



# **UNDERSTANDING AND TACKLING THE COMPLEXITY OF THE MYCOTOXIN PROBLEM IN SUB-SAHARAN AFRICA: REGULATIONS AND DECONTAMINATION STRATEGIES**



**Limbikani Matumba**

**Promoters**

**Prof. Dr. Sarah De Saeger**

**Dr. Christof Van Poucke**

**2014**

Thesis submitted in fulfillment of the requirements for the Degree of Doctor in Pharmaceutical Sciences

Proefschrift voorgelegd tot het bekomen van de graad van Doctor in de Farmaceutische Wetenschappen





FACULTEIT FARMACEUTISCHE WETENSCHAPPEN

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Cover photo: Map of Africa showing sub-Saharan region (marked by maize and groundnuts; three glass receptors show groundnut oil)-Art by Limbikani Matumba and Jeroen Walravens.

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**“Life is really simple, but we insist on making it complicated”**

**Confucius (551–479 BC)**

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## LIST OF ABBREVIATIONS

<b>Abbreviation</b>	<b>Definition</b>
A/I-ratio	peak area of analyte divided by peak area of internal standard (tailor made abbreviation)
15–AcDON	15–acetyldeoxynivalenol
3–AcDON	3–acetyldeoxynivalenol
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AFM1	aflatoxin M1
AFPA	aspergillus flavus and parasiticus agar
AFs	aflatoxins
ALARA	as low as reasonably achievable
ALT	altenuene
AME	alternariol methyl ether
ANOVA	analysis of variance
AOH	alternariol
BGYF	bright greenish yellow fluorescence
BMDL	benchmark dose lower limit
bw	body weight
C <sub>12</sub> H <sub>22</sub> O <sub>11</sub>	sucrose
CAC	Codex Alimentarius Commission
CAST	Council for Agricultural Science Technology
CIT	citrinin
CO	company
COMESA	Common Market for Eastern and Southern Africa
CONTAM Panel	Panel on Contaminants in the Food Chain
DAS	diacetoxyscirpenol
DOM	deepoxy–deoxynivalenol
DON	deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
ENNs	enniatins
EPA	Extension Planning Area
ESI	electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization
FAS	Foreign Agricultural Service
FB1	fumonisin B1
FB2	fumonisin B2



<b>Abbreviation</b>	<b>Definition</b>
FB3	fumonisin B3
FBs	fumonisin
FLD	fluorescence detection
FUS-X	fusarenon-X
GAP	good agricultural practices
GC	gas chromatography
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis and Critical Control Point
HBV+	hepatitis B virus positive
HCC	hepatocellular carcinoma
HPLC	high performance liquid chromatography
HSD	honestly significant difference
HT2	HT2 toxin
IAC	immunoaffinity-column
IARC	International Agency for Research on Cancer
IFAD	International Fund for Agricultural Development
IQR	interquartile range
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
LTD	limited
M	mole
Max	maximum
min	minutes
MIP	molecular imprinted polymers
ML	maximum level
mL	milliliters
MOEs	margins of exposure
MRM	multiple reaction monitoring
NaCl	sodium chloride
NaHCO <sub>3</sub>	sodium hydrogen carbonate
NEO	neosolaniol
ng	nanogram
NIV	nivalenol
NOAEL	no observed adverse effect level
OECD	Organization for Economic Cooperation and Development
OTA	ochratoxin A

<b>Abbreviation</b>	<b>Definition</b>
OVOP	One Village One Product
PCD	post column derivatization
PDI	probable daily intake
pH	potential hydrogen
PMTDI	provisional maximum tolerable daily intake
ROQ C	roquefortine C
SCF	Scientific Committee for Food of the European Commission
sMRM	scheduled multiple reaction monitoring
SPE	solid-phase extraction
SPSS	Statistical Package for the Social Sciences
STERIG	sterigmatocystin
T2	T2-toxin
TDI	tolerable daily intake
TLC	thin-layer chromatography
TTC	threshold of toxicological concern
U.S.FDA	United States Food and Drug Administration
UNFPA	United Nations Population Fund
USDA	United States Department Of Agriculture
V	volts
WFP	World Food Programme
WHO	World Health Organisation
WTO	World Trade Organixzation
ZAN	zearalanone
ZEN	zearalenone
µg	microgram
µL	microlitre



## **FRAMEWORK AND RESEARCH AIMS**

Maize is a major staple cereal in sub-Saharan Africa. Groundnuts also play an integral role in the livelihoods of many through provision of dietary proteins and income. However, due to climatic conditions in sub-Saharan Africa these commodities are ineluctably contaminated with mycotoxins, the toxic secondary metabolites produced by filamentous fungi. Worse still, in this region, the majority of the population are subsistent consumers who have little or no access to mycotoxin testing. Moreover, discarding a mycotoxin contaminated food lot is not a ready option in Africa due to persistent food shortage. In case of groundnuts, hand sorting is often employed and high quality nuts are often exported to high value markets and the bad nuts are consumed locally. Realizing the dangers of such a practice, recently there have been some efforts to divert bad nuts to vegetable oil production, however elsewhere there have been reports of carry-over of the toxins into the vegetable oil. In this light, efficient and cost-effective mycotoxin decontamination methods are required to salvage contaminated maize at household level and assess the feasibility of using contaminated nuts for vegetable oil production at local industrial level.

### **Aims of the study and work packages**

The aims of this study were two-fold: First, was to further understand the complexity of the problem of mycotoxin contamination of foods in sub-Saharan Africa and secondly was to explore feasible mycotoxin decontamination means particularly for shelled maize. This task was accomplished by:

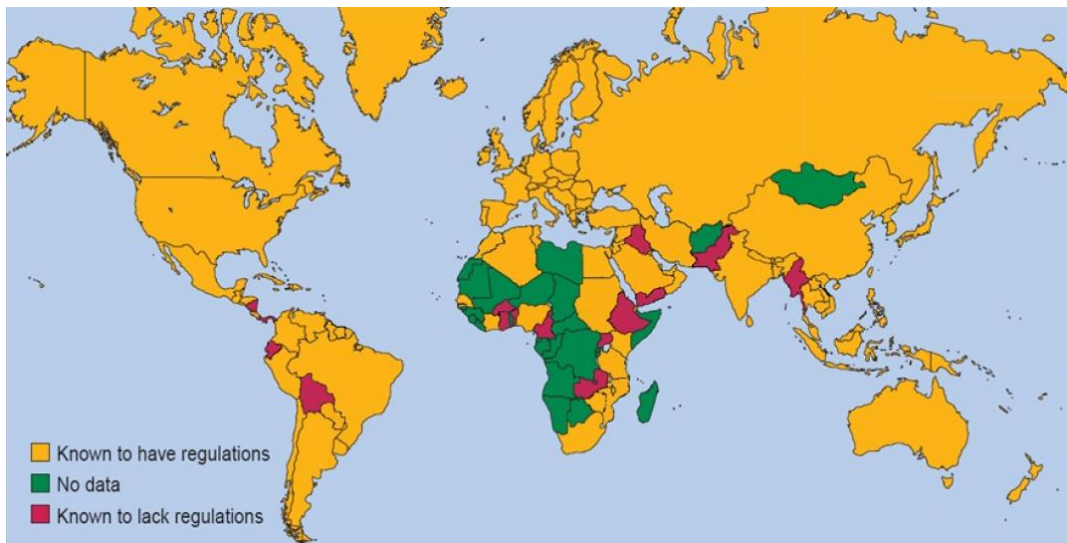
- critically reviewing sub-Saharan Africa legislation of mycotoxins in food with respect to Africa's agrarian setup, food insecurity and analytical challenges (**Chapter 1**)
- studying the influence of micro-climatic conditions on the mycotoxin pattern and exposures using a case study of maize from Malawi (small sub-Saharan country with four distinct climatic zones) (**Chapter 2**)
- performing a meta-analysis of mycotoxin contamination data of locally consumed products and products destined for exports in Malawi (**Chapters 3 and 4**)
- assessing the incidence of mycotoxins in small-scale mechanically pressed groundnut oils (**Chapter 5**)
- studying the effect of a sodium hydrogen carbonate-maize cake baking procedure on the stability of mycotoxins (**Chapter 6**)
- exploring physical mycotoxin decontamination methods for shelled maize (**Chapter 7**).



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## CHAPTER ONE

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Countries known to regulate mycotoxins in food and feed (yellow), those where it is unknown whether regulations exist (green) and known to have no specific regulations (red) (FAO, 2004)

Following the discovery of aflatoxins in the early 1960s, there have been many studies leading to the uncovering of many mycotoxins and the understanding of associated health effects in animals and humans. Consequently, there has been a global increase in the number of countries with mycotoxin regulations in foods. However, many African countries have only regulations for aflatoxins (or a few other mycotoxins) in specific foods or no regulations at all. Chapter 1 critically looks at the challenges thwarting the establishment of mycotoxin regulations and their impacts on human dietary mycotoxin exposure in Africa. In this chapter, the agrarian setup, food insecurity and mycotoxin analytical challenges in African countries are discussed; and more feasible mycotoxin dietary exposure reduction strategies weighed. Chapter 1 is reproduced from a submitted manuscript ‘**Keeping mycotoxins away from the table: Does the existence of regulations have any impact in Africa? (Critical Reviews in Food Science and Nutrition)**’ written by Matumba, L., Van Poucke, C., Ediage, E.N., and De Saeger, S.



## **Chapter 1: Keeping mycotoxins away from the food: Does the existence of regulations have any impact in Africa?**

### **1.1. Introduction**

Fungi are ubiquitous in nature and are capable of colonizing a wide range of substrates including food crops. Under favourable conditions, some of the fungi produce low molecular weight secondary metabolites called mycotoxins. Mycotoxins have been proven under laboratory experiments not to be necessary for the fungi's growth and have been thought to aid in competition against other organisms in the habitat (Shwab and Keller, 2008).

Mycotoxins are generally heat stable and are not destroyed during most normal cooking processes (Bullerman and Bianchini, 2007; Raters and Matissek, 2008 ). They can potentially lead to carcinogenicity, mutagenicity, teratogenicity, oestrogenicity, neurotoxicity and immunotoxicity based on the kind of toxins dose and the sex, health, age and nutritional status of the exposed individual (Hussein and Brasel, 2001; Ready et al., 2010). Acute exposures to mycotoxins can potentially lead to sudden death (Lewis et al., 2005).

Mycotoxins have probably existed since the origin of mankind and have an extremely ancient history (Dugan, 2008) but were not identified and fully characterized until the early 1960s when aflatoxin was first discovered (Lancaster et al., 1961; Nesbit et al., 1962). Since then, there has been an increased attention that has led to the discovery of about 400 more mycotoxins (Hussein and Brasel, 2001; Bennett and Klich, 2003), but there probably do exist thousands. However to date only a few mycotoxins are considered to be of toxicological relevance either because they have been proven so or due to insufficient toxicological data.

Mycotoxins have been classified in many ways by chemists, biologists, biochemists and clinicians based on chemical structure, fungal species that produce them, biosynthetic origins and affected organ respectively (Bennett and Klich, 2003). Some of the most studied mycotoxins (classes) include aflatoxins, fumonisins, zearalenone, trichothecenes, citrinin, ergot alkaloids, ochratoxin and patulin. Recently, the so called 'emerging Fusarium-mycotoxins' which include fusaproliferin, beauvericin, enniatins, and moniliformin have received much attention (Jestoi, 2008). To date, there exist many reviews that cover biosynthesis, the chemistry and toxicokinetics of mycotoxins (Pohland, 1993; Betina, 1989;

Hussein and Brasel, 2001; Bennett and Klich, 2003; Santin, and Diaz, 2005; Bräse et al., 2012). Likewise there are hundreds and thousands of reviews and articles respectively on the occurrence of mycotoxins in several food commodities on regional and global levels.

### **1.2. Production of mycotoxins in food**

The formation of mycotoxins depends on the interaction of several factors such as nutritional composition of the substrate (Luchese and Harrigan, 1993; Abbas et al., 2009), genetic susceptibility of the host plant or commodity to fungi infestation (Brown et al., 1999; Munkvold, 2003; Somers et al., 2003), moisture content, humidity, water activity ( $a_w$ ), aeration, temperature, pH value (Marin et al., 1995; Dorner et al., 1989), fungal populations (Cotty and Mellon, 2006), physical damage of grain due to insect pests and other stress factors (Widstrom, 1979; Schatzki and Ong, 2001). Climate is thought to be the most critical driving factor for fungal colonization and mycotoxin production as it has influence on most of the factors listed above (Paterson and Lima, 2010; Magan et al., 2011). Mycotoxin contamination in food may occur while crops are in the fields, during storage or processing or may be carried-over into milk, meat or eggs, when farm animals are fed on mycotoxins contaminated feed (Gareis and Wolff, 1999; MacLachlan, 2011).

