lemongrass EO as an antifungal agent against the *Fusarium* isolates is probably related to its chemical composition (84.8% citral), which differs from those of spearmint (87.9% R-carvone) and thyme (63.1% thymol and 21.3% linalool) (Combrinck *et al.*, 2011). There were also significant differences in the sensitivity of the different *Fusarium* isolates to the EOs, with *F. equiseti* being the most susceptible and *F. solani* being the least susceptible to spearmint and thyme EOs. Although there is no literature available for direct comparison, differences in the susceptibility of *Fusarium* spp. towards various EOs have been reported before. For example, Sahab *et al.* (2014) reported on the effect of rocket, rosemary and tea tree EOs on *Fusarium* spp. isolated from maize in Egypt. They

found *F. solani* to be the most sensitive and *F. oxysporum* to be the least sensitive towards the EOs tested. Similarly, Sreenivasa *et al.* (2011) evaluated selected EOs (clove, cedar wood, lemongrass, peppermint, citronella and neem) against fungi isolated from maize and sorghum and found that they were most effective against *F. solani*, followed by *F. anthophilum*, *F. graminearum*, *F. proliferatum*, *F. sporotrichioides*, *F. pallidoroseum*, *F. lateritium* and finally *F. verticilliioides*.

Table 6.7: Average percentage inhibition of mycelial growth of selected *Fusarium* isolates by lemongrass, spearmint and thyme essential oils

Isolate	Concentrations of essential oils in $\mu\text{L/L}$					
	100	500	750	1 000	1 500	2 000
Lemongrass						
<i>F. brachygibbosum</i>	36.2 $\pm$ 1.4 ^{a, C}	100 ^{b, F}	100 ^{b, D}	100 ^{b, C}	100 ^{b, A}	100 ^{b, A}
<i>F. equiseti</i>	59.6 $\pm$ 6.0 ^{a, D}	100 ^{b, F}	100 ^{b, D}	100 ^{b, C}	100 ^{b, A}	100 ^{b, A}
<i>F. oxysporum</i>	40.1 $\pm$ 1.4 ^{a, C}	100 ^{b, F}	100 ^{b, D}	100 ^{b, C}	100 ^{b, A}	100 ^{b, A}
<i>F. solani</i>	22.7 $\pm$ 3.3 ^{a, B}	100 ^{b, F}	100 ^{b, D}	100 ^{b, C}	100 ^{b, A}	100 ^{b, A}
<i>F. verticilliioides</i>	21.1 $\pm$ 5.0 ^{a, B}	100 ^{b, F}	100 ^{b, D}	100 ^{b, C}	100 ^{b, A}	100 ^{b, A}
Spearmint						
<i>F. brachygibbosum</i>	43.6 $\pm$ 1.2 ^{a, C}	63.6 $\pm$ 6.4 ^{b, C}	100 ^{c, D}	100 ^{c, C}	100 ^{c, A}	100 ^{c, A}
<i>F. equiseti</i>	40.8 $\pm$ 2.9 ^{a, C}	100 ^{b, F}	100 ^{b, D}	100 ^{b, C}	100 ^{b, A}	100 ^{b, A}
<i>F. oxysporum</i>	20.9 $\pm$ 4.9 ^{a, B}	47.9 $\pm$ 4.2 ^{b, A}	59.9 $\pm$ 3.9 ^{c, A}	100 ^{d, C}	100 ^{d, A}	100 ^{d, A}
<i>F. solani</i>	42.1 $\pm$ 7.6 ^{a, C}	62.3 $\pm$ 6.3 ^{b, BC}	64.1 $\pm$ 4.0 ^{b, B}	66.6 $\pm$ 7.2 ^{b, A}	100 ^{c, A}	100 ^{c, A}
<i>F. verticilliioides</i>	41.2 $\pm$ 6.9 ^{a, C}	54.1 $\pm$ 12.2 ^{b, AB}	77.2 $\pm$ 4.5 ^{c, C}	100 ^{d, C}	100 ^{d, A}	100 ^{d, A}
Thyme						
<i>F. brachygibbosum</i>	35.3 $\pm$ 7.0 ^{a, C}	65.1 $\pm$ 6.1 ^{b, CD}	100 ^{c, D}	100 ^{c, C}	100 ^{c, A}	100 ^{c, A}
<i>F. equiseti</i>	55.7 $\pm$ 7.8 ^{a, D}	100 ^{b, F}	100 ^{b, D}	100 ^{b, C}	100 ^{b, A}	100 ^{b, A}
<i>F. oxysporum</i>	6.67 $\pm$ 5.9 ^{a, A}	62.9 $\pm$ 8.9 ^{b, BC}	78.2 $\pm$ 2.8 ^{c, C}	100 ^{d, C}	100 ^{d, A}	100 ^{d, A}
<i>F. solani</i>	38.9 $\pm$ 0.8 ^{a, C}	74.3 $\pm$ 4.6 ^{b, E}	78.7 $\pm$ 2.5 ^{bc, C}	83.3 $\pm$ 4.2 ^{c, B}	100 ^{d, A}	100 ^{d, A}
<i>F. verticilliioides</i>	42.1 $\pm$ 9.0 ^{a, C}	74.1 $\pm$ 8.3 ^{b, DE}	78.5 $\pm$ 4.0 ^{bc, C}	83.9 $\pm$ 1.9 ^{c, B}	100 ^{d, A}	100 ^{d, A}

Means with the same upper-case letter did not differ significantly within a column ($p>0.05$), means with the same lower-case letter did not differ significantly within a row ($p>0.05$); the values after ($\pm$) are standard deviations and grey fill indicates complete inhibition

Since lemongrass EO showed the best potential, especially towards *F. verticilliioides* (which produces fumonisins), it was selected for the follow-up experiment, where its inhibitory effect was determined at smaller concentration intervals against a wider range of fusaria isolated from maize and soil (Table 6.8). At a cost of approximately

\$74/L, lemongrass is also the most economical option (spearmint and thyme EOs cost \$112/L and \$88/L, respectively)¹².

Table 6.8: Average percentage inhibition of mycelial growth of *Fusarium* isolates from soil and maize by lemongrass essential oil

Isolates	Code	Concentration of lemongrass essential oil (μL/L)				
		100	200	300	400	500
<i>F. brachygibbosum</i>	S10c77	36.2±1.4 ^{a, CDE}	57.8±2.4 ^{b, C}	100 ^{c, G}	100 ^{c, A}	100 ^{c, A}
<i>F. equiseti</i>	S9c71	59.6±6.0 ^{a, H}	100 ^{b, F}	100 ^{b, G}	100 ^{b, A}	100 ^{b, A}
<i>F. oxysporum</i>	L3M-44	32.9±1.3 ^{a, BC}	40.2±8.8 ^{a, A}	75.9±2.3 ^{b, C}	100 ^{c, A}	100 ^{c, A}
<i>F. oxysporum</i>	L16C3M	40.1±1.4 ^{a, DE}	69.1±0.7 ^{b, E}	100 ^{c, G}	100 ^{c, A}	100 ^{c, A}
<i>F. solani</i>	S9c70	22.7±3.3 ^{a, A}	58.0±5.9 ^{b, C}	60.9±11.4 ^{b, A}	100 ^{c, A}	100 ^{c, A}
<i>F. verticillioides</i>	MRC 826	21.1±5.0 ^{a, A}	39.8±6.1 ^{b, A}	100 ^{c, G}	100 ^{c, A}	100 ^{c, A}
<i>F. verticillioides</i>	FM 9	34.9±1.2 ^{a, C}	67.9±4.3 ^{b, E}	79.2±2.5 ^{c, CDE}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	FM 15	33.3±1.9 ^{a, BC}	64.8±5.4 ^{b, DE}	83.6±3.7 ^{c, E}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L9-125	54.3±0.6 ^{a, G}	68.7±0.8 ^{b, E}	100 ^{c, G}	100 ^{c, A}	100 ^{c, A}
<i>F. verticillioides</i>	L10-127	37.1±1.6 ^{a, CDE}	60.9±1.0 ^{b, CD}	79.0±1.3 ^{c, CDE}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L12SS	49.3±5.5 ^{a, F}	69.2±1.3 ^{b, E}	89.7±0.5 ^{c, F}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L13-25	58.1±1.8 ^{a, GH}	69.6±0.8 ^{b, E}	80.4±1.2 ^{c, CDE}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L14-61	40.5±0.6 ^{a, E}	68.2±0.1 ^{b, E}	82.6±0.6 ^{c, DE}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L15SS	29.1±1.9 ^{a, B}	58.7±1.2 ^{b, C}	78.5±0.8 ^{c, CD}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L15-35	45.4±1.5 ^{a, F}	50.9±4.3 ^{b, B}	67.8±1.3 ^{c, B}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L17SS	45.3±0.7 ^{a, F}	58.5±1.2 ^{b, C}	68.0±0.2 ^{c, B}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L17C3T	35.9±1.5 ^{a, CD}	50.1±0.9 ^{b, B}	69.3±0.5 ^{c, B}	100 ^{d, A}	100 ^{d, A}
<i>F. verticillioides</i>	L18C1B	40.0±1.3 ^{a, DE}	58.6±1.1 ^{b, C}	80.2±1.0 ^{c, CDE}	100 ^{d, A}	100 ^{d, A}