### **1.3. Prevention and control of mycotoxin contamination in food**

There are many pre- and post- harvest prevention strategies for mycotoxins in several crops (Bruns, 2003; Magan and Aldred, 2007; Choudhary and Kumari, 2010; Guo et al., 2009; Chulze, 2010; Lehoczki-Krsjak et al., 2010). These strategies are based on good agricultural practices (GAP) which represent the primary line of defense against contamination of food commodities with mycotoxins while in the field, followed by the implementation of good manufacturing practices (GMP) during the handling, storage, and distribution of the food commodities. Complementary to this management system is Hazard Analysis and Critical Control Point (HACCP), a preventive system based on the systematic identification of hazards, establishing controls and monitoring these controls (Park et al., 1999). To this effect, the Codex Alimentarius Commission (CAC), an intergovernmental body established to implement the Joint FAO/WHO Food Standards Programme, has developed several codes of practice for the reduction of mycotoxins in a range of food commodities (CAC, 2003, 2004, 2009, 2013). However, it is impractical to totally preclude mycotoxin contamination because, as already highlighted, most critical factors for mycotoxin production are extrinsic to man's

control. Consequently there are continued reports of incidences of mycotoxin contamination in various food commodities particularly in the tropics where high ambient humidity makes the control of commodity moisture difficult (Chulze, 2010). Besides this in some parts of Africa there are additional compounding social factors such as theft of food commodities while still standing in the fields (McCall, 1985) which may compel farmers to harvest and store products before adequate drying thus increasing the risk of fungal colonization and mycotoxin contamination.

#### **1.4. Managing mycotoxin contaminated commodities in a food chain**

Although mycotoxins can never be completely removed from the food supply, it is possible to keep the levels low. This could be achieved by physical, chemical and microbiological decontamination strategies which have been extensively explained (Park, 1993; Charmley et al., 1994; Bata and Lásztity, 1999; Galvez et al., 2002; Galvez et al., 2003; Siwela et al., 2005; Van der Westhuizen, 2011; Grenier et al., 2014).

The other way of achieving this is through institutionalisation of mycotoxin regulations. By 2003, about 100 countries, including fifteen African countries, had mycotoxin regulations for at least the aflatoxins (FAO, 2004). By early 2007, the European Union (EU) had implemented the most extensive and detailed regulations for food mycotoxins worldwide that encompassed 13 different mycotoxins or groups and for 40 different food combinations (Van Egmond et al., 2007). Due to complexity of obtaining similar data for Africa, the exact state of affairs remains unknown. However, in several recent publications, authors report the inexistence of regulations for specific mycotoxins (eg fumonisins, zearalenone, ochratoxin, deoxynivalenol) in their countries and made reference to maximum tolerable guidelines set by international food safety bodies or regulations set by other states (Kimanya et al., 2010; Warth et al., 2012; Mohale et al., 2013; Matumba et al., 2014a, Adetunji et al., 2014; Ediage et al., 2014), an indication of little or no improvement at all as regards to the mycotoxin regulatory situation reported in 2003. Perhaps what is also interesting is the absence of fumonisin regulations in South Africa, a country where fumonisin research was pioneered and where there is a clear evidence of high dietary exposures from maize (Dutton, 2003; Shephard et al., 2007; Shephard, 2013; Leroux, 2014). What could be the reasons?

Scientific as well as socio-economic factors are considered when establishing mycotoxin regulation and these include 1) toxicological data, 2) exposure data, 3) availability of analytical facilities and methods, 4) distribution of mycotoxins within a lot, 5) food sufficiency, and 6) legislation in other countries with which trade contacts exist (FAO, 2004). Equally important is the influence of societal set-up (industrial or agrarian) and the food supply system. The subsequent sections critically analyse these factors to understand the disparity between Europe's and Africa's scenarios as regards to institutionalization of mycotoxin regulations and further examine the impact of existing regulations in Africa.

#### *1.4.1. Toxicological data on mycotoxins*

Regulations are primarily made on the basis of toxicity of a particular mycotoxin as different mycotoxins exhibit different toxicities and at varying doses. All over the world toxicologists are actively trying to understand risks associated with various mycotoxins *in vitro* or using animal models. The majority of animal *in vivo* data on toxicity of mycotoxins are limited to studies of laboratory animals such as mice, rats and guinea pigs. These animal studies principally aim at determining the (1) kinds of adverse effects (hazard identification); (2) the potency or sensitivity of effects (dose–response assessment); (3) the no observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) (Faustman and Omenn, 2001; Boermans and Leung, 2007). Data from such studies are pooled together and scientifically evaluated by national or international scientific teams of experts. For a toxin, where the effect shows a threshold, tolerable daily intake (TDI) (measure of the amount of a contaminant that can be ingested on a daily basis over a lifetime without an appreciable health risk) is established (WHO, 1987). On a global level, these evaluations are carried out by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) whereas in the EU, these evaluations are performed by the European Food Safety Authority (EFSA). Most of the time the TDIs established by JECFA and EFSA are the same or comparable. For instance provisional maximum tolerable daily intake (PMTDI) or TDI for zearalenone, deoxynivalenol and fumonisins are universally 0.5, 1.0 and 2.0 µg/kg body weight (bw)/day respectively (JECFA 2000, 2002; SCF, 2003).

However, the TDIs are not applicable for toxins where carcinogenicity is the basis for concern, as is the case with aflatoxins. In 1993, the International Agency for Research on Cancer (IARC) assessed and classified naturally occurring mixtures of aflatoxins as class 1



human carcinogens (IARC, 1993). For aflatoxin B1, exposure of as little as <1 ng/kg bw/day can contribute to a risk of liver cancer (SCF, 1994) and because of this JECFA failed to establish a numerical TDI for aflatoxins (JECFA, 1999). Therefore it is recommended that levels of aflatoxins should be as low as technologically feasible or as low as reasonably achievable (ALARA). Nevertheless, TDIs of <1 ng/kg bw/day have been used in risk assessments elsewhere (Kuiper-Goodman, 1995; Leblanc et al., 2004; Sekiyama et al., 2005, Ediage et al., 2014)

#### *1.4.2. Exposure data, availability of analytical facilities and sampling criteria*

Also critical in risk assessment of mycotoxins is exposure estimation. For this reliable and sufficient data on the occurrence of mycotoxins in various food commodities and consumption data are needed to estimate the probable daily intake (PDI). Usually mycotoxin occurrence data are never sufficient, however for most developing parts of the world including Africa these are almost non-existent (FAO, 2004). Unlike in the EU where there are regular surveys of occurrence of multi mycotoxins, until now there are several countries in Africa where hardly any survey has been carried out to investigate the incidence of mycotoxins on a national basis. Literature search revealed that for Africa, with exception of Morocco (Zinedine and Mañes, 2009), even in cases where there is at least a record of mycotoxin survey, it is often a limited survey (few samples) and mostly only focusing on aflatoxins in food commodities on a market (Daniel, et al., 2011; Elshafie et al., 2011; Kamika et al., 2011; Babana et al., 2013; Chala et al., 2013; Asiki et al., 2014; Matumba et al., 2014b).

Recently the use of biomarkers in assessing mycotoxin exposure is rapidly becoming a complimentary approach to traditional food analysis (Lattanzio et al., 2011; Solfrizzo et al., 2011; Turner et al., 2011; Ediage et al., 2012; Warth et al., 2013). However, with the exception of fumonisins biomarker research work that has been carried out in South Africa, comprehensive mycotoxin biomarker exposure assessments in Africa have involved exportation of urine or blood samples to advanced laboratories overseas (Wild et al., 1992; Gong et al., 2004; Ediage et al., 2012; Shephard et al., 2007a, 2013). The exportation of specimens may be constrained by ethical challenges and may also not be sustainable in a long-term.

The lack of mycotoxin surveys in Africa is undoubtedly linked to the limitations in analytical capabilities. Typically, mycotoxin analysis involves a sequence of five discrete steps and these are sampling, sample preparation, extraction, clean-up, separation and determination. Sample preparation involves grinding, homogenisation or slurry preparation and this requires laboratory mills, blenders and homogenizers. Sample extraction usually involves mixtures of water and polar organic solvents and mechanical shaking or high speed blending. Sample clean-up involves removal of non-mycotoxin "interfering" compounds through liquid extraction, solid-phase extraction (SPE), immunoaffinity-column (IAC), molecular imprinted polymers (MIP) or aptamers (Razzazi-fezeri and Reiter, 2011). Mostly mycotoxin quantitation involves chromatographic techniques such as thin-layer chromatography (TLC), gas chromatography (GC) or high-performance liquid chromatography (HPLC) coupled to a photo diode array, UV-Vis or fluorescent detector, or liquid chromatography with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) (Shephard, 2011; Spanjer, 2011). Exception to the above analytical scheme are 'dilute and shoot' methods that bypass sample clean-up but require the use of more sensitive LC-MS/MS equipment (Sulyok et al., 2007; Razzazi-fezeri and Reiter, 2011).

The realization of the fact about mycotoxins synergism in humans and animals (Pedrosa and Borutova, 2011; Speijers and Speijers, 2004) coupled with technological advancement has caused a rapid shift from single mycotoxin targeted analysis to LC-MS/MS based multi-mycotoxin analysis (Garon et al., 2006; Sulyok et al., 2006; Sulyok et al., 2007; Spanjer et al., 2008; Vishwanath et al., 2009; Rasmussen et al., 2010; Monbaliu et al., 2010; Ediage et al., 2011; Spanjer, 2011; Streit et al., 2013). In spite of all these developments at global level, Africa remains constrained with lack of mycotoxin laboratory infrastructure and overall shortage of trained mycotoxicologists and analytical chemists to support the laboratory services. It is generally argued that food safety programmes are not fully appreciated by most African governments, as such they are not given priority and hence have reduced budgets (WHO Regional Office for Africa, 2004).

It is therefore not surprising that until now there are hardly any mycotoxin sampling plans tailored for African setting. The distribution of mycotoxins in food is highly heterogeneous, requiring statistically based sampling plans are required to obtain a representative laboratory-sized sample (Whitaker, 2006). Mycotoxin sampling criteria depend on the type of

mycotoxin, food type, volume and nature of a lot (i.e. field, truck, warehouse, pack, ...) (Whitaker, 2006; Cheli et al., 2009). The EU has laid down the most detailed statistically-based sampling plans for a wide range of mycotoxins in different foods and in different scenarios for official control/enforcement purposes (EC, 2006). Unfortunately the EU sampling plans can not just be exported to Africa as they may not represent the African setting where small lots dominate and besides there are differences in food types and probably in the spectra of mycotoxins in these two regions.

#### *1.4.3. Food sufficiency or food safety? The Africa dilemma*

According to the World Summit on Food Security held in 2008, food security is defined as all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life (World Food Summit, 2009). Ironically FAO experts estimate that 25% of the world food crops are affected by mycotoxins annually (CAST, 1989). This is even more complex in Africa, particularly in sub-Saharan Africa which faces persistent food shortage (Clover, 2003; Baro and Deubel, 2006; Smith et al., 2006; FAO, IFAD and WFP, 2013). Although there has been a gradual improvement, sub-Saharan Africa has been persistently ranked highest in human undernourishment worldwide with rates estimated between 31% in 2000-2002 and 25% in 2011-2013 (FAO, IFAD and WFP, 2013). Given the clear evidence of food shortage and high incidence of mycotoxins in food in Africa (Lewis et al., 1995; Matumba et al., 2013; Mohale et al., 2013; Adetunji, et al., 2014; Ediage et al., 2014 ), is there any room for reaching a compromise between achieving food sufficiency and food safety in Africa?

#### *1.4.4. Trade clusters*

Most African countries rely heavily on exports of agricultural commodities for a large share of their export revenues (Diao et al., 2007). Exports may include food commodities even in situations where millions of citizens are going hungry (Clover, 2003). Given the challenges faced by African countries in developing and enforcing mycotoxin regulations, it is more likely that the establishment of some of the existing regulations could have been influenced by the existence of regulations in trade partnering countries. A typical example is a case of Malawi, which during the 2003 FAO mycotoxin regulation survey was reported to have aflatoxin B1 regulation for peanuts (5 µg/kg) specified for 'exports' and had none for the local market (FAO, 2004). The 5 µg/kg aflatoxin B1 regulation for peanuts matched that of long

time Malawi's peanut importer and former colonial master, Great Britain. Wu and Guclu (2012) recently offered a detailed account of the existence of maize trade clusters among countries with similar aflatoxin regulations. However, in the process of meeting importers' regulations, agricultural countries tend to concentrate mycotoxins in food for the locals (FAO, 2004).

#### *1.4.5. Societal set-up: Agrarian vs industrial*

In industrialized societies a vast majority of the population lives in urban settings (UNFPA 2007) and heavily relies on supermarkets for their food supply (Hawkes, 2008). In case of the EU, most of the food commodities are imported from elsewhere (WTO, 2013) and usually undergo thorough safety inspection at the European border (Otsuki, et al., 2001; Kleter et al., 2009). Within the EU, there are also regular food safety inspections at manufacturing and retail level by government food agencies and recently this system is increasingly being complemented by private food safety regulations by supermarkets (Havinga, 2006).

In contrast, in spite of sub-Saharan Africa's unprecedented urban growth rate, estimated at 3% and the highest in the world (UNFPA, 2007; White et al., 2008), about 63% of sub-Saharan Africa's population still lived in the rural setting by 2012 (World Bank, 2012). In this region, almost all rural dwellers and a significant proportion of the urban dwellers rely on subsistence farming for their livelihoods (Arku et al., 2012). While there has been significant supermarket expansion due to the rapid urbanization in sub-Saharan Africa, informal food sources such as street trades, vendors, and spazas remain vital as supermarket shelves are unaffordable for the vast majority of urban dwellers (Crush and Frayne, 2011).