Means with the same upper-case letter did not differ significantly within a column ($p>0.05$), means with the same lower-case letter did not differ significantly within a row ($p>0.05$); the values after ($\pm$) are standard deviations and grey fill indicates complete inhibition

The MIC of lemongrass EO was 400 μL/L for 13 of the 18 *Fusarium* isolates tested. An MIC of 300 μL/L was obtained for four isolates, while an MIC of 200 μL/L was obtained for only one isolate. These values are in concordance with Seseni *et al.* (2015), who reported an MIC of 300 μL/L for *F. oxysporum* and values ranging from 400-700 μL/L for *F. circinatum* isolates. Sreenivasa *et al.* (2011) obtained higher MIC values for lemongrass towards fusaria isolated from sorghum and maize, with *F. solani* having the lowest MIC (500 μL/L) and MIC values of >2 500 μL/L for

¹² <http://www.holisticemporium.co.za/> [Accessed 22/03/2018]

F. oxysporum and *F. verticillioides*. Fandohan *et al.* (2004) obtained a MIC of 1 300 $\mu\text{L/L}$ when testing lemongrass EO against a *F. verticillioides* isolate from maize. On the contrary, Linde *et al.* (2010) reported that the growth of *F. oxysporum* isolated from maize was totally inhibited at a much lower concentration of lemongrass EO (50 $\mu\text{L/L}$).

The antifungal activity of EOs do not only depend on its concentration and composition, but is also affected by factors like water activity and incubation time (Sahab *et al.*, 2014). There were slight differences in the experimental protocols used in the different studies. For example, in the current study 0.05% Tween[®] 20 was added as emulsifier and the incubation temperature was 25 °C, whereas Sreenivasa *et al.* (2011) used 5% Tween[®] 20 (100 x more) and incubated at 28 °C. Fandohan *et al.* (2004) incubated at 25 °C and did not use an emulsifier. At higher concentrations, non-ionic surfactants such as Tween[®] 20 may cause changes in the physicochemical properties of the test system (Mann and Markham, 1998). However, the fungal growth inhibition was expressed relative to the negative control and therefore the effect of differences in the experimental conditions is compensated for. Differences in the MIC values are possibly due to genetic differences between the *Fusarium* isolates, which enable some isolates to adapt better to adverse environments. This was also evident from the results in Table 6.8. For example, two *F. verticillioides* isolates (MRC 826 and L9-125) had an MIC of 300 $\mu\text{L/L}$ lemongrass EO, whereas the rest of the *F. verticillioides* isolates had an MIC of 400 $\mu\text{L/L}$.

The potential of lemongrass EO to inhibit the growth of *Fusarium* spp. isolated from maize and soil was confirmed by this study. Because the EO has an adverse effect on kernel germination (Fandohan *et al.*, 2004), it is not recommended for use as an antifungal during storage of maize used for seeds. However, it may have potential for preventing fungal growth in stored maize destined for food or feed and even to use as a disinfectant in silo's and mills.

6.3.4 Effect of lemongrass essential oil on expression of *FUM* genes and fumonisin production

Although EOs are effective inhibitors of fungal growth, mycotoxin production is not always inhibited (Kumar *et al.*, 2016). Therefore, the effect of lemongrass EO on the expression of *FUM* genes and fumonisin production had to be established. Two concentrations were assessed in a preliminary experiment, namely 400 $\mu\text{L/L}$ (the MIC) and 100 $\mu\text{L/L}$ (25% of the MIC) and the effect was determined in two isolates (MRC 826 and FM 9) over a period of 7 days (Tables 6.9 and 6.10).

Table 6.9: Effect of lemongrass essential oil on *FUM1* and *FUM21* gene expression in *Fusarium verticillioides* isolate MRC 826 over 7 days

Time (days)	Rep	<i>FUM1</i> (ΔC_t)			<i>FUM21</i> (ΔC_t)		
		Control	100 $\mu\text{L/L}$	400 $\mu\text{L/L}$	Control	100 $\mu\text{L/L}$	400 $\mu\text{L/L}$
0	1	-*	-	-	-0.414 $\pm$ 2.06	-	1.71 $\pm$ 1.27
	2	-	0.222 $\pm$ 1.20	-	-	-	-
	3	2.84 $\pm$ 3.58	-	0.853 $\pm$ 1.33	-	2.12 $\pm$ 0.74	-
	Avg	-	-	-	-	-	-
	2ΔC_t	-	-	-	-	-	-
	2$\Delta\Delta\text{C}_t$	-	-	-	-	-	-
1	1	5.21 $\pm$ 1.95	10.9 $\pm$ 5.71	-	-0.059 $\pm$ 0.46	4.59 $\pm$ 0.74	-
	2	4.32 $\pm$ 0.90	-	6.18 $\pm$ 5.93	-1.42 $\pm$ 0.70	2.80 $\pm$ 0.53	-
	3	5.93 $\pm$ 0.93	-	-	-	4.23 $\pm$ 1.04	-
	Avg	5.15	-	-	-0.712	3.87	-
	2ΔC_t	0.028	-	-	1.64	0.068	-
	2$\Delta\Delta\text{C}_t$	1.00	-	-	1.00	0.042	-
3	1	0.440 $\pm$ 1.78	-0.129 $\pm$ 1.11	-	1.78 $\pm$ 0.51	4.84 $\pm$ 0.38	4.25 $\pm$ 0.58
	2	0.212 $\pm$ 0.92	-2.04 $\pm$ 0.59	5.74 $\pm$ 3.94	0.084 $\pm$ 0.59	1.34 $\pm$ 0.55	-
	3	1.41 $\pm$ 2.32	-2.05 $\pm$ 0.43	4.74 $\pm$ 3.08	-0.125 $\pm$ 1.42	0.557 $\pm$ 0.40	-
	Avg	0.395	-1.40	5.24	0.581	2.25	-
	2ΔC_t	0.760	2.65	0.026	0.668	0.211	-
	2$\Delta\Delta\text{C}_t$	1.00	3.48	0.035	1.00	0.316	-
5	1	2.06 $\pm$ 0.43	0.902 $\pm$ 0.540	-	3.87 $\pm$ 1.70	3.90 $\pm$ 0.39	-
	2	2.93 $\pm$ 0.21	1.28 $\pm$ 0.65	-	4.46 $\pm$ 0.20	3.61 $\pm$ 1.80	-
	3	6.30 $\pm$ 0.80	3.24 $\pm$ 1.00	-	0.699 $\pm$ 0.851	4.95 $\pm$ 0.27	-
	Avg	3.76	1.81	-	3.01	4.16	-
	2ΔC_t	0.074	0.286	-	0.124	0.056	-
	2$\Delta\Delta\text{C}_t$	1.00	3.88	-	1.00	0.452	-
7	1	2.50 $\pm$ 0.28	0.236 $\pm$ 0.47	-	5.78 $\pm$ 0.61	2.42 $\pm$ 0.54	-
	2	2.55 $\pm$ 0.45	-0.938 $\pm$ 0.27	-	2.62 $\pm$ 0.47	2.28 $\pm$ 0.56	-
	3	2.39 $\pm$ 0.09	1.97 $\pm$ 0.49	-	2.94 $\pm$ 0.16	5.00 $\pm$ 0.41	-
	Avg	2.48	0.42	-	3.78	3.23	-
	2ΔC_t	0.179	0.747	-	0.073	0.106	-
	2$\Delta\Delta\text{C}_t$	1.00	4.17	-	1.00	1.46	-

*Target gene $\text{C}_t > 38$, therefore not possible to calculate ΔC_t

For some of the treatments (especially at Days 0 and 1), the ΔC_t values could not be calculated due to the C_t values being >38 for the target gene. This could be expected during the early stages of the incubation period, since the samples were inoculated with a spore suspension and the fungus would not have had time to grow actively. The same phenomenon was observed for isolate FM 9 (Table 6.10).

Table 6.10: Effect of lemongrass essential oil on *FUM1* and *FUM21* gene expression in *Fusarium verticillioides* isolate FM 9 over 7 days

Time (days)	Rep	<i>FUM1</i> (ΔC_t)			<i>FUM21</i> (ΔC_t)		
		Control	100 μ L/L	400 μ L/L	Control	100 μ L/L	400 μ L/L
0	1	-*	2.46 $\pm$ 0.41	-	2.73 $\pm$ 0.62	2.44 $\pm$ 0.71	2.11 $\pm$ 0.23
	2	4.55 $\pm$ 4.26	0.558 $\pm$ 0.720	-	2.66 $\pm$ 0.53	0.618 $\pm$ 1.92	-
	3	-	0.723 $\pm$ 1.12	-	2.33 $\pm$ 0.65	-	-
	Avg	-	1.25	-	2.57	-	-
	2ΔC_t	-	0.421	-	0.168	-	-
	2$\Delta\Delta C_t$	-	-	-	1.00	-	-
1	1	6.72 $\pm$ 0.68	5.69 $\pm$ 0.85	-	4.82 $\pm$ 0.42	7.63 $\pm$ 1.37	-
	2	6.65 $\pm$ 1.21	6.26 $\pm$ 0.66	3.03 $\pm$ 0.81	4.33 $\pm$ 0.36	6.48 $\pm$ 0.51	-
	3	10.1 $\pm$ 4.93	7.95 $\pm$ 0.82	-	6.12 $\pm$ 0.93	7.93 $\pm$ 0.99	-
	Avg	7.81	6.63	-	5.09	7.35	-
	2ΔC_t	0.004	0.010	-	0.029	0.006	-
	2$\Delta\Delta C_t$	1.00	2.27	-	1.00	0.209	-
3	1	-1.04 $\pm$ 0.19	-2.24 $\pm$ 0.15	-	4.40 $\pm$ 0.16	5.75 $\pm$ 0.28	-
	2	-3.45 $\pm$ 0.61	-0.391 $\pm$ 0.07	4.19 $\pm$ 0.44	3.90 $\pm$ 0.55	4.77 $\pm$ 0.38	-
	3	-1.13 $\pm$ 0.24	-0.161 $\pm$ 0.51	-	4.26 $\pm$ 0.32	4.94 $\pm$ 0.13	-
	Avg	-1.87	-0.930	-	4.19	5.15	-
	2ΔC_t	3.66	1.91	-	0.055	0.028	-
	2$\Delta\Delta C_t$	1.00	0.520	-	1.00	0.513	-
5	1	4.23 $\pm$ 0.46	2.86 $\pm$ 0.41	-	3.85 $\pm$ 1.24	5.22 $\pm$ 0.49	-
	2	4.03 $\pm$ 0.64	3.92 $\pm$ 0.67	-	4.09 $\pm$ 0.75	-	1.62 $\pm$ 3.23
	3	5.28 $\pm$ 0.52	4.00 $\pm$ 2.61	-	3.74 $\pm$ 0.30	6.12 $\pm$ 2.55	-
	Avg	4.51	3.59	-	3.89	5.67	-
	2ΔC_t	0.044	0.083	-	0.067	0.02	-
	2$\Delta\Delta C_t$	1.00	1.89	-	1.00	0.291	-
7	1	4.14 $\pm$ 0.65	4.36 $\pm$ 0.51	4.64 $\pm$ 0.99	3.63 $\pm$ 0.61	7.25 $\pm$ 0.31	-
	2	4.28 $\pm$ 0.22	4.94 $\pm$ 0.45	5.16 $\pm$ 2.89	4.49 $\pm$ 0.19	7.79 $\pm$ 0.51	-
	3	5.04 $\pm$ 0.27	3.86 $\pm$ 0.37	-	4.20 $\pm$ 0.33	6.93 $\pm$ 0.55	-
	Avg	4.49	4.39	4.90	4.11	7.32	-
	2ΔC_t	0.045	0.048	0.034	0.058	0.006	-
	2$\Delta\Delta C_t$	1.00	1.07	0.752	1.00	0.108	-

*Target gene C_t >38, therefore not possible to calculate ΔC_t

On solid agar medium, *F. verticillioides* grows sufficiently within 7 days to determine gene expression (Fanelli *et al.*, 2013); however, the growth of the fungus is slower in nutrient-poor liquid media, therefore requiring a longer incubation time (typically

up to 28 days (López-Errasquín *et al.*, 2007). The *FUM* genes were also not detected in the fungi exposed to 400 µL/L lemongrass oil over the period of 7 days, which is foreseeable, as there was no visible fungal growth at this concentration. For the reference culture, MRC 826, there was an increase in the expression of both *FUM1* and *FUM21* over time. However, this trend was not observed for FM 9. In the follow-up experiment, the incubation time was extended to 28 days and the effects of 100 µL/L (25% of the MIC) and 200 µL/L (50% of the MIC) lemongrass EO on the *FUM* gene expression in the two isolates (MRC 826 and FM 9) were determined (Tables 6.11 and 6.12).

Table 6.11: Effect of lemongrass essential oil on *FUM1* and *FUM21* gene expression in *Fusarium verticillioides* isolate MRC 826 over 28 days

Time (days)	Rep	<i>FUM1</i> (ΔC_t)			<i>FUM21</i> (ΔC_t)		
		Control	100 µL/L	200 µL/L	Control	100 µL/L	200 µL/L
3	1	0.072±0.40	2.53±0.64	3.07±0.59	2.34±0.32	-	4.24±0.62
	2	1.66±0.35	0.636±0.210	3.47±0.64	2.77±0.16	1.86±0.16	3.65±0.56
	3	2.37±0.43	4.12±0.46	2.58±0.45	3.21±0.43	2.74±0.13	-
	Avg	1.37	2.43	3.04	2.77	2.30	3.95
	2ΔC_t	0.388	0.185	0.121	0.146	0.203	0.065
	2$\Delta\Delta C_t$	1.00	0.478	0.313	1.00	1.39	6.84
7	1	3.98±1.09	2.10±0.17	4.77±0.15	3.17±0.97	4.31±0.19	6.26±0.14
	2	6.60±0.30	2.50±0.14	1.50±0.09	4.37±0.18	4.61±0.22	4.86±0.12
	3	5.36±0.17	7.02±0.16	3.23±0.16	4.94±0.06	4.07±0.18	6.42±0.77
	Avg	5.31	3.87	3.16	4.16	4.33	5.85
	2ΔC_t	0.25	0.068	0.112	0.056	0.050	0.017
	2$\Delta\Delta C_t$	1.00	2.71	4.43	1.00	0.888	0.209
14	1	2.57±0.10	0.937±0.38	-2.95±0.38	3.66±0.12	2.79±1.74	-1.31±0.18
	2	3.89±0.82	-2.37±0.24	-2.67±0.21	2.15±0.34	1.50±0.24	-3.58±0.22
	3	5.60±2.5	4.93±0.14	-3.20±0.18	1.81±0.93	3.04±0.06	-3.05±0.80
	Avg	4.04	1.16	-2.94	2.54	2.44	-2.65
	2ΔC_t	0.062	0.466	7.67	0.172	0.184	6.27
	2$\Delta\Delta C_t$	1.00	7.22	124	1.00	1.07	48.2
21	1	0.449±0.69	1.65±0.36	2.33±0.62	-	3.91±0.28	-
	2	-	2.38±0.81	0.007±0.80	1.58±0.94	2.70±0.34	1.44±1.18
	3	-	-	-0.580±2.38	1.97±1.47	-	-
	Avg	-	2.02	0.59	1.78	3.31	-
	2ΔC_t	-	0.247	0.667	0.292	0.101	-
	2$\Delta\Delta C_t$	-	-	-	1.00	0.35	-
28	1	5.33±0.480	5.47±2.68	2.21±0.56	3.62±0.33	2.36±1.53	0.617±0.22
	2	5.73±0.73	4.75±0.92	1.20±0.42	2.61±0.23	2.27±0.67	0.850±0.74
	3	4.52±0.25	4.75±0.66	2.38±0.28	4.71±0.95	2.98±0.84	0.457±0.33
	Avg	5.19	4.99	1.93	3.65	2.54	0.64
	2ΔC_t	0.027	0.031	0.262	0.080	0.172	0.641
	2$\Delta\Delta C_t$	1.00	1.15	9.61	1.00	2.16	9.13

*Target gene $C_t > 38$, therefore not possible to calculate ΔC_t

The highest expression of the *FUM1* and *FUM21* genes in isolate MRC 826 occurred at an incubation time of 14 days in the 200 μ L/L lemongrass treatment. For isolate FM 9 the highest gene expression was also observed at 14 days incubation time, but in the 100 μ L/L treatment (Table 6.12).