From the above one can clearly see the relevance of food safety regulations in the EU and the ease with which the regulations would be established and enforced. This further explains the reasons behind existence of detailed and efficient mycotoxin regulations in the EU (Van Egmond et al., 2007) and the converse hold for Africa.

### **1.5. Are the existing or borrowed regulatory standards protective enough?**

There have been suggestions to harmonize regulatory limits for mycotoxins across the world (Berg, 2003; Whitaker, 2003). Proponents argue that standardized mycotoxin regulation may facilitate international trade and offer improved consumer protection. While standardization

of regulatory limits for mycotoxins may indeed work in that way for countries with similar food consumption patterns and food security status such as the EU countries, it remains impractical to achieve the same legal framework while maintaining universal food safety due to differences in food consumption patterns across the world. Nonetheless the Codex Alimentarius Commission has international standards laid down for different mycotoxins in different products (CAC, 2013).

Dietary aflatoxin B1 exposure is known to increase hepatocellular carcinoma (HCC) risk particularly among hepatitis B virus positive (HBV+) carriers (McGlynn et al., 1995). Recognising this, Wu et al. (2013) recently examined the level of protection offered in reducing HCC risk by the existing aflatoxin regulation for maize and peanuts (mostly between 4 and 20µg/kg) around the globe taking into consideration the prevailing HBV+ rates. They found most existing regulatory standards do not adequately protect even, if enforced, particularly in countries where large amounts of maize and peanuts are consumed which include sub-Saharan Africa.

Surprisingly, Kenya, a country within sub-Saharan Africa with a record of acute aflatoxicosis and probably with the highest HBV+ prevalence rate (11-15%) in the world (Liu and Wu, 2010) maintains the aflatoxin regulatory limit at 20µg/kg for maize and groundnuts despite the high dietary consumption rate associated. Could it be that the decision for setting the regulatory limit was heavily influenced by Kenya's food insecurity situation (FAO, IFAD and WFP, 2013) or that it was just borrowed from elsewhere without performing risk assessment? This question holds for all existing regulations in Africa. If these regulations are borrowed, say from the EU, or just adopted from the CAC recommendations, do they serve the purpose (i.e. are they protective)?

Given the high maize consumption in sub-Saharan Africa where daily average intake for an adult can be as high as 500g (Dowswell et al., 1996; Shephard et al., 2013) the borrowing of *Fusarium* mycotoxin regulatory limits from anywhere would not suffice. For instance the EU regulatory limits for fumonisins, deoxynivalenol and zearalenone for raw unprocessed maize are 4000, 1750 and 200 µg/kg respectively (EC, 2007, 2010). Assuming an average weight of 60 kg and a daily intake of 400g of maize for an adult, the consumption of maize containing these mycotoxins at the maximum limits would result in an exposure of roughly 13, 12 and 3

times JECFA’s guided TDIs for fumonisins (2 µg/kg bw/day or 120 µg per 60 kg-person/day), deoxynivalenol (60µg per 60kg-person/day) and zearalenone (30 µg per 60 kg-person/day) respectively (JECFA 2000, 2002) (Figure 1).

Conversely, using the TDI approach only maximum limits of less than 300, 150 and 75µg/kg for fumonisins, deoxynivalenol and zearalenone respectively could adequately protect 60kg adults of consumers with a daily intake of 400g (Figure 1). As regards to nivalenol which still has not been assigned a regulatory limit by the EU but has a TDI of 72 µg per 60 kg-person/day (EFSA, 2013), an average intake of 400g would require a maximum limit of less than 180 µg/kg (Figure 1.1).

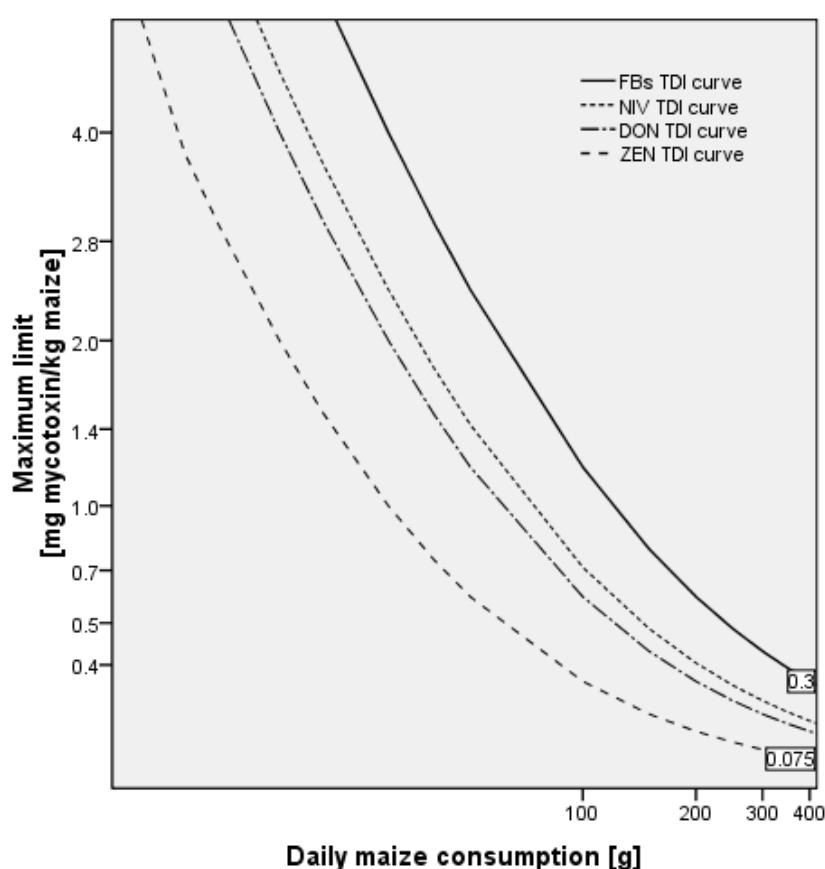


Figure 1.1: TDI curves for fumonisins (FBs), deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEN) for a 60kg adult based on JECFA’s guided PMTDIs or TDIs for fumonisins (2.0 µg/kg body weight (bw)/day), deoxynivalenol (1.0 µg/kg bw/day), nivalenol (1.2 µg/ kg bw/day) and zearalenone (0.5 µg/kg bw/day). Area under each curve represents “safe area”, in contrast area above the curve represents the “unsafe area”. The numbered points in small white boxes (0.3 and 0.075) illustrate maximum limits in mg/kg level for fumonisins and zearalenone respectively that could be set to protect a 60kg adult with an average maize intake of 400g.

Based on the available occurrence data for these mycotoxins (Doko et al., 1996; Bankole et al., 2013; Mohale et al., 2013; Shephard et al., 2013; Adetunji et al., 2014 ; Ediage et al., 2014; Matumba et al, 2014a), the above calculated maximum limit would seriously exacerbate starvation in sub-Saharan Africa if enforced. Making the situation even more complicated is that the climate in sub-Saharan Africa favours co-occurrence of multiple mycotoxins. Considering the mycotoxin synergism, wide margin of safety may be required (Egmond et al., 2007).

### **1.6. Conclusions and perspectives**

From the above it can be clearly seen that the existence of mycotoxin regulations in an agrarian setting of Africa would have very little impact. In as much as governments need to establish and enforce regulations in order to protect the minority that rely on supermarkets, prevent the influx of contaminated foodstuffs from elsewhere and facilitate exports (Wu et al, 2013), there is need for much more to be done in order to protect citizens from high dietary mycotoxin exposures. The subsistence nature of food production in sub-Saharan Africa offers huge opportunities to a consumer in ensuring food safety as he/she has almost full control over intrinsic pre-and post-harvest management factors. In that regard, governments in agrarian countries need to invest a lot of effort in augmenting farmers' knowledge about the health hazards and prevention strategies highlighted earlier in this paper. As opposed to the approach followed by most governments by emphasizing only on achieving quality of exports as means of survival strategy, augmenting farmers' knowledge about the health hazards would more likely improve the quality of exports as producers (and at the same time consumers) would be more careful than when the ultimate goal of food safety would be to export. Perhaps the biggest opportunity in an agrarian setting is the application of mycotoxin decontamination strategies. Physical decontamination methods which include hand sorting, washing, dehulling, screening, density separation and fractionation would certainly work better on raw food products than processed food due to their complexity (Chapter 7). In this sense, an agrarian consumer has an advantage. However, most mycotoxin decontamination methods require optimization before wide dissemination and usage. The other important thing is the need to explore feasible ways of diverting contaminated food fractions, otherwise contaminated food will still find its way onto the table of an agrarian consumer.

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## CHAPTER TWO



Fungal colonization, growth and mycotoxin production are greatly affected by environmental factors. Different fungal species have specific temperature and water activity ranges for optimal growth and mycotoxin production. Consequently, different mycotoxins have been generally linked to macroclimatic regions; for instance aflatoxins (AFs) and zearalenone (ZEN) are generally linked to tropical and temperate regions, respectively. As highlighted already in Chapter 1, sub-Saharan Africa lacks the capacity to monitor mycotoxin occurrences and these only focuses on aflatoxin (based on the above stated theory). Using Malawi, a small sub-Saharan country (94,079 sq km land size) with four micro-climatic zones as a case example, Chapter 2 seeks to verify if aflatoxins are really the major problem for sub-Saharan Africa and further tries to assess if a country could be treated as one block as far as mycotoxin management in an agrarian setting is concerned. Chapter 2 is reproduced from an accepted manuscript **‘Fungal metabolites diversity in maize and associated human dietary exposures relate to micro-climatic patterns in Malawi’** (World Mycotoxin Journal) written by Matumba, L., Sulyok, M., Monjerezi, M., Biswick, T. and Krska, R.



## **Chapter 2: Fungal metabolites diversity in maize and associated human dietary exposures relate to micro-climatic patterns in Malawi.**

### **2.1. Introduction**

Fungal colonization, growth and mycotoxin production are greatly affected by environmental factors such as temperature and humidity (Sanchis and Magan, 2004). Different fungal species have specific temperature and water activity ranges for optimal growth and mycotoxin production. Consequently different mycotoxins have been generally linked to climatic regions (Paterson and Lima, 2010, 2011; Magan et al., 2011; Wu et al., 2011). However, the occurrence of the mycotoxins has generally been linked to macro-climates disregarding micro-climatic variations. For instance aflatoxins (AFs) and zearalenone (ZEN) are generally linked to tropical and temperate regions, respectively. Likewise, mycotoxin monitoring programs target the expected mycotoxins in a particular macro-climatic region. Such targeted monitoring programs might disadvantage subsistence populations living in extreme micro-climatic zones where a different spectrum of mycotoxins may be prevalent other than those that are generalized for a particular macro-climatic region. Beside, despite the existence of 300-400 mycotoxins (Hussein and Brasel, 2001; Bennett and Klich, 2003) only a few mycotoxins have been adequately studied and their favourable ecological production conditions understood.

Malawi is a small country with a total land area of 94,079 sq km. It has a general tropical climate with a wide spatial variation in rainfall, temperature and relative humidity. The climate is highly influenced by the topography and surface fluxes (Torrance, 1972), and as such the country is divided into four distinct agro-ecological zones based on micro-climatic conditions namely: lower Shire valley, Lake Shore, middle upper Shire, mid elevation (Malawi Government 2002; Figure 2.1). The country's tropical climate is characterized by well-defined wet and dry seasons (Figure 2.2).