Table 6.12: Effect of lemongrass essential oil on *FUM1* and *FUM21* gene expression in *Fusarium verticillioides* isolate FM 9 over 28 days

Time (days)	Rep	<i>FUM1</i> (ΔC_t)			<i>FUM21</i> (ΔC_t)		
		Control	100 μ L/L	200 μ L/L	Control	100 μ L/L	200 μ L/L
3	1	4.48 $\pm$ 0.27	9.50 $\pm$ 0.21	6.18 $\pm$ 0.45	2.90 $\pm$ 0.27	5.98 $\pm$ 0.13	6.86 $\pm$ 0.52
	2	8.71 $\pm$ 0.33	5.92 $\pm$ 0.32	5.49 $\pm$ 0.33	6.54 $\pm$ 0.10	7.46 $\pm$ 0.42	6.49 $\pm$ 0.44
	3	6.01 $\pm$ 0.39	10.0 $\pm$ 0.45	5.52 $\pm$ 0.64	3.33 $\pm$ 0.11	7.34 $\pm$ 0.17	7.06 $\pm$ 1.14
	Avg	6.40	8.47	5.73	4.26	6.93	6.80
	2ΔC_t	0.012	0.003	0.019	0.052	0.008	0.009
	2$\Delta\Delta C_t$	1.00	0.24	1.60	1.00	0.16	0.14
7	1	5.86 $\pm$ 0.47	4.91 $\pm$ 0.58	6.84 $\pm$ 0.58	6.96 $\pm$ 0.46	7.88 $\pm$ 0.72	6.73 $\pm$ 0.17
	2	6.69 $\pm$ 0.26	9.55 $\pm$ 0.31	5.57 $\pm$ 0.58	7.67 $\pm$ 0.28	7.86 $\pm$ 0.13	7.16 $\pm$ 0.71
	3	8.18 $\pm$ 0.63	8.66 $\pm$ 0.16	6.35 $\pm$ 0.24	7.78 $\pm$ 0.43	8.04 $\pm$ 0.13	8.30 $\pm$ 0.36
	Avg	6.91	7.71	6.25	7.47	7.93	7.40
	2ΔC_t	0.008	0.005	0.013	0.006	0.004	0.006
	2$\Delta\Delta C_t$	1.00	0.577	1.58	1.00	0.727	0.560
14	1	7.52 $\pm$ 0.49	6.91 $\pm$ 0.95	5.16 $\pm$ 0.99	5.64 $\pm$ 0.30	5.85 $\pm$ 0.41	5.90 $\pm$ 0.98
	2	8.23 $\pm$ 0.62	4.30 $\pm$ 1.42	5.38 $\pm$ 1.12	8.16 $\pm$ 0.53	4.34 $\pm$ 1.85	5.11 $\pm$ 0.64
	3	8.48 $\pm$ 0.89	6.18 $\pm$ 0.30	7.81 $\pm$ 0.63	6.62 $\pm$ 0.25	5.65 $\pm$ 1.27	6.10 $\pm$ 0.42
	Avg	8.08	5.90	6.12	6.81	5.28	5.70
	2ΔC_t	0.004	0.018	0.014	0.009	0.026	0.019
	2$\Delta\Delta C_t$	1.00	4.86	3.90	1.00	2.89	1.63
21	1	4.11 $\pm$ 0.60	-	-	4.32 $\pm$ 0.87	-	4.96 $\pm$ 1.19
	2	3.57 $\pm$ 0.96	-	-	6.26 $\pm$ 0.71	6.21 $\pm$ 1.60	-
	3	-	-	-	-	4.93 $\pm$ 1.02	-
	Avg	3.84	-	-	5.29	5.57	-
	2ΔC_t	0.070	-	-	0.026	0.021	-
	2$\Delta\Delta C_t$	1.00	-	-	1.00	0.83	-
28	1	-	8.02 $\pm$ 2.37	5.57 $\pm$ 0.63	1.38 $\pm$ 0.50	2.85 $\pm$ 0.48	1.81 $\pm$ 0.62
	2	-	8.92 $\pm$ 0.17	7.18 $\pm$ 0.72	3.23 $\pm$ 0.37	1.91 $\pm$ 0.19	3.61 $\pm$ 0.31
	3	-	-	5.10 $\pm$ 0.52	2.63 $\pm$ 0.44	2.46 $\pm$ 0.31	3.71 $\pm$ 0.16
	Avg	-	8.47	5.95	2.41	2.41	3.04
	2ΔC_t	-	0.003	0.016	0.188	0.189	0.121
	2$\Delta\Delta C_t$	-	-	-	1.00	1.00	0.41

*Target gene $C_t > 38$, therefore not possible to calculate ΔC_t

Fumonisin production by *Fusarium* spp. is regulated by the fumonisin biosynthetic gene cluster (*FUM*) (Proctor *et al.*, 2013). The *FUM1* gene encodes the polyketide synthase enzyme, which is responsible for the synthesis of the linear polyketide backbone structure of the fumonisin molecule (Rocha *et al.*, 2015; Medina *et al.*, 2016). The backbone is subsequently modified by other enzymes, namely amino transferase (*FUM8*), C-3 carbonyl reductase (*FUM13*) and cytochrome P450 oxygenase (*FUM2*, *FUM3*, *FUM6* and *FUM15*). Tricarballic acid ester esterification is regulated by *FUM7*, *FUM10*, *FUM11*, *FUM14* and *FUM16*, whereas *FUM17*, *FUM18* and *FUM19* are longevity assurance factors. *FUM21* is a DNA-binding transcription factor which regulates *FUM* gene expression. Currently there is no specific information available in literature on the effect of lemongrass EO on *FUM* gene expression. With regard to the effect of EOs on genes coding for other mycotoxins, there are reports that thyme oil (at the MIC) down-regulated the expression of the *Tri4* gene in *F. oxysporum* (Divband *et al.*, 2017) and the EOs of fennel, cardamom, chamomile, rosemary, anise and celery reduced ochratoxin A production and the expression of the genes associated with its biosynthesis (although there was only slight fungal growth reduction) (El Khoury *et al.*, 2016). The relationship between the relative expression of *FUM1* and *FUM21* genes and fumonisin production by the isolates MRC 826 and FM 9 in the current experiment is illustrated in Figure 6.6.

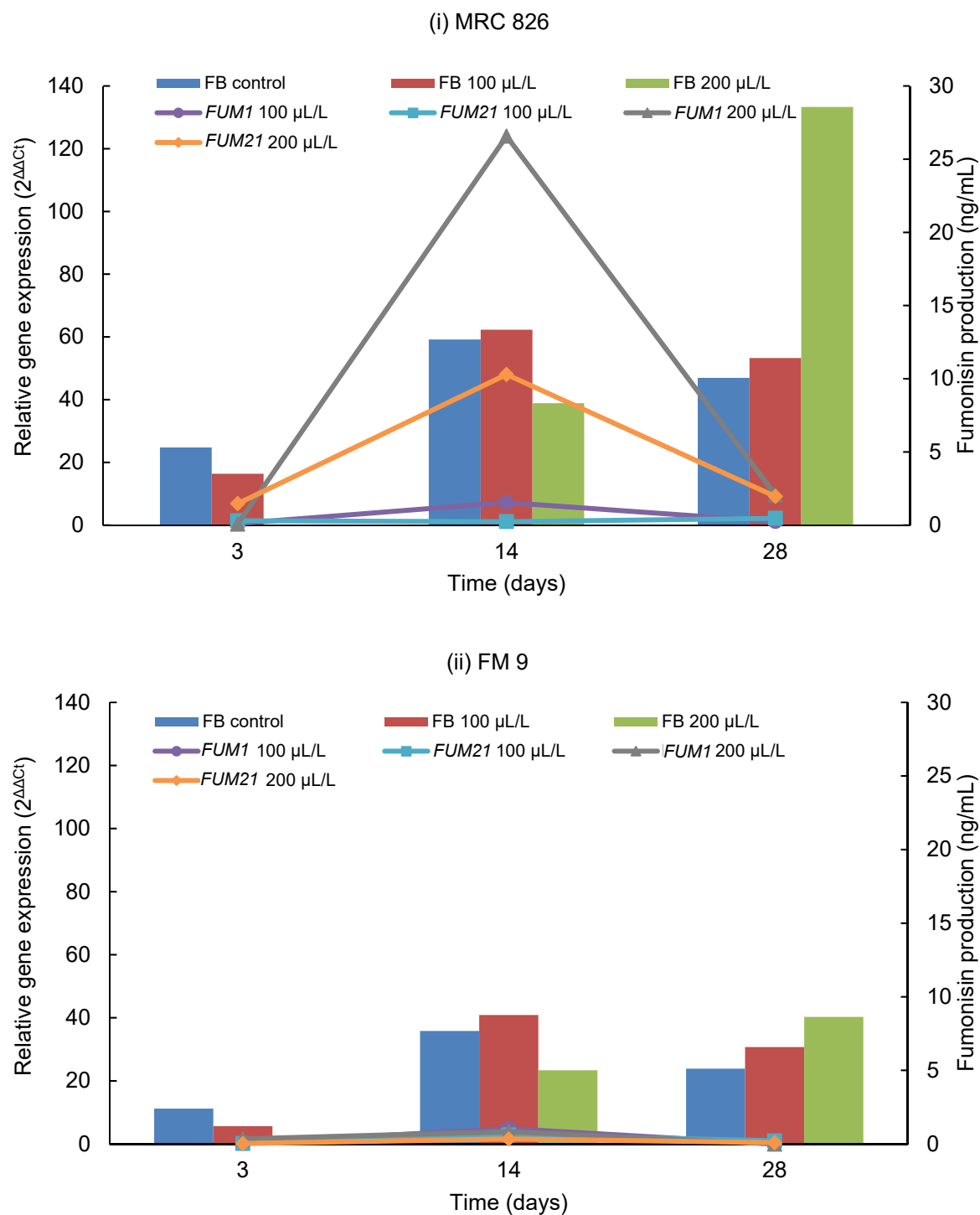


Figure 6.6: Effect of lemongrass essential oil on fumonisin (FB) production and mean relative expression of *FUM1* and *FUM21* genes in *Fusarium verticillioides* isolates MRC 826 (i) and FM 9 (ii)

The results demonstrate that initially, on Day 3, the 200 μ L/L lemongrass treatment prevented fumonisin production in both isolates and that the controls had slightly higher fumonisin levels than the 100 μ L/L lemongrass treatments. There was a marked increase in fumonisin production and *FUM* gene expression at Day 14 for

both isolates, although the values were higher for MRC 826 than FM 9. At 28 days, in both isolates, the fumonisin content in the 200 μ L/L lemongrass treatment increased to such an extent that it became higher than the control. The treatment with 200 μ L/L lemongrass resulted in final FB concentrations of 28.6 ng/mL and 8.62 ng/mL in isolates MRC 826 and FM 9, respectively. Concentrations of EOs lower than the MIC may induce stress, leading to increased toxin production by the fungus (Kumar *et al.*, 2016). Furthermore, growth inhibition and fumonisin production is not necessarily correlated. One study found that, although lemongrass EO was an effective inhibitor of *F. verticillioides* growth, it only inhibited fumonisin production at a water activity of 0.995 and temperature of 30 °C and under certain conditions toxin production was actually stimulated (Velluti *et al.*, 2004b). In the current study, the addition of lemongrass EO at concentrations lower than the MIC also stimulated toxin production over time.

The growth of toxigenic *Fusarium* spp. and the production of fumonisin mycotoxins are multifaceted biological processes that are affected by interactions between several abiotic and biotic factors (Gong *et al.*, 2015). It is well-known that the amount of genomic DNA correlates with the concentration of mycotoxins. For example, Ncube *et al.* (2011) reported a positive correlation ($r = 0.93$) between the target DNA (specifically *FUM1*) of fumonisin-producing *Fusarium* spp. determined by the real-time polymerase chain reaction and the concentration of fumonisins in maize (Ncube *et al.*, 2011). However, gene expression is more complex, since it is regulated by various specific transcription factors for the RNA polymerase binding to promoters (Gong *et al.*, 2015). Under some conditions (e.g. pH, water activity and salinity), the expression of *FUM1* and *FUM21* genes is reported to mirror fumonisin production (Fanelli *et al.*, 2013). In the current study, a direct relationship between the expression of the *FUM1* and *FUM21* genes and fumonisin production over time was not established. The highest gene expression was observed at 14 days, and the highest fumonisin content only at 28 days. However, the time window of transcription may not coincide with toxin production and it is possible that post-transcriptional regulation could modulate fumonisin production (Fanelli *et al.*, 2013).

Fumonisin-producing *Fusarium* spp. are adaptable to a broad range of environmental conditions (Fanelli *et al.*, 2013). In order to develop strategies to

reduce the presence of fumonisins in maize and manage the associated human health risk, a better understanding of the biosynthetic mechanism is required (Brown *et al.*, 2007). The results of the current study revealed that the concentration of EO and incubation period influence the expression of genes that code for fumonisin production.

6.4 CONCLUSIONS

A total of 25 *Fusarium* isolates were obtained from soil and maize samples. The soil did not contain any fumonisin-producing fusaria, as only *F. equiseti*, *F. solani* and *F. brachygibbosum* were detected. Two species of *Fusarium* were isolated from maize, namely *F. oxysporum* and *F. verticillioides*; with *F. verticillioides* being the most prevalent. The ability of lemongrass oil to control the growth of *Fusarium* spp. associated with maize was confirmed. At a concentration of 400 $\mu\text{L/L}$, lemongrass oil was able to prevent the growth of 18 *Fusarium* cultures isolated from maize and soil, as well as the reference culture MRC 826. The expression of *FUM1* and *FUM21* genes, which are involved in the biosynthesis of fumonisins, were also prevented at the MIC. Concentrations lower than the MIC (100 $\mu\text{L/L}$ and 200 $\mu\text{L/L}$) resulted in an increase in *FUM1* and *FUM21* gene expression and after 28 days the highest fumonisin concentration (28.6 ng/mL) was detected in the treatment with 200 $\mu\text{L/L}$ (MRC 826 isolate). The results obtained are of concern since they imply that EO treatments aimed at controlling pathogenic fungi may actually stimulate mycotoxin production in fungi that survive. This could hold health risks for consumers. Future work may include an investigation of environmental factors, such as temperature and/or water activity, which could influence the anti-mycotoxigenic activity of EOs.

CHAPTER 7

GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 ACHIEVEMENT OF OBJECTIVES

This study aimed to gain a better understanding of the risks to the health of humans and animals associated with fumonisins in South African maize. All the objectives as set out in Chapter 1, to achieve the aim, were accomplished. The first objective was to evaluate the RTL-W1 trout liver cell line and the zebrafish embryo as models to assess fumonisin toxicity. The study demonstrated that FB₁ exhibited a very low cytotoxicity towards the RTL-W1 cell line when tested without pH adjustment (IC₅₀ 1 260 µg/mL) and no cytotoxicity when the pH was adjusted to physiological conditions. The mycotoxins FB₁, FB₂, FB₃ and HFB₁ were not genotoxic towards the RTL-W1 liver cell line. The zebrafish embryo test was used to complement cell-based assays for the determination of the acute toxicity of FB. The FET also yielded high LC₅₀ values for FB₁ (765 and 277 mg/L at 48 and 96 h, respectively), reflecting a low acute toxicity. For FB₁ solutions with the pH adjusted to physiological conditions, the LC₅₀ could only be obtained after 96 hpf using the concentrations tested and the value (682 mg/L) was 2.5 times higher than the corresponding concentration without pH adjustment (277 mg/L). This indicates that the acute toxicity towards zebrafish embryos was reduced, but not eliminated when adjusting the pH. In the zebrafish FET assay, HFB₁ proved to be less toxic than FB₁. The teratogenicity index for FB₁ (with and without pH adjustment) and for HFB₁ (without pH adjustment) were <2, indicating a lack of teratogenicity towards zebrafish embryos. The results of the current study demonstrate the importance of pH in toxicity testing. The hypothesis that the FET is a suitable toxicity screening tool for fumonisins is confirmed.

The second objective was to investigate the interaction of FB with maize and maize macrocomponents (starch, protein and their respective monomers) and the formation of cryptic fumonisins under different pH and temperature conditions. None of the treatments (pH 4, 7 and 10) or temperatures (30 and 90 °C) resulted in a

significant decrease in the recovery of FB₁ from starch, glucose, zein or maize meal spiked with FB. These results confirm the stability of FB₁ in maize porridge and thereby support the findings of Shephard and co-workers (Shephard *et al.*, 2002; Shephard *et al.*, 2012). Although the recoveries of FB₁ from the mixture of amino acids and from glutamic acid were low, the UPLC-QToF-HDMS results demonstrated that no modified fumonisins were formed in the presence of glutamic acid at ambient temperature. The hypothesis that fumonisins will react with maize and maize macrocomponents to form masked/cryptic fumonisins is therefore rejected.

The third objective was to assess the *in vitro* bioaccessibility of fumonisins from South African maize porridge. This was achieved by subjecting fumonisin-contaminated stiff maize porridge to an *in vitro* digestion procedure using the TNO Gastrointestinal Model of the stomach and small intestine (tiny-TIM). The results demonstrate that FB₁, FB₂ and FB₃ are released rapidly from stiff maize porridge made from naturally contaminated maize meal. Within the first 120 min, 47% of the FB₁ and 59% FB₂ and FB₃ were released from the maize porridge and detected in the dialysate. After 360 min, 68%, 94% and 87% of FB₁, FB₂ and FB₃, respectively, were bioaccessible, thereby supporting the hypothesis that FB has a high bioaccessibility. The results of the current study are also in agreement with the findings of a study in which a dynamic *in vitro* digestion procedure simulating the digestive tract of pigs was used to analyse mycotoxin-contaminated feed containing maize and wheat (Avantaggiato *et al.*, 2007). The fact that the FB in contaminated maize porridge become available for absorption rapidly during digestion, implies that it is likely that the intestine could be exposed to high concentrations of FB after consuming porridge made from contaminated maize meal. It confirms that the low bioavailability is not due to strong association of the toxin with the intestinal content, but that it is in fact rapidly released from maize porridge.

The fourth and final objective was to evaluate the potential of selected EOs for the biocontrol of soil and maize *Fusarium* spp. A total of 25 *Fusarium* isolates were obtained from soil and maize samples. The soil did not contain any fumonisin-producing fusaria, as only *F. equiseti*, *F. solani* and *F. brachygibbosum* were detected. Soil analysis confirmed that the samples represented a variety of textural

classes, and that there were differences in the pH and the trace mineral composition of the soil samples collected from different locations. Two species of *Fusarium* were isolated from maize, namely *F. oxysporum* and *F. verticillioides*, with *F. verticillioides* being the most prevalent. Lemongrass EO was more efficient than spearmint and thyme in inhibiting the growth of the *Fusarium* spp. *in vitro*. At a concentration of 400 $\mu\text{L/L}$, lemongrass oil was able to prevent the growth of 18 *Fusarium* cultures isolated from maize and soil, as well as the reference culture MRC 826. This confirms the hypothesis that plant EOs are effective fungicides against *Fusarium* spp. isolated from maize and soil. The expression of *FUM1* and *FUM21* genes, which are involved in the biosynthesis of fumonisins, was also prevented at the MIC. However, exposure to concentrations lower than the MIC (100 $\mu\text{L/L}$ and 200 $\mu\text{L/L}$) resulted in an increase in *FUM1* and *FUM21* gene expression, and after 28 days, the highest fumonisin concentration (28.6 ng/mL) was detected in the treatment with 200 $\mu\text{L/L}$ (MRC 826 isolate).