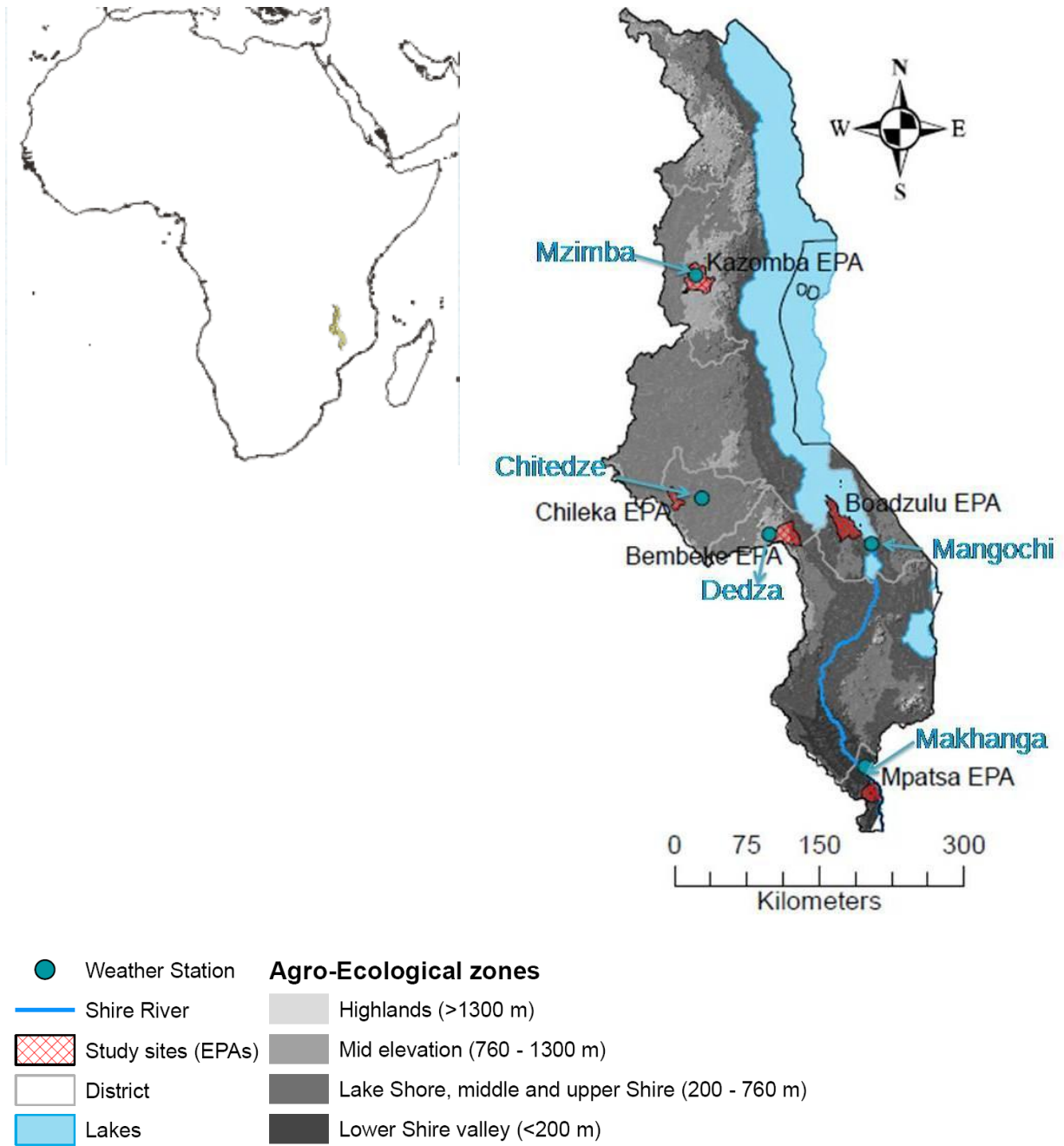


Figure 2.1: Study sites in different agro-ecological zones of Malawi



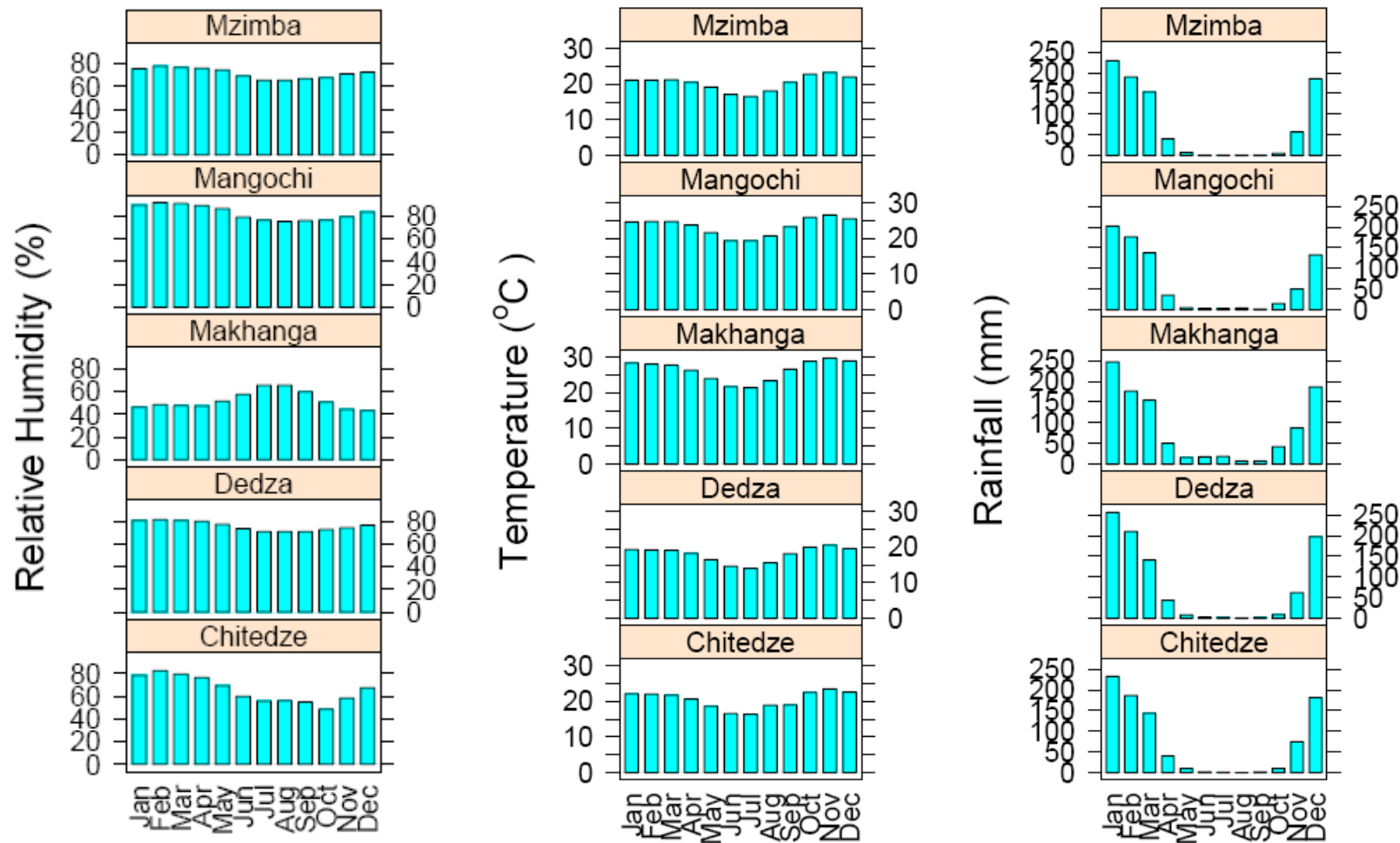


Figure 2.2: Average monthly relative humidity, temperature and rainfall for the sampled agro-ecological zones (1960-2009).

Maize (*Zea mays*), is the third most important cereal staple food crop worldwide after wheat and rice. In Malawi, maize contributes significantly to diets of more than 80% of the population with per capita consumption of 382 grams/day (Ecker and Qaim, 2011). With such high dependency on the crop, associated dietary mycotoxin risks are most likely. In fact, previous surveys indicated widespread aflatoxin contamination in foodstuffs (Khonga, 1985; Matumba et al. 2011, 2013, 2014a, 2014b; Monyo et al., 2012), consequently attention to aflatoxin issues from the policy community has increased in recent years. The Malawi government and development partners are collaboratively trying to raise public knowledge on management of AFs in food and to that effect guidelines have been published and distributed to all government agricultural frontline staff (Matumba et al., 2012). However, focusing on AFs only cannot guarantee dietary safety because of the putative presence of other undetected mycotoxins. In this context, the present study was undertaken with the aim of providing for the first time, information on I) the diversity of mycotoxins in maize from four agro-ecological zones in Malawi, and II) the influence of (micro-) climatic conditions on the mycotoxin pattern and dietary exposures in order to identify low risk zones for maize production. The data presented in this study will be useful in facilitating improved dietary risk management.

## **2.2. Materials and methods**

### *2.2.1. Sample collection and preparation*

A total of 90 shelled maize samples (intended for human consumption) were collected (bought or given for free) from farmsteads from four agro-ecological zones of Malawi at the end of July 2011. The timing of the sample collection was aimed at including the bad maize grains (shriveled grains and sometimes visually mouldy grains) that are usually consumed during the earlier months after harvest due to poor storability (Singano et al., manuscript in preparation). The farmsteads were selected from *Bembeke* Extension Planning Area (EPA) in Dedza district and *Kazomba* EPA in Mzimba district representing highlands agro-ecology (cool); *Chileka* EPA in Lilongwe district representing mid elevation agro-ecology (warm); *Boadzulo* EPA in Mangochi district representing lake shore, middle and upper and mid Shire agro-ecology (hot); and *Mpatsa* EPA in Nsanje district representing lower Shire valley agro-ecology (hottest) (Figure 2.1).

Shelled maize samples were systematically collected from a second house of every randomly selected village (one house per village) within an EPA. If the selected household was found to be storing their maize on cobs (unshelled), the household was excluded and the sampling process continued to the next house. The shelled maize samples were drawn from woven polypropylene sacks containing approximately 50 kg of maize and the bags were usually stacked in a room within a house. If the selected household had 10 bags of maize or less, all bags were sampled otherwise 10 bags were randomly sampled from each household. From each selected bag, maize was drawn by using an improvised cylindrical bag sampler (approximately 1.1 m long with 40 mm external diameter) (Annex I). The bag sampler was pushed twice into a bag through both tips of farmer sewn-end (to minimize damaging the bag) diagonally into a horizontally laying bag. The bag sampler was pushed into the bag with the intake aperture facing down and was then turned 180°, agitated in order to fill the bag sampler and withdrawn. All increments were then pooled and mixed thoroughly and a 5 kg maize sample (Campbell et al 1986), was collected in khaki paper bag. The maize samples were transported to Chitedze Agricultural Research Station where the samples were immediately milled. To ensure sample homogeneity, the whole samples were first ground using a laboratory blender (Waring Products, New Hartford, Connecticut) and further fine milled using a laboratory mill (Christy and Norris Ltd, Suffolk, UK) to pass sieve #20 (aperture size of 0.841 mm) (Annex II). Homogenized sub-samples were then transported to Austria for analysis.

### *2.2.2. Chemicals and materials for mycotoxin analysis*

Methanol and acetonitrile (both LC gradient grade) were supplied by Merck (Darmstadt, Germany) and VWR (Leuven, Belgium), respectively. Ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma-Aldrich (Vienna, Austria). Purified water was obtained from a Milli-Q plus system (Millipore, Molsheim, France). Commercially available standards of fungal and bacterial metabolites were procured from Biopure Referenzsubstanzen GmbH (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland) and LGC Promochem GmbH (Wesel, Germany). Stock solutions of each analyte were prepared and stored as previously outlined by Vishwanath et al. (2009).

### *2.2.3. Sample extraction and dilution*

To determine the mycotoxins, 5g of fine ground maize sample was extracted with 20 ml of a mixture of acetonitrile/water/acetic acid (79:20:1, v/v/v) in 50 ml polypropylene centrifuge tubes with conical bottom according to Sulyok et al. (2007). Samples were shaken using a rotary shaker (GFL 3017, GFL; Burgwedel, Germany) for 90 minutes in horizontal position and left to settle for at least 3 minutes. A supernatant of 500  $\mu$ L of the raw extract was transferred into autosampler vials and diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v). The diluted extracts were vortexed and 5  $\mu$ L injected into the LC-MS/MS system.

### *2.2.4. Liquid chromatography–tandem mass spectrometry conditions*

The method used in this study was described by Vishwanath et al. (2009). Briefly, the analysis was carried out using an Agilent 1200 Series HPLC System (Agilent, Waldbronn, Germany) coupled to a QTrap 5500 (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray electrospray ionization (ESI) source and controlled by Analyst Software 1.5.1 (Applied Biosystems, Foster City, CA, USA). Chromatographic separation was achieved using a Gemini<sup>®</sup> C18-column, 150 $\times$ 4.6 mm i.d., 5  $\mu$ m particle size, protected by a C18 guard column, 4 $\times$ 3 mm i.d. (all from Phenomenex, Torrance, CA). A binary gradient elution was performed with methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B) respectively. Both mobile phases contained 5 mM ammonium acetate. After an initial time of 2 minutes at 100% eluent A, eluent B was increased linearly to 50% within 3 minutes and further increased to 100% within 9 minutes, and held constantly for 4 minutes followed by a 2.5 minutes column re-equilibration at 100% eluent A. The flow rate was maintained at 1 mL/minute.

Data acquisition was performed in the scheduled multiple reaction monitoring (sMRM) mode in both positive and negative polarity using two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte (to yield 4 identification points as required by European commission (EC, 2002)). The analytical method simultaneously investigated the presence of 235 fungal and 40 bacterial metabolites. ESI-MS/MS parameters were as follows: source temperature 550°C; curtain gas 30 psi; ion source gas 1 (sheath gas) 80 psi; ion source gas 2 (drying gas) 80 psi; collision gas (nitrogen) medium; ion-spray voltage  $-4500$  V and  $+5500$  V respectively. Quantification was performed using external calibration taking into

account the apparent recoveries determined in the course of method validation (Malachova et al., 2014). In addition, the accuracy of the method was further verified by regular participation in a proficiency test scheme organized by BIPEA (Gennevilliers, France). For 27 of the 29 test results submitted for five maize samples the z-score was in the satisfactory range between -2 and 2.

#### *2.2.5. Data analysis*

All values below the limit of detection (LOD) were treated as missing data (Helsel, 2006). Values of half the limit of quantification (LOQ) were assigned to analytical values in the range  $LOD \leq \text{value} < LOQ$ . Mean and median values were calculated based on positive data only except during estimation of margins of exposure (MOEs) for aflatoxin B1 (AFB1) where non-detects were assigned half the value of limit of detection. Analysis of variance (ANOVA) and Tukey's HSD test were used to compare the means of mycotoxins in different districts. Since mycotoxin concentration data were not normally distributed, data were log transformed before analyses. The analyses were conducted using SPSS version 15 (SPSS inc., Chicago, IL, USA).