7.2 CONTRIBUTION OF THE STUDY

This is the first study in which the zebrafish FET was used to assess FB toxicity, and the findings have broadened the scientific knowledge concerning the toxic effects of FB and the role of pH in fumonisin toxicity. The current project was also one of only a few fundamental studies which investigated the effect of pH and temperature on the binding of FB to maize and to its macrocomponents during processing systematically under conditions simulating the preparation of traditional maize porridge. Under these conditions, no evidence was found for the formation of modified FB. Another novel contribution was the use of the tiny-TIM dynamic model simulating human digestion to determine the bioaccessibility of FB in maize porridge. The results provide new knowledge and insight into the kinetics of FB release from maize porridge during digestion. The finding that FB is rapidly released from porridge prepared from fumonisin-contaminated maize in the small intestine, thus exposing it to fumonisins, has implications for human and animal health that are not yet understood. This aspect requires more attention. The ability of lemongrass EO to control the growth *Fusarium* spp. associated with maize was confirmed and the effect of lemongrass EO on the expression of *FUM* genes was

determined, which has never been reported before in literature. The increase in fumonisin production when the MRC 826 isolate was exposed to concentrations of lemongrass EO below the MIC is concerning. This finding suggests that fungus surviving after application of the oil may be stimulated to produce higher concentrations of toxic metabolites than would have otherwise been the case. This finding also highlights the fact that more research into the effects of EO application to control mycotoxin-producing fungi is required.

7.3 CRITICAL EVALUATION OF THE STUDY

Although all the objectives set out in Section 1.5 were achieved, the following shortcomings may be identified:

- Due to the cost and lack of availability of fumonisin standards, toxicity testing at higher concentrations was only done for FB₁ and not for FB₂ and FB₃. However, due to the similarity in the chemical structures, it is reasonable to assume that FB₂ and FB₃ will have toxicities in the same range as FB₁ (EFSA, 2018).
- The results of this study did not provide a clear understanding as to why the recoveries of FB₂ and FB₃ from starch, zein and maize meal samples spiked with FB were lower than that of FB₁.

7.4 RECOMMENDATIONS FOR FURTHER RESEARCH

The following recommendations for future research arose from the results of the current study:

- Investigating the hepatotoxicity of FB at sub-lethal concentrations using transgenic zebrafish, in which liver abnormalities can be observed using a fluorescent microscope.
- Determining the ratio of sphinganine to sphingosine in zebrafish embryos after exposure to sub-lethal concentrations of FB.
- Behavioural analysis of zebrafish exposed to doses lower than the LC₅₀ to assess neurotoxicity.
- Studying the toxicity of combinations of mycotoxins, e.g. FB and DON or ZEA which often co-occur in South African maize.
- Determining the bioaccessibility of FB in combined meal, e.g. stiff maize porridge with vegetables.
- Investigating environmental factors, such as temperature and/or water activity, which could influence the anti-mycotoxigenic activity of EOs and the expression of *FUM* genes.
- Application of lemongrass EO as disinfectant in a food processing environment.

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APPENDICES

Appendix 1: UPLC-MS/MS calibration data for fumonisins in different matrices

Matrix	Calibration curve data*					
	FB ₁		FB ₂		FB ₃	
	Equation	R ²	Equation	R ²	Equation	R ²
Neat	y = 20.31x - 4.934	0.9962	y = 15.22x - 8.228	0.9844	y = 17.02x - 7.267	0.9871
Starch	y = 16.82x - 12.36	0.9980	y = 26.61x - 30.44	0.9994	y = 21.63x - 45.97	0.9985
Glucose	y = 18.93x - 10.80	0.9969	y = 23.92x + 0.7319	0.9987	y = 15.67x - 2.672	0.9968
Zein	y = 37.56x - 10.60	0.9973	y = 42.10x - 37.15	0.9983	y = 40.45x - 37.71	0.9969
Amino acid mix**	y = 1.999x - 10.40	0.9632	y = 0.3387x - 1.105	0.8897	y = 1.460x - 12.52	0.9703
Alanine	y = 37.59x - 5.053	0.9977	y = 41.02x + 3.191	0.9954	y = 39.17x - 24.90	0.9981
Glutamic acid**	y = 3.568x - 24.36	0.9676	y = 0.0594x - 0.2152	0.9566	y = 0.4201x - 2.886	0.9737
Leucine	y = 35.33x + 6.153	0.9969	y = 38.34x - 37.42	0.9967	y = 36.27x - 16.86	0.9965
Proline	y = 38.13x + 2.343	0.9972	y = 42.64x + 30.66	0.9974	y = 39.18x + 22.70	0.9989
Maize meal	y = 10.44x + 0.3508	0.9970	y = 17.09x - 10.64	0.9993	y = 14.71x - 14.71	0.9976

*Weighted linear regression (1/x)

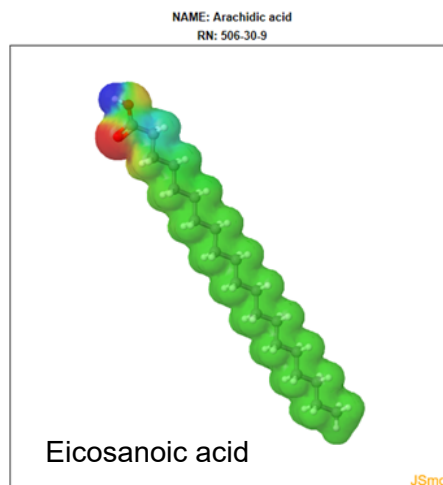
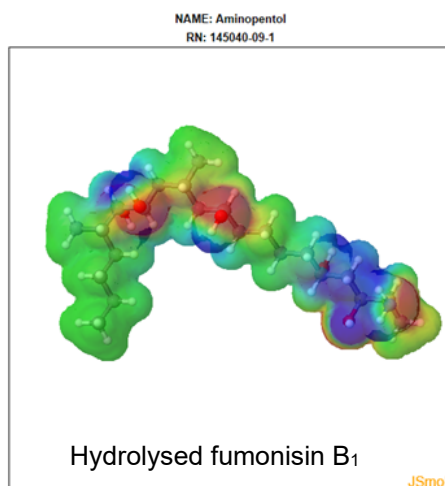
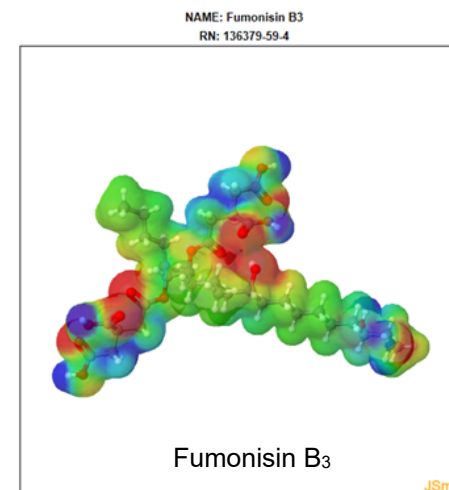
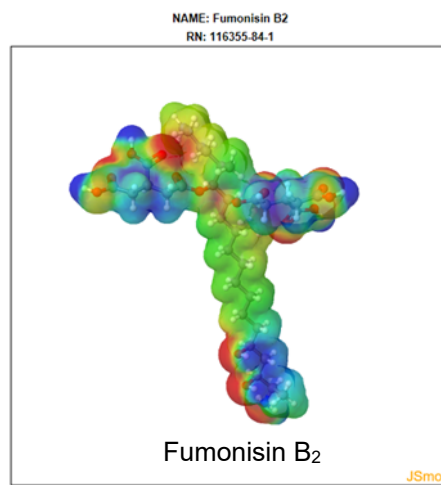
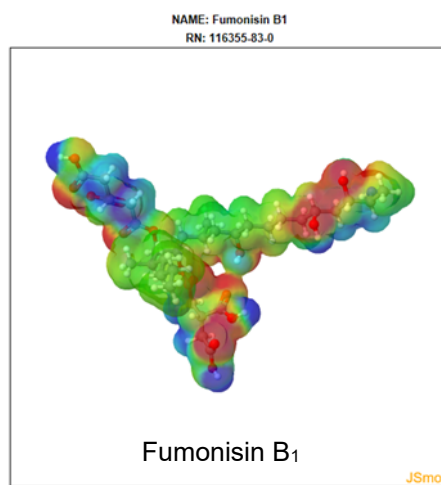
**Calibration data not reliable, some of the data points (especially at lower concentrations) were scattered and therefore excluded from the standard curve

Appendix 2: UPLC-QToF-HDMS calibration data for fumonisins

Mycotoxin	Equation*	R ²
FB ₁	$0.5597x^2 + 22.17x + 0.4162$	0.9996
FB ₂	$0.1416x^2 + 6.141x - 0.2165$	0.9987
FB ₃	$0.6354x^2 + 19.82x + 1.075$	0.9999
HFB ₁	$-0.1268x^2 + 38.38x - 5.298$	0.9975

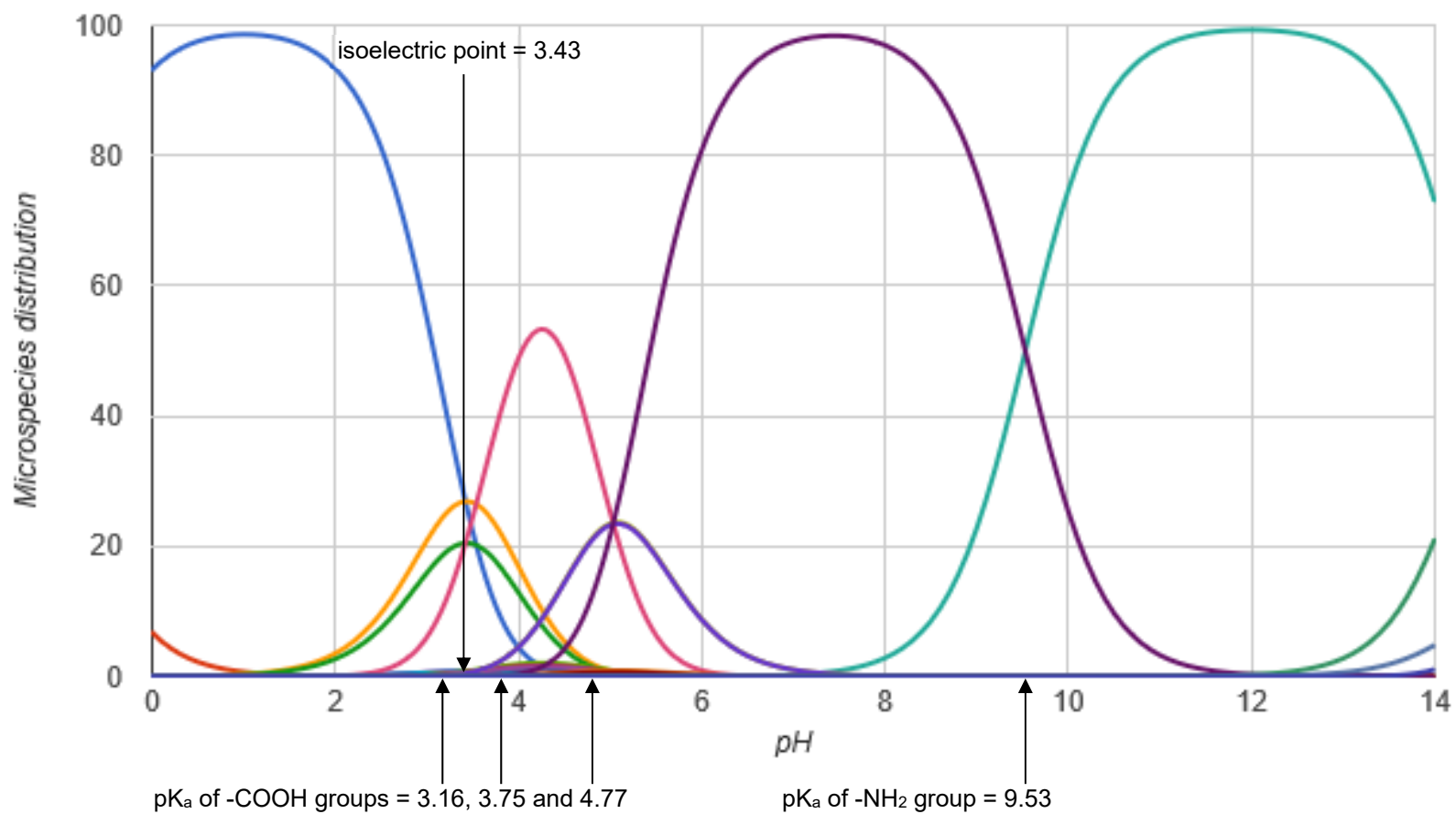
*2nd order curve fitting, no weighting

Appendix 3: The three-dimensional structures of FB₁, FB₂, FB₃ and HFB₁ compared to eicosanoic acid¹³



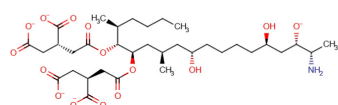
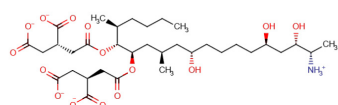
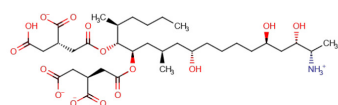
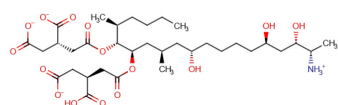
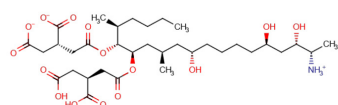
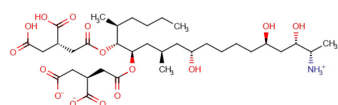
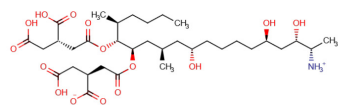
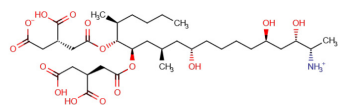
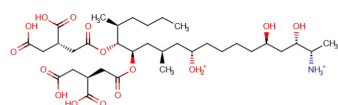
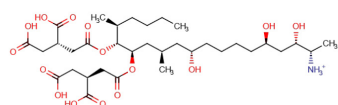
¹³ <https://chem.nlm.nih.gov/chemidplus/> [Accessed 28/02/2018]

Appendix 4: The microspecies distribution of fumonisin B₁ at different pH's¹⁴

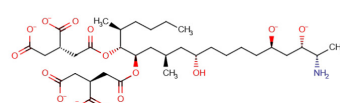
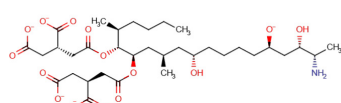
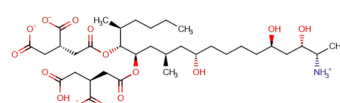
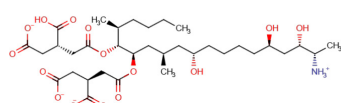
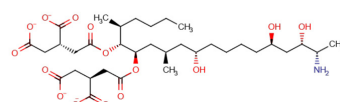
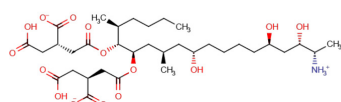
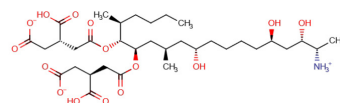
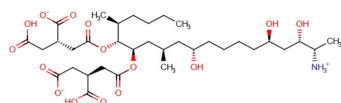
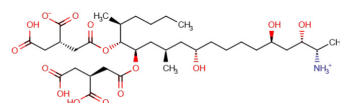
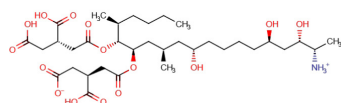


¹⁴ <https://chemicalize.com> [Accessed 06/03/2018]

Appendix 4: The microspecies distribution of fumonisins B₁ at different pH's (continued)