Dietary mycotoxin exposures for adults were computed for each sample by considering the mycotoxin concentration, the per capita maize consumption for Malawi (382 grams per day) (Ecker and Qaim, 2011) and an average adult body weight of 60 kg (EFSA, 2007). It is recognized that a probabilistic approach would have provided more realistic exposure estimates, however there is hardly consumption data to perform such. Estimation of AFB1 exposures in the different agro-ecological zones considered mean AFB1 concentrations of the samples (including samples with concentrations  $< LOD$ ). MOEs for AFB1 were estimated by dividing the benchmark dose lower limits (BMDL) for 10% increased cancer risk using rodent data (170 ng kg/body weight/day) (EFSA, 2007). It is noteworthy that despite the fact that dehulling maize significantly reduces the mycotoxin content (Siwela et al., 2005; Fandohan et al., 2006) all the maize was assumed to have been consumed non-dehulled (without removing bran) as it was not known whether the farmers dehulled the maize or not.

## 2.3. Results and discussion

### 2.3.1. Metabolite occurrence

A total of 65 different fungal metabolites were detected in the maize samples (Table 2.1) whereas there were no detected bacterial metabolites. AFs, citrinin (CIT), curvularin, kojic acid, griseofulvin and dechlorogriseofulvin were detected in at least 25% of the samples from relatively warm and hot agro-ecological zones and were distinctly absent in samples from the highlands. AFs predominantly occurred in samples from hotter agro-ecologies (Lake Shore agro-ecological zone, 60%; lower Shire valley, 80%) and in a few samples (15%) from the mid elevation zone and were not detected in samples from the highlands. A similar trend of AFs distribution was also observed in maize samples surveyed in 2012 (Matumba et al., 2013). On the other hand, culmorin and deoxynivalenol-3-glucoside were widespread in samples from the highlands (50% and 83%, respectively) but distinctly absent in warm and hot agro-ecological zones. Similarly, enniatins (ENNs) were limited to samples from cool and warm agro-ecologies only.

Only 35 (54%) of the metabolites were detected in samples from all the four agro-ecological zones (Table 2.1). Among these, beauvericin, aurofusarin and deoxynivalenol (DON) were the most common contaminants across the agro-ecologies and were detected in 99% of the samples followed by equisetin (97%), moniliformin (94%), fumonisins (FBs) (90%) and nivalenol (NIV) (84%) (Table 2.1). However, there were differences in average metabolite concentrations across the zones (Table 2.1). Mean concentrations for FBs, 3-nitropropionic acid, monocerin and equisetin were significantly higher in samples from hot agro-ecological zones than in cool zones (Table 2.1). The concentrations of beauvericin, DON, aurofusarin, moniliformin, fusaric acid, fusaproliferin, NIV, ZEN, were higher in samples from the cool zones than in warm and hot ecologies. It is noteworthy that metabolite concentrations varied considerably from few  $\mu\text{g}/\text{kg}$  (in case of ENNs, apicidin and diacetoxyscirpenol (DAS)) to about 9 mg/kg (in a case of kojic acid and CIT).

Table 2.1: Occurrence and comparison of mycotoxins and other fungal metabolites detected in the samples, percentage of positive samples, and means and maximum (Max) concentrations in µg/kg. Analytes are listed in order of prevalence (N= 90 samples)

Metabolite / Mycotoxin	Agro-ecological zone														
	Total (all zones) (N=90)			Highlands (N=30)			Mid elevation (N=20)			Lake Shore, middle and upper Shire (N=20)			Lower Shire valley (N=20)		
	% pos	Means ±SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% Pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)
Beauvericin	99	28±7	415	97	74±18 <sub>a</sub>	415	100	5±3 <sub>b</sub>	58	100	8±4 <sub>b</sub>	67	100	6±2 <sub>b</sub>	35
Deoxynivalenol	99	312±40	2328	97	600±103 <sub>a</sub>	2328	100	182±15 <sub>b</sub>	338	100	170±8 <sub>b</sub>	242	100	167±8 <sub>b</sub>	231
Aurofusarin	99	181±53	2896	100	469±138 <sub>a</sub>	2896	100	94±57 <sub>b</sub>	1096	95	2±1 <sub>c</sub>	7	100	8±3 <sub>bc</sub>	38
Equisetin	97	59±16	843	97	9±3 <sub>c</sub>	83	90	70±44 <sub>bc</sub>	791	100	47±14 <sub>ab</sub>	194	100	135±52 <sub>a</sub>	843
Moniliformin	94	249±35	1624	97	456±82 <sub>a</sub>	1624	90	140±34 <sub>b</sub>	475	95	180±45 <sub>b</sub>	600	95	105±21 <sub>b</sub>	344
Total Fumonisin	90	1745±180	6475	73	757±188 <sub>b</sub>	3457	100	2514±429 <sub>a</sub>	6261	100	1424±230 <sub>a</sub>	4834	100	2384±412 <sub>a</sub>	6475
Fumonisin B1	90	1264±129	4638	70	545±132 <sub>b</sub>	2350	100	1797±302 <sub>a</sub>	4545	100	1031±170 <sub>a</sub>	3627	100	1720±296 <sub>a</sub>	4638
Fumonisin B2	90	369±40	1340	70	181±47 <sub>b</sub>	870	100	533±99 <sub>a</sub>	1314	100	295±45 <sub>a</sub>	896	100	488±88 <sub>a</sub>	1340
Fumonisin B3	90	128±13	502	70	58±14 <sub>b</sub>	238	100	184±31 <sub>a</sub>	502	100	98±15 <sub>a</sub>	312	100	175±29 <sub>a</sub>	497
Nivalenol	84	94±34	2220	100	194±81 <sub>a</sub>	2220	55	85±40 <sub>ab</sub>	409	95	8±2 <sub>b</sub>	34	80	16±5 <sub>ab</sub>	69
Fusaric acid	72	122±20	1020	87	151±27 <sub>a</sub>	554	25	243±196 <sub>ab</sub>	1020	95	46±8 <sub>b</sub>	148	75	129±28 <sub>ab</sub>	415
Fusaproliferin	70	167±48	2056	87	382±102 <sub>a</sub>	2056	50	18±5 <sub>b</sub>	46	65	17±4 <sub>b</sub>	63	70	15±4 <sub>b</sub>	58
Zearalenone	68	129±49	2025	97	147±71 <sub>a</sub>	1566	75	235±142 <sub>ab</sub>	2025	35	5.2±3 <sub>b</sub>	25	50	7.9±4 <sub>b</sub>	40
3-Nitropropionic acid	61	233±49	2172	43	73±36 <sub>c</sub>	466	60	100±30 <sub>bc</sub>	297	55	123±20	267 <sub>ab</sub>	95	489±117 <sub>a</sub>	2172
Hydrolysed FB1	61	3±1	30	7	0.2±0 <sub>n</sub>	0.2	80	4±2 <sub>n</sub>	30	85	2±1 <sub>n</sub>	11	100	4±1 <sub>n</sub>	24
15-Hydroxyculmorin	52	8±1	43	70	13±3 <sub>a</sub>	43	40	3±1 <sub>c</sub>	6	40	4±1 <sub>bc</sub>	7	50	6±3 <sub>ab</sub>	25
Zearalenone-4-Sulfate	50	7±2	80	97	6±3 <sub>ab</sub>	80	25	19±10 <sub>a</sub>	58	25	1±1 <sub>b</sub>	3.6	30	3±2 <sup>ab</sup>	13
Monocerin	46	104±38	1308	10	30±20 <sub>n</sub>	68	15	13±6 <sub>n</sub>	23	85	136±54 <sub>n</sub>	944	90	102±72 <sub>n</sub>	1308

Mycotoxin concentration means were calculated based on positive data only. Means in the same row followed by the same letter are not significantly different (P≤0.05) according to Tukey's HSD test. (-) means the statistic is not applicable because the metabolite was not detected.

Table 2.1(continue): Occurrence and comparison of mycotoxins and other fungal metabolites detected in the samples, percentage of positive samples, and means and maximum (Max) concentrations in µg/kg. Analytes are listed in order of prevalence (N= 90 samples)

Metabolite / Mycotoxin	Agro-ecological zone														
	Total (all zones) (N=90)			Highlands (N=30)			Mid elevation (N=20)			Lake Shore, middle and upper Shire (N=20)			Lower Shire valley (N=20)		
	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% Pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)
Butenolid	44	143±35	1276	67	204±67 <sub>n</sub>	1276	30	63±25 <sub>n</sub>	166	30	129±27 <sub>n</sub>	220	40	62±14 <sub>n</sub>	143
Pestalotin	42	8 ±2	62	17	16±10 <sub>n</sub>	57	35	12±83 <sub>n</sub>	62	70	4.7±0.6 <sub>n</sub>	9.2	60	7±2 <sub>n</sub>	28
Macrosporin A	41	10±2	61	30	17±8 <sub>n</sub>	61	50	10±2 <sub>n</sub>	23	50	7±3 <sub>n</sub>	29	40	3±1 <sub>n</sub>	8
Citrinin	40	882±304	8867	0	-	-	25	65±46 <sub>b</sub>	244	70	1651±709 <sub>a</sub>	8867	85	490±201 <sub>ab</sub>	3044
Emodin	38	5±1	21	3	3	3	25	2±1 <sub>n</sub>	4	60	7±2 <sub>n</sub>	21	80	4±1 <sub>n</sub>	20
Diacetoxyscirpenol	37	3±1	17	23	5±2 <sub>n</sub>	17	30	3±0 <sub>n</sub>	3	35	3±0 <sub>n</sub>	2.9	65	3±0.3 <sub>n</sub>	6
total Aflatoxins (B1+B2+G1+G2)	34	164±45	878	0	-	-	15	42±8 <sub>n</sub>	56	60	310±101 <sub>n</sub>	878	80	78±19 <sub>n</sub>	289
Aflatoxin B1	34	91±26	592	0	-	-	15	7.0±5 <sub>n</sub>	17	60	168±57 <sub>n</sub>	592	80	50±18 <sub>n</sub>	272
Aflatoxin B2	24	11±3	54	0	-	-	5	3 <sub>n</sub>	3	40	23±6.6 <sub>n</sub>	54	65	4.7±1.9 <sub>n</sub>	22
Aflatoxin G1	30	68±20	417	0	-	-	15	31±3 <sub>n</sub>	36	50	136±46 <sub>n</sub>	417	70	27±7.1 <sub>n</sub>	77
Aflatoxin G2	23	9±3	44	0	-	-	15	4±1 <sub>n</sub>	5	40	20±44 <sub>n</sub>	44	50	3±1 <sub>n</sub>	7
Cyclopiazonic acid	33	147±26	730	7	61±6.2 <sub>n</sub>	67	20	239±164 <sub>n</sub>	730	45	177±42 <sub>n</sub>	356	75	116±19 <sub>n</sub>	315
Radiciol	32	24±6.8	170	33	24±12 <sub>n</sub>	119	80	28±9.9 <sub>n</sub>	170	0	-	-	15	5.1±0.5 <sub>n</sub>	6
Fusarenon-X	32	109±30	664	50	172±53 <sub>n</sub>	664	30	60±12 <sub>n</sub>	100	15	33±2.3 <sub>n</sub>	38	25	26±4.7 <sub>n</sub>	40
Aspyrone	28	99±12	226	30	118±26 <sub>n</sub>	226	30	113±19 <sub>n</sub>	157	15	56±0 <sub>n</sub>	56	35	80±16 <sub>n</sub>	158
Deoxynivalenol-3-glucoside	28	60±13	268	83	61±13	269	0	-	-	0	-	-	0	-	-
Chanoclavine	27	0.2±0	0.8	13	0.3±0.1 <sub>n</sub>	0.6	40	0.4±0.1 <sub>n</sub>	1	10	0.1±0 <sub>n</sub>	0.1	50	0.1±0 <sub>n</sub>	0.2

Mycotoxin concentration means were calculated based on positive data only. Means in the same row followed by the same letter are not significantly different (P≤0.05) according to Tukey's HSD test. (-) means the statistic is not applicable because the metabolite was not detected



Table 2.1(continue): Occurrence and comparison of mycotoxins and other fungal metabolites detected in the samples, percentage of positive samples, and means and maximum (Max) concentrations in µg/kg. Analytes are listed in order of prevalence (N= 90 samples)

Metabolite / Mycotoxin	Agro-ecological zone														
	Total (all zones) (N=90)			Highlands (N=30)			Mid elevation (N=20)			Lake Shore, middle and upper Shire (N=20)			Lower Shire valley (N=20)		
	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% Pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)
Kojic Acid	22	2699±581	8741	0	-	-	0	-	-	50	2218±791 <sub>n</sub>	8741	50	3180±866 <sub>n</sub>	8216
Apicidin	22	11±3.3	56	37	17±5 <sub>n</sub>	56	25	4±1 <sub>n</sub>	7	0	-	-	20	3±1 <sub>n</sub>	49
Phycion	21	88±26	454	3	29± <sub>n</sub>	29	15	68±41 <sub>n</sub>	147	40	87±32 <sub>n</sub>	302	35	106±60 <sub>n</sub>	454
Total Enniatins	20	1.4±0.5	9.5	33	1.3±0.9 <sub>n</sub>	9.5	40	1.5±0.5 <sub>n</sub>	5	0	-	-	0	-	-
Enniatin A1	20	0.6±0.2	3.7	33	0.5±0.4 <sub>n</sub>	4	40	0.6±0.2 <sub>n</sub>	2	0	-	-	0	-	-
Enniatin B1	18	0.2±0.1	1	27	0.2±0.1 <sub>n</sub>	1	40	0.2±0.1 <sub>n</sub>	1	0	-	-	0	-	-
Enniatin A	17	0.8±0.3	4.8	23	0.9±0.6 <sub>n</sub>	5	40	0.8±0.2 <sub>n</sub>	2	0	-	-	0	-	-
Enniatin B	2	0.04±0	0.04	3	0.04	0.04	5	0.04	0	0	-	-	0	-	-
Chaetoglobosin A	19	4±1	14	27	5±1 <sub>n</sub>	14	25	4±0 <sub>n</sub>	4	5	3.5	3.5	15	3.5±0 <sub>n</sub>	4
Curvularin	19	31±14	197	0	-	-	20	19±10 <sub>n</sub>	49	35	29±24 <sub>n</sub>	174	30	40±31 <sub>n</sub>	197
Aflatoxin M1	19	7±1	22	0	-	-	10	2.5±0 <sub>b</sub>	2	35	12±3	22	40	4±1 <sub>a</sub>	10
β-zearalenol	18	29±10	124	30	27±13 <sub>n</sub>	95	30	37±19 <sub>n</sub>	123	0	-	-	5	2.3	3
Culmorin	17	21±4	53	50	21±4.0	53	0	-	-	0	-	-	0	-	-
Dechlorogriseofulvin	13	28±16	196	0	-	-	25	23±9 <sub>n</sub>	45	0	-	-	35	32±27 <sub>n</sub>	196
Griseofulvin	10	21±11	101	0	-	-	20	20±7 <sub>n</sub>	32	0	-	-	25	22±20 <sub>n</sub>	101
Viomellein	10	77±27	253	3	22	22	5	37	37	10	44±39 <sub>n</sub>	83	25	109±44 <sub>n</sub>	253
Aspinolid B	10	1±0	1	3	1	1	35	1±0	1	0	-	-	5	1	1

Mycotoxin concentration means were calculated based on positive data only. Means in the same row followed by the same letter are not significantly different (P≤0.05) according to Tukey's HSD test. (-) means the statistic is not applicable because the metabolite was not detected

Table 2.1(continue): Occurrence and comparison of mycotoxins and other fungal metabolites detected in the samples, percentage of positive samples, and means and maximum (Max) concentrations in µg/kg. Analytes are listed in order of prevalence (N= 90 samples)

Metabolite / Mycotoxin	Agro-ecological zone														
	Total (all zones) (N=90)			Highlands (N=30)			Mid elevation (N=20)			Lake Shore, middle and upper Shire (N=20)			Lower Shire valley (N=20)		
	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)	% Pos	Means ± SE (µg/kg)	Max (µg/kg)	% pos	Means ± SE (µg/kg)	Max (µg/kg)
Aspinolid B	10	1±0	1	3	1	1	35	1±0	1	0	-	-	5	1	1
Alternariol	8	6±2	17	3	2	2	10	6±2 <sub>n</sub>	8	15	10±5 <sub>n</sub>	17	5	2	2
Averufin	8	20±5	52	0	-	-	0	-	-	25	22±7.3	52	10	15±0	15
α-zearalenol	7	31±7.9	56	10	30±10 <sub>n</sub>	42	10	45±11 <sub>n</sub>	56	0	-	-	5	6	6
Sterigmatocystin	6	2±0	3	0	-	-	0	-	-	5	1	1	20	2±0	3
Averufanin	6	5±1	5	0	-	-	0	-	-	25	5±0	5	0	-	-
Terphenyllin	4	79±45	163	0	-	-	0	-	-	15	105±52	163	5	2.6	3
O-Methylsterigmatocystin	3	4±2	7	0	-	-	0	-	-	10	2±1	4	5	7	8
Gibberellic acid	3	9±0	9	7	9±0	9	0	-	-	5	9	9	0	-	-
Rugulosin	3	13±4.2	21	0	-	-	10	15±6.3	21	5	9	9	0	-	-
T2 Tetraol	3	53±35	123	3	122±123	123	0	-	-	0	-	-	10	18±0	18
Elymoclavine	2	0.4±0.2	0.6	3	0.3±0.3	0.3	0	-	-	5	0.6	0.6	0	-	-
Citreoviridin	1	6	6	3	6±5.7	5.7	0	-	-	0	-	-	0	-	-
Ochratoxin A	1	16	16	0	-	-	0	-	-	5	16	16	0	-	-
Ochratoxin B	1	1	1	0	-	-	0	-	-	5	1.3	1.3	0	-	-
Fusarielin A	1	29	29	0	-	-	0	-	-	0	-	-	5	29	29
Mycophenolic acid	1	16	16	0	-	-	0	-	-	0	-	-	5	16	16

Mycotoxin concentration means were calculated based on positive data only. Means in the same row followed by the same letter are not significantly different ( $P \leq 0.05$ ) according to Tukey's HSD test. (-) means the statistic is not applicable because the metabolite was not detected

All samples were co-contaminated with up to 41 different metabolites (Figure 2.3). The median number of metabolites co-occurring in a same sample was 20 for all zones except for lower Shire valley where it was 26 (Figure 2.3).

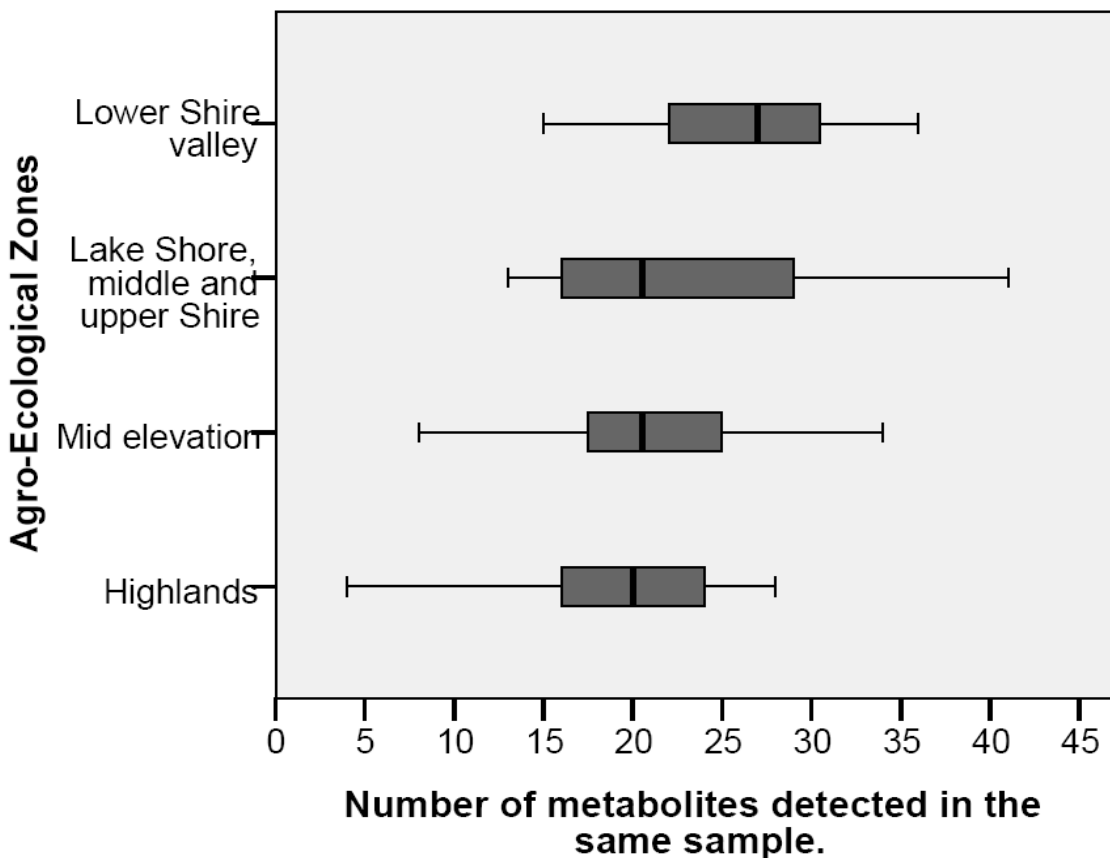


Figure 2.3: Box and whisker plot showing distribution of numbers of metabolites detected in the same sample by agro-ecological zone. A vertical line within the box represents the median. The front and back ends of the box represent first quartile (25<sup>th</sup> percentile) and the third quartile (75<sup>th</sup> percentile) respectively. The front whisker extends from first quartile to the smallest non-outlier in the data set (25<sup>th</sup> percentile - 1.5 \* (interquartile range, IQR)), and the back whisker goes from third quartile to the largest non-outlier (75<sup>th</sup> percentile + 1.5 \* IQR)

As with regard to the regulated mycotoxins, 29%, 11%, 2% and 9% of all the tested samples contained total AFs (aflatoxin B1+B2+G1+G2), total FBs (fumonisin B1+B2), DON and ZEN, respectively exceeding the European Union (EU) maximum level (ML) (Table 2.2). 15%, 40% and 75% of the samples from mid elevation, lake Shore and lower Shire River valley, respectively, exceeded the EU ML for maize subject to sorting or other physical treatment.

Table 2.2: Number and percentage of samples exceeding EU maximum levels set for different mycotoxins in different agro-ecological zones

Agro-Ecological Zone	Number and percentage of samples of exceeding European Union (EU) maximum levels							
	Total Aflatoxins		Total Fumonisin		Deoxynivalenol		Zearalenone	
	10 µg/kg <sup>a</sup>	0.1 µg/kg <sup>b</sup>	4000 µg/kg <sup>c</sup>	200 µg/kg <sup>b</sup>	1750 µg/kg <sup>c</sup>	200 µg/kg <sup>b</sup>	200 µg/kg <sup>c</sup>	20 µg/kg <sup>b</sup>
Highlands (N=30)	0	0	0	16(53%)	2(7%)	23(77%)	4(13%)	13(43%)
Mid elevation (N=20)	3(15%)	3(15%)	5(25%)	19(95%)	0	7(35%)	4(20%)	4(20%)
Lake Shore, middle and upper Shire (N=20)	8(40%)	12(60%)	1(5%)	20(100%)	0	4(20%)	0	1(5%)
Lower Shire valley (N=20)	15(75%)	16(80%)	4(20%)	19(95%)	0	2(10%)	0	1(5%)
<b>Total (N=90)</b>	<b>26(29%)</b>	<b>31(34%)</b>	<b>10(11%)</b>	<b>74(82%)</b>	<b>2(2%)</b>	<b>36(40%)</b>	<b>8(9%)</b>	<b>19(21%)</b>

<sup>a</sup> EU ML for maize subject to sorting or other physical treatment.

<sup>b</sup> Regulatory limit for different mycotoxins in maize products covered by Commission Regulation (EU) No. 165/2010 and (EC) No. 1126/2007 specifically for infants and young children

<sup>c</sup> Regulatory limit for different mycotoxins in maize products covered by Commission Regulation (EU) No. 165/2010 and (EC) No. 1126/2007 specifically for adults

Forty-two percent (42%) of all tested maize samples contained either total AFs, total FBs (FB1+FB2), DON, ZEN; or a combination thereof in concentrations exceeding the EU regulatory limits set for adults (Figure 2.4). Since the EU mycotoxin regulations for infant and baby food are much more stringent (EC, 2007, 2010), almost all (97%) the samples tested above the limits and there were generally no differences in percentages among the agro-ecologies (Figure 2.4). Considering the agro-ecological zones individually, the hotter agro-ecological zone of lower Shire valley comparatively had more (75%) samples that contained the regulated mycotoxins above the regulatory limits for adults whereas the cool zone contained the least (17%) (Figure 2.4).