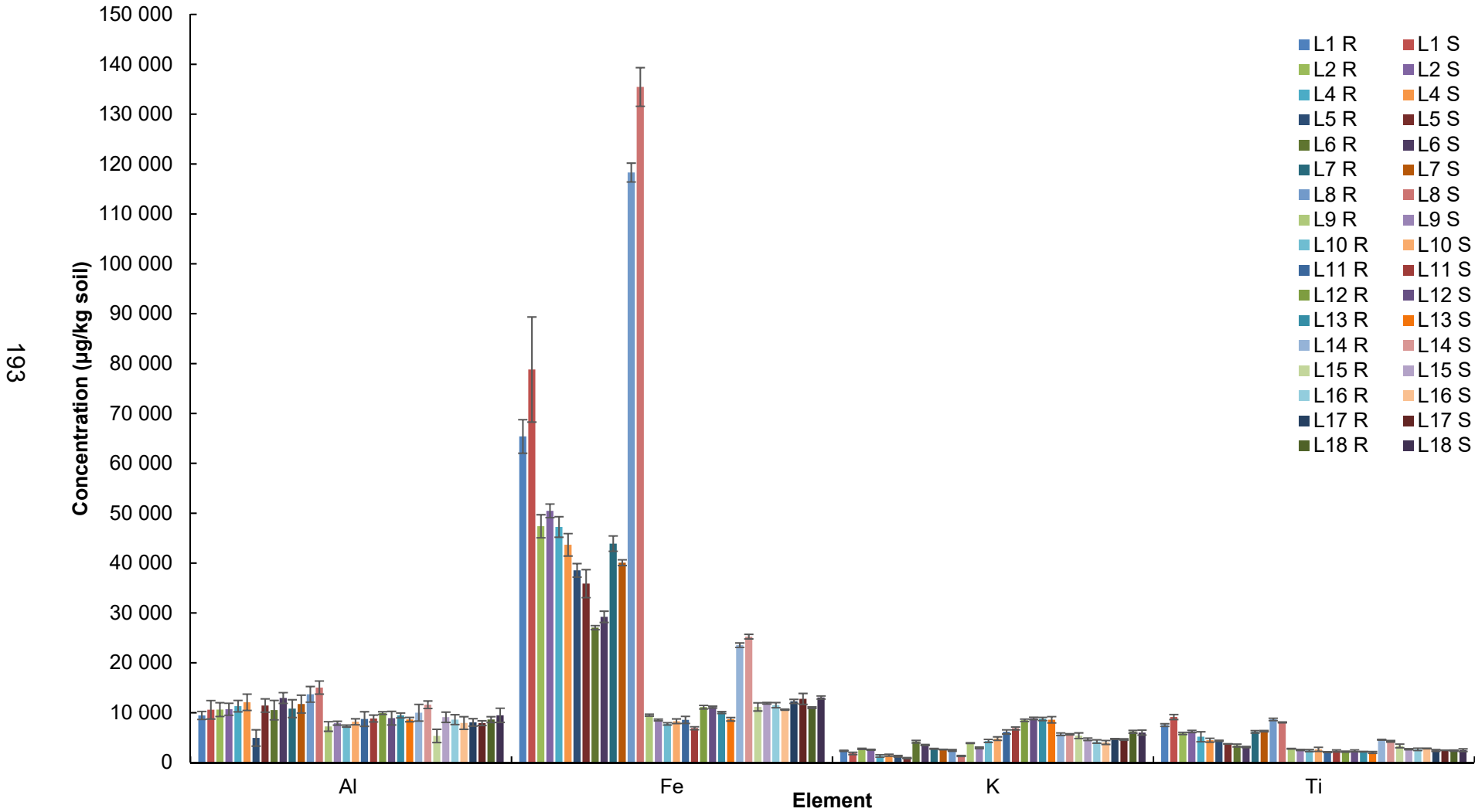
Appendix 4: The microspecies distribution of fumonisins B₁ at different pH's (continued)



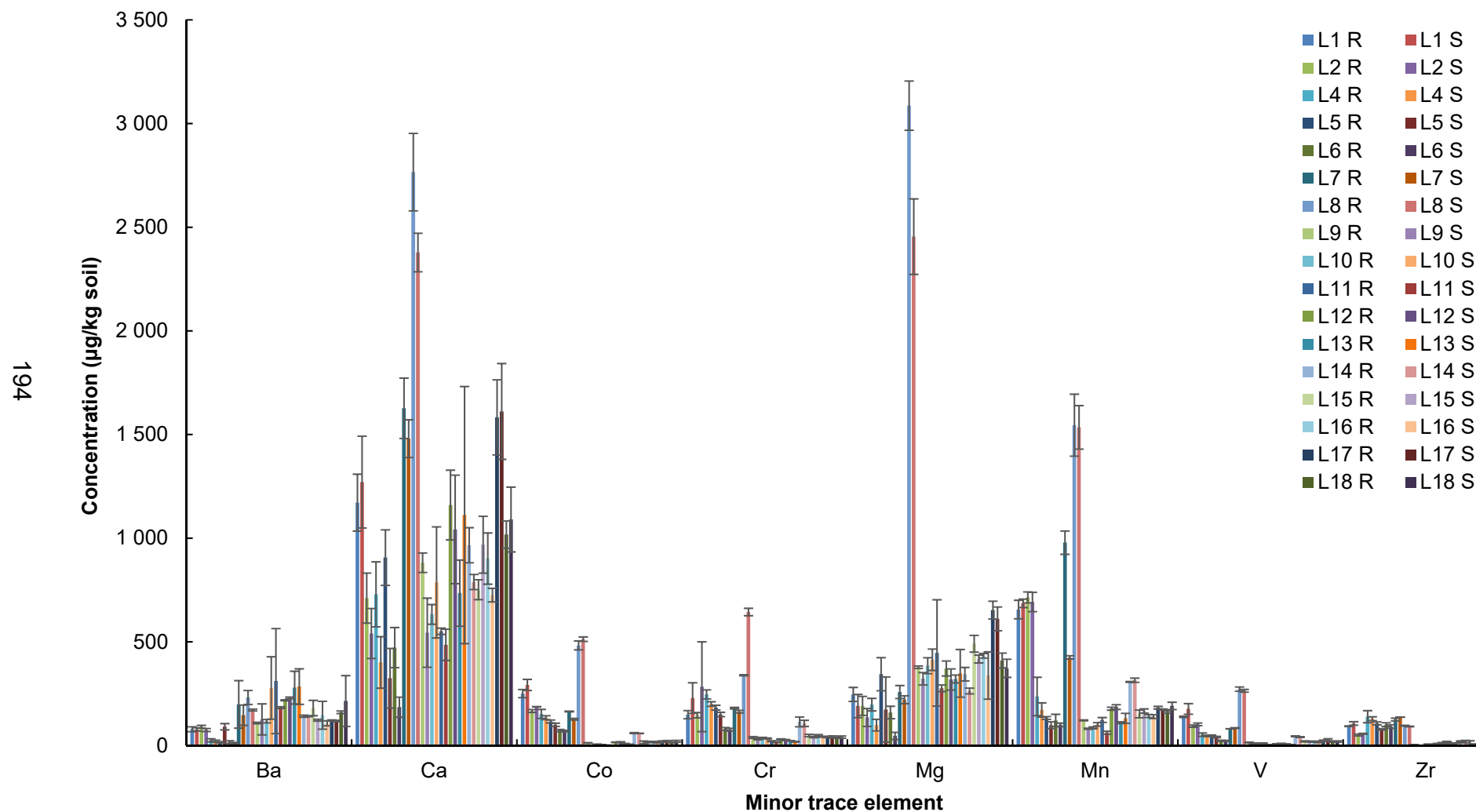
Appendix 5: ICP calibration data for trace elements

Element	Wavelength (nm)	Equation	R ²
Al	394.4	$y = 2\,055x + 2\,276$	0.9916
Ba	455.4	$y = 19\,222x + 6\,750$	0.9916
Ca	393.4	$y = 458\,750x + 313\,003$	0.9909
Co	238.9	$y = 8\,841x + 4\,950$	0.9919
Cr	283.6	$y = 4\,646x + 2\,838$	0.9916
Cu	324.8	$y = 9\,452x + 4\,332$	0.9910
Fe	238.2	$y = 13\,066x + 11\,201$	0.9923
K	766.5	$y = 557x + 910$	0.9898
Mg	280.4	$y = 45\,705x + 25\,301$	0.9916
Mn	257.6	$y = 19\,512x + 10\,983$	0.9913
Na	589.0	$y = 7\,515x + 44\,695$	0.9906
Sr	407.8	$y = 227\,046x + 107\,535$	0.9926
Ti	334.2	$y = 6\,835x + 3\,163$	0.9910
V	292.5	$y = 10\,598x + 4\,690$	0.9918
Zn	206.2	$y = 7\,024x + 7\,803$	0.9896
Zr	343.8	$y = 14\,067x + 6\,460$	0.9909

Appendix 6: Major trace elements in rhizosphere (R) and surface (S) soil from locations (L) in the Mpumalanga and Free State provinces



Appendix 7: Minor trace elements in rhizosphere (R) and surface (S) soil from locations (L) in the Mpumalanga and Free State provinces



Appendix 8: Efficiency of qPCR in *FUM* gene expression experiment

Target gene	Equation	R ²	Reaction efficiency
<i>TUB2</i>	$y = 30.99 - 3.42x$	0.9990	96.06%
<i>FUM1</i>	$y = 35.12 - 3.26x$	0.9820	102.7%
<i>FUM21</i>	$y = 35.48 - 3.22x$	0.9840	104.4%

Appendix 9: Presentations and papers

Oral presentation:

Du Plessis, B., Regnier, T. & Combrinck, S. *Bioaccessibility of fumonisins in maize porridge*. Paper presented at the Innovations in Food Analytics International Conference and Expo, 19-21 September 2018, Munich, Germany.

Poster presentations:

Du Plessis, B., Regnier, T. & Combrinck, S. *Fumonisin interaction with maize and maize macrocomponents under different pH and temperature conditions*. Poster presented at the SAAFoST 22nd Biennial International Congress and Exhibition, 3-6 September 2017, Cape Town, South Africa.

Du Plessis, B., Regnier, T. & Combrinck, S. *Fumonisin interaction with the major amino acids of maize protein*. Poster presented at the NMISA Africa Food Safety Workshop, 4-5 June 2018, Pretoria, South Africa.

Du Plessis, B., Regnier, T., Combrinck, S. Heinrich, P. & Braunbeck, T. *The zebrafish embryo assay and RTL-W1 trout liver cell line as models to assess the toxicity of fumonisins*. Poster presented at the Innovations in Food Analytics International Conference and Expo, 19-21 September 2018, Munich, Germany.