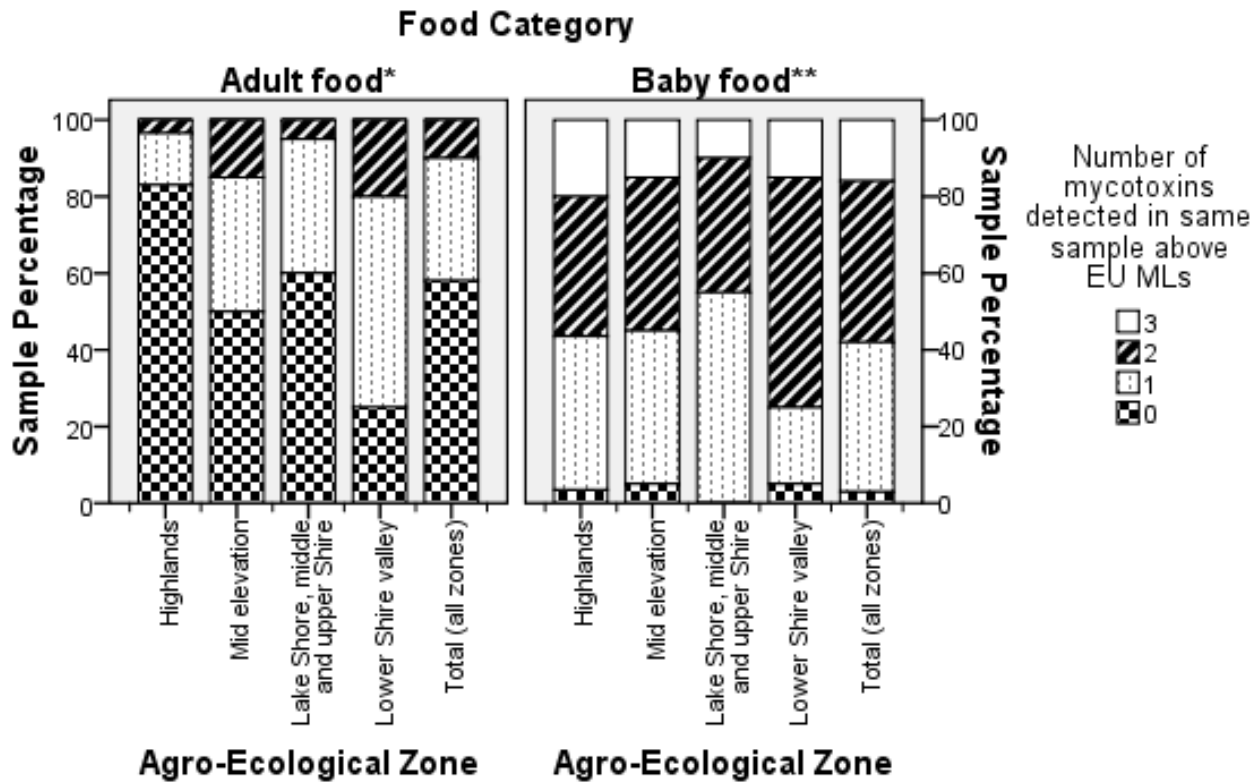


Figure 2.4: Percentage of samples containing either AFs (AFB1, AFB2, AFG1 and AFG2), FBs (FB1+FB2), DON, ZEN; or a combination thereof in levels above EU regulatory limit in different agro-ecological zones. \*Maximum limits as covered by Commission Regulation (EU) No. 165/2010 and (EC) No. 1126/2007 specifically for adult food. \*\* Maximum limits as covered by Commission Regulation (EU) No. 165/2010 and (EC) No. 1126/2007 specifically for infants and baby food.

The differences in occurrence patterns of the metabolites across the agro-ecological conditions presented in this study are attributable to the differences in the climatic zones. The low-lying agro-ecological zones (lake shore and lower Shire River valley agro-ecological zones) have all-year round high ambient temperatures and humidity. Rainfall in these zones is often erratic during silking and grain filling stages of maize crop particularly in lower Shire River valley. These conditions predispose maize to fungal infection and mycotoxin production including AFs in the field and during storage (Jones et al., 1980; Cotty and Jaime-García 2007). On the other hand, the highlands experience heavy conventional rainfall during the growing season and maturation, harvesting and drying of maize coincides with relatively cold winters with periodic showers. The mid elevation zones experience moderate rainfall spread through the growing season. Maize harvesting coincides with relatively moderate temperature dry winters.

AFs are produced optimally at temperatures between 28 and 30 °C (OBrian et al., 2007); CIT between 25 and 30 °C (Wu et al., 1974); and kojic acid between 30 and 35°C (Davis, 1963). These optimal temperatures are typical for low-lying agro-ecological zones of the lake shore and lower Shire River valley and are generally higher than average temperatures for the highlands. This explains the absence of AFs, CIT and kojic acid in the samples collected in the highlands. These findings agree with the low prevalence of AFs and CIT in Europe (which is a cold region) (Alborch et al., 2012; EFSA, 2012; Battilani et al., 2012; Streit et al., 2013). As for FBs, the low incidence in the cool agro-ecological zone is similar to studies from the USA (Murphy et al., 1993). FBs are produced optimally under a wide range of temperatures (20-28 °C) (Alberts et al., 1990). It is therefore not surprising that despite significant mean concentration differences across the zones, high FBs concentrations were widespread.

Similarly, beauvericin, DON, aurofusarin, fusaric acid, fusaproliferin, NIV and ZEN, which were found in significantly higher concentrations in the cool highlands than in the hot agro-ecologies in the current study are in agreement with the existing data. Much higher concentrations of these metabolites have been reported for several cereals from European countries where temperatures are much lower (Uhlig et al., 2004; Jestoi, 2008).

Interestingly, despite the significant differences in DON and NIV concentrations across the agro-ecologies, there were no apparent differences in co-occurrence patterns for these two metabolites. Co-occurrence of DON and NIV was observed in 85% of the DON positive samples. In 95% of the NIV positive samples, DON concentrations were higher than NIV and the ratio of DON level/NIV level varied between 1.5 and 552.0 (median 23.5). On the other hand, in 4% of these samples, the NIV/DON ratio varied from 1.3 to 5.2, and NIV was detected in absence of DON only in one sample. This pattern is similar to that observed by the EFSA CONTAM Panel (EFSA, 2013). Similarly for FBs, an occurrence ratio of 383: 112: 39: 1 was observed for FB1: FB2: FB3: hydrolyzed FB1. Furthermore, with an exception of one sample which contained a higher FB2 concentration than FB1, in all tested samples the concentration of FB1 > FB2 > FB3 > hydrolyzed FB1. The occurrence ratio for FB1: FB2: FB3 found in this study (10:3:1) is consistent with an evaluation of natural occurrence of FBs made by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), (JECFA, 2002).

As regards to the general co-occurrence patterns of some metabolites, about 80% of the AFB1 positive samples were also contaminated with cyclopiazonic acid. Production of cyclopiazonic acid, aflatoxin A, AFB1 and AFB2 has been reported to be regulated by the same gene (*veA*) in *Aspergillus flavus* (Duran et al., 2007). However in the present study, aflatoxin A was not detected and there were no significant correlations between cyclopiazonic acid and AFB (AFB1 and AFB2) which may suggest differences in fungal strains and environmental factors between the two studied regions (present study and that reported by Duran et al., 2007).

Aflatoxin M1 (AFM1), a potent carcinogen (Cullen et al., 1987), occurred in at least 35% of the samples collected from hot agro-ecologies and AFM1 concentration ranged up to 22 µg/kg. All the samples (N=17) that had AFM1 also contained Aflatoxin Bs and Gs. A statistically significant correlation ( $P < 0.01$ ;  $R^2 = 0.92$ ) was observed between the AFM1 and the total AF concentration with a regression equation as follows:  $AFM1 = 0.023 * \text{Total AFs}$ . AFM1 is a hydroxy-metabolite form of AFB1 that has mostly been reported in animal products (Mohammadi, 2011), but recently there has been rise of reports on occurrence of the metabolite in plant products (Sulyok et al., 2007; Ezekiel et al., 2012b; Streit et al., 2013). *A. flavus* and *A.*

*parasiticus* are known to produce AFM1 through a synthesis chain that does not involve AFB1 (Dutton et al., 1985; Yabe et al., 1988; Yabe et al., 2012). The presence of AFM1 in plant tissues has not been thoroughly studied. The AFM1 detected in the present study may have been produced by the *Aspergillus* strains (Yabe et al., 2012) or may have resulted from AFB1 metabolism by insect pests nourishing on AFB1 contaminated grains (Lee and Campbell, 2000) or both. The most important insect pests of maize in sub-Saharan Africa include stem borer in the field, and both the maize weevil (*Sitophilus zeamais*) and larger grain borer (*Prostephanus truncates*) in storage (Tefera et al. 2010). Unfortunately the current study did not investigate if these pests were connected to the AFM1 contamination. All the AFM1 levels reported herein exceeded the maximum tolerable level range (0.05 to 0.5 µg/kg) for milk, milk products and meat that existed in 58 countries (97% of countries that had AFM1 regulations) at the end of 2003 (FAO. 2004). It is noteworthy that AFM1 limits being referred to here were set for contamination in foods of animal origin in which the levels of contamination are relatively lower than found in cereals. However, given the significant correlation highlighted it may not be necessary to establish separate AFM1 regulations for cereals such as maize as adhering to aflatoxin B and G regulations could already keep AFM1 low in human diet.

### 2.3.2. Dietary exposure estimates and risks

Maize is a main staple food for the population across the four agro-ecologies of Malawi with per capita consumption of 382 grams/day (Ecker and Qaim, 2011). It is mostly consumed as a thick porridge (*nsima*), which is prepared from fine flour and normally served with relish during lunch and supper. The flour is made from either dehulled or non-dehulled maize. However in Malawi there is currently no data on how much each kind of the maize flour is consumed, as such mycotoxin exposure estimates were done with the assumption that the maize was consumed non-dehulled. Mycotoxin dietary exposure estimates reflected the occurrence data that have been shown in Table 2.1. For instance, for all the samples from lake Shore and lower Shire River valley, 95% of samples from mid elevation and 70% samples from highlands FBs (B1+B2+B3) daily intakes estimates were higher than the Joint FAO/WHO Expert Committee on Food Additives' (JECFA) and the Scientific Committee for Food of the European Commission's (SCF) guided provisional maximum tolerable daily intake (PMTDI) or tolerable daily intake



(TDI) of 2.0 µg/kg body weight (bw)/day (SCF, 2003) (Figure 2.5). These FB dietary exposures are comparable to estimates made for populations in Former Transkei, South Africa (Shephard et al., 2013). While DON daily intake dietary estimates for Former Transkei, South Africa were found to be below TDI, daily intake associated with 90% of samples collected from the cool highlands in Malawi and at least 50% of the samples from mid elevation, lake shore middle and upper shire and lower Shire valley agro-ecologies, were well above the JECFA's PMTDI of 1.0 µg/kg bw/day for DON (JECFA, 2002; SCF, 2002). Estimated daily intakes for more than 10% of the samples from mid elevation and 28% from highlands were greater than EFSA CONTAM Panel's TDI of 1.2 µg/kg bw/day for NIV (EFSA, 2013). For at least 20% of samples from highland and mid elevation zones, the daily intakes for ZEN exceeded JECFA's PMTDIs of 0.5 µg/kg bw/day (JECFA, 2000). Due to an inadequate data base, the EFSA (CONTAM Panel) set a level of no concern for nephrotoxicity at 0.2 µg/kg bw/day for CIT (EFSA, 2012). However, this was exceeded for more than 50% of the samples collected from the hot lake shore middle and upper shire and lower Shire valley agro-ecological zones. For samples collected from the lake shore middle and upper shire, a daily intake of as high as 56.5 µg/kg bw/day for CIT was estimated. As for alternariol, despite the relatively low occurrence (Table 2.1), all daily intake estimates (11-110 ng/kg bw/day) were far above the threshold of toxicological concern (TTC) decided by EFSA CONTAM Panel of 2.5 ng/kg bw/day (EFSA, 2011a). For total AFs, estimated median daily intakes for hot lake shore and lower Shire River valley zones were 1.2 and 0.4 µg/kg bw/day respectively and ranged up to 5.6 µg/kg bw/day (lake shore).

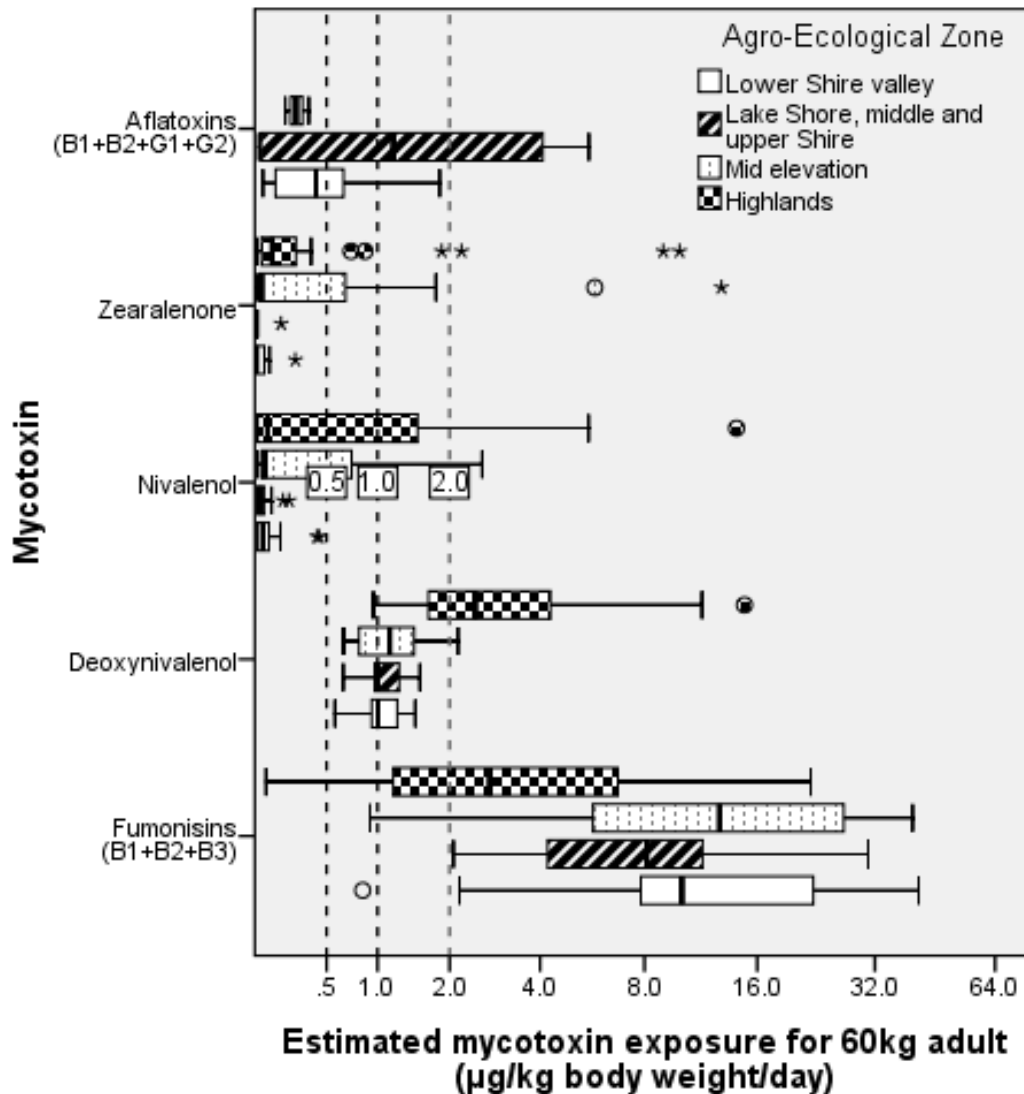


Figure 2.5: Box and whisker plot showing mycotoxin exposure estimates for a 60 kg adult ( $\mu\text{g}/\text{kg}$  body weight (bw)/day) assuming an ingestion of 382g of maize flour made from the analysed samples. A vertical line within the box represents the median. The front and back ends of the box represent first and third quartiles, respectively. The front and back whiskers extend from the box to the smallest or largest non-outliers in the data set (relevant quartile  $\pm 1.5 \times$  (interquartile range, IQR)). Circles depict mild outliers ( $1.5 \times$  IQR) and asterisks depict extreme outliers ( $3 \times$  IQR). Vertical dotted lines indicate JECFA's PMTDIs for zearalenone ( $0.5 \mu\text{g}/\text{kg}$  bw/day), DON ( $1.0 \mu\text{g}/\text{kg}$  bw/day) and FBs (sum of fumonisin B1 and B2) ( $2.0 \mu\text{g}/\text{kg}$  bw/day). TDI derived by the EFSA CONTAM Panel for nivalenol is  $1.2 \mu\text{g}/\text{kg}$  bw/day (not shown).

FBs dietary exposure is linked to oesophageal cancer (Rheeder et al., 1992; Sun et al., 2007) and Malawi has the highest prevalence rate (24.2 per 100,000 population) of oesophageal cancer in the world (Ferlay et al., 2013). Therefore the high FBs daily intakes estimated in the present study could be linked to high incidence of oesophageal cancer in Malawi. Unfortunately until now there has not been any attempt to study if the two are linked in Malawi. As for the high CIT daily intakes, it could be a contributing factor to the high HIV-associated nephropathy prevalence in Malawi (Struik et al., 2011). As for AFs, the dietary exposures found in the hot agro-ecological zones are worrisome since as little as <1 ng/kg bw/day can induce liver cancer (SCF, 1994), a reason behind JECFA's failure to establish a numerical TDI for AFs (JECFA, 1999). Nonetheless, according to the risk characterization by EFSA, (2007), MOEs for AFB1 (derived using BMDL for 10% increased cancer risk using rodent data (170 ng kg/body weight/day)) of less than 10000 indicate a health concern. In present study mean MOEs for highlands; medium elevation; lake shore, upper and mid Shire River valley; and lower Shire River valley agro-ecological zones were estimated to be 136; 117±11(mean±SE); 56±15 and 25±11 respectively. These MOEs are generally smaller (more risky) than most MOEs estimated for other populations elsewhere as summarized by (Andrade et al., 2013) probably due to high maize consumption.

Toxicological evaluations of most metabolites found in the present study have not been thoroughly performed due to lack of sufficient exposure assessment data; as such PMTDI or TDI have not been established. Worse still, incidence data for most of these metabolites are also scarce. In fact in some cases non-regulated metabolites have proven to be more toxic than mycotoxins addressed by regulatory limits. For instance, DAS (found in 37% of the tested samples) has been found to be more toxic than DON and NIV (D'Mello et al., 1999) however it has received less attention perhaps due to its low prevalence. Nonetheless, an extensive review of toxicities of some of these has been provided by Streit et al., (2013) and an additional summary together with incidence data from elsewhere have been provided in Table (2.3) for comparison with the current data. It is important to stress that the mycotoxin daily intake estimates presented in the present study could be relatively higher than for many other regions due to the high dependency on maize by Malawians. For instance despite comparatively higher incidence of

ZEN in Canada, Denmark and Norway and the USA, daily intakes estimated in the present study were higher than the population exposure estimates for Canada, Denmark and Norway (20 ng/kg bw/day) and the USA (30 ng/kg bw/day) (Zinedine et al., 2007). In Malawi maize porridge is an important complementary and weaning food (Hotz and Gibson, 2001), as such much higher dietary intakes of the toxins in babies/infants are expected due to their low bodyweights. Worse still, babies/infants are more susceptible to toxins due to their higher metabolic rate and lower detoxification ability (Sherif et al., 2009). Moreover some mycotoxins have already been linked with malnutrition in children. Notably, aflatoxins are known to cause decreased transport of soluble nutrients (Fink-Gremmels, 2008), disrupt protein, carbohydrate and lipid metabolism (Cheeke and Shull, 1985), alter growth factor expression and impair child growth (Gong et al 2008; Khlangwiset et al., 2011). Similarly Kimanya et al. (2010), reported that FB exposure through maize based complementary food was inversely associated with linear growth of infants in Tanzania. It is likely that many more metabolites that were detected in the present study would have similar effects. Therefore, the incidence of mycotoxins in the maize may exacerbate the incidence of stunting among children estimated at 50% in Malawi (ORC Macro, 2006).

The safety evaluations of the maize food samples in the current study are complicated due to co-occurrence as toxicological synergism exists among the metabolites (Speijers and Speijers, 2004; Pedrosa and Borutova, 2011). Mycotoxin synergisms among regulated and non-regulated toxins have been observed. Notably, kojic acid augments the toxicity of AFB1 (Dowd, 1988); beauvericin interacts synergistically with FB1 (Klarić et al., 2006) and DON (Ruiz et al., 2011); fusaric acid interacts synergistically with DON (Smith et al., 1997) and FB1 (Bacon et al., 1995); culmorin augments the toxicity of DON (Dowd et al., 1989). In addition, some metabolites detected in this study are just emerging and their toxicokinetics remain largely unexplored. Therefore, it is difficult to evaluate the overall toxicological effects of the co-occurring metabolites found in this study. The co-occurrence of as much as 41 different metabolites in the same foodstuff and the health effects deriving from the exposure to such a cocktail of fungal metabolites require further investigations.

Table 2.3. Occurrence data, effects and toxicities of some less well-known metabolites

Metabolite	Occurrence	Some effects and toxicities
3-Nitropropionic acid	71-682 µg/kg in 100% of fonio millet samples from Nigeria (Ezekiel et al., 2012a); up to 392 µg/kg in 23% of feed and feed ingredients from Austria (Streit et al., 2013)	Induces excitotoxicity (Hamilton et al., 1987); reduces motor performance (Beal et al., 1993; Roitberg et al., 2002); irreversibly inhibits succinate dehydrogenase thereby, negatively affecting oxidative energy production (Scarlet et al., 2003).
Apicidin	Up to 21 µg/kg in 90% of poultry feeds samples from Cameroon (Abia et al. 2012); up to 160 µg/kg in 55% of feed and feed ingredients from Austria (Streit et al., 2013)	Causes death of experimental rats accompanied with weight loss; hemorrhage in the stomach, intestines, and bladder (Park et al., 1999).
Aurofusarin	Up to 4.2 mg/kg has been reported in wheat (Kotyk and Trufanova, 1998).	Lowered docosahexaenoic acid proportion in the phospholipid, cholesteryl ester and free fatty acid fractions of the egg yolk (Dvorska et al., 2003).
Butenolid	Up to 430 µg/kg in wheat and barley samples from Japan (Yoshizawa, 1984); up to 1490 µg/kg in 52% of feed and feed ingredients of samples from Austria (Streit et al., 2013).	Induces rat cardiotoxicity and causes marked oxidative damage in myocardial cells (Mei et al., 2009).
Chaetoglobosin A	Up to 130 mg/kg in wooden wall scrapings from Slovakia and Austria (Vishwanath et al., 2009).	Demonstrated cytotoxic effects in chick embryos (Ohtsubo et al. 1978; Veselý et al. 1995).
Cyclopiazonic acid	Up to 9 mg/kg in 80% maize samples from Indonesia (Widiastuti et al., 1988).	caused pathological injuries in livers and kidneys of experimental chickens (Malekinejad et al., 2011).
Curvularin	Up to 19 µg/kg in 85% in poultry feeds samples from Cameroon (Abia et al., 2013); up to 484 µg/kg in 35% of feed and feed ingredients samples from Austria (Streit et al., 2013).	Produced hepatic necrosis in experimental rats (Rout et al., 1989).
Diacetoxyscirpenol	Up to 0.8, 51, 118 and 120 µg/kg in various cereals from Turkey, Nigeria, Poland and Brazil respectively (Yazar and Omurtag, 2008); up to 31500 µg/kg in 6% of tested samples across the world (Bottalico, 1998).	More toxic than deoxynivalenol and nivalenol (D'Mello et al., 1999); synergistic interaction with fusarenon X and butenolid has been demonstrated (Bhavanishankar et al., 1988); causes immunosuppression and reproductive and developmental toxicity (Pronk et al., 2002).
Emodin	Up to 38 µg/kg in 100% of poultry feeds samples from Cameroon (Abia et al., 2013); up to 1570 µg/kg in 89% of feed and feed ingredients from Austria (Streit et al., 2013).	Has demonstrated genotoxic potency (Mueller et al., 1999).

Table 2.3(continue). Occurrence data, effects and toxicities of some less well-known metabolites

Metabolite	Occurrence	Some effects and toxicities
Equisetin	Up to 25µg/kg in 100% of poultry feeds samples from Cameroon (Abia et al., 2012); detected up to 2833µg/kg in 100% of fonio millet samples from Nigeria (Ezekiel et al., 2012a).	Has anti-HIV integrase activity (Hazuda et al., 1999; Singh et al., 2005); toxic to mitochondria through inhibition of substrate anion carriers (Konig et al., 1993)
Fusarenon X	In cereals at levels up to 1000µg/kg (Pronk et al., 2002). Absent in poultry feeds samples from Cameroon (Abia et al., 2013).	Possesses antibiotic properties (Kobayashi et al., 1995).
Fusaric Acid	Up to 295µg/kg in 63% of fonio millet samples from Nigeria Nigeria (Ezekiel et al., 2012a); up to 13,593 µg/kg in 22% of feed and feed ingredients from Austria (Streit et al., 2013).	Altered rat brains and pineal neurotransmitters resulting lethargy (Porter et al., 1995); depressed weight gain of immature pigs when fed together with deoxynivalenol (Smith et al., 1997); interacts synergistically with FBs in chicken eggs (Bacon et al., 1995).
Griseofulvin	Up to 12 µg/kg in 19% of fonio millet samples from Nigeria (Ezekiel et al., 2012a); up to 399 µg/kg in 5% of feed and feed ingredients from Austria (Streit et al., 2013).	Disturbs porphyrin metabolism in humans (Knasmüller et al., 1997).
Kojic acid	Up to 7480 µg/kg in 97% of peanut cake samples from Nigeria (Ezekiel et al., 2012b); 35-61353 µg/kg in 100% of poultry feeds samples from Cameroon (Abia et al., 2013); up to 3172 µg/kg in 10% of feed and feed ingredients from Austria (Streit et al., 2013).	Augments effects of aflatoxins B1 (Dowd, 1988).
Moniliformin	Up to 0.95mg/kg and 2 mg/kg in cereal grains from Norway and Austria respectively (Filek and Lindner, 1996; Uhlig et al., 2004); up to 3 µg/kg in 38% of fonio millet samples (Ezekiel et al., 2012); up to 774 µg/kg in 50% maize samples from USA (Gutema et al., 2000).	Causes cardiotoxicity of chickens (Nagaraj et al., 1996); linked to Keshan disease, a human myocardial impairment (Chen et al., 1990); <5 µM selectively inhibited mitochondrial pyruvate and α-ketoglutarate oxidations by 50 per cent (Thiel, 1978).
Rugulosin	Up to 76 µg/kg in 70% of poultry feeds samples from Cameroon (Abia et al., 2013).	Causes retardation of growth in budworms (Miller et al., 2008); causes chronic toxicity and hepatocarcinogenicity in mouse (Ueno et al., 1980).
Sterigmatocystin	Up to 83 µg/kg in 25% of barley, wheat, buckwheat and rye samples from Latvia (Veršilovskis et al 2008).	Is a class 2B carcinogen (IARC, 1987).

## 2.4. Conclusions

This study demonstrated that the mycotoxin distribution pattern is influenced by the micro-climatic conditions in Malawi. Significant metabolite quality and quantity differences existed across the four agro-ecological zones of Malawi with the hot agro-ecology of the lower Shire River valley being the most risky zone and the cool zones being the least risky as far as the number of metabolites found in a sample and concentrations are concerned. Therefore risk assessors need to continuously monitor occurrence of these metabolites and develop and promote site (agro-ecological) specific pre- and post-harvest management strategies in order to reduce mycotoxin contamination and likely dietary risks. Limiting maize production to cool and warm climatic zones and sparing the hot climate zones for less susceptible crops such as cassava (CAST, 2003) and shifting from monotonous maize diets to more diverse diets that could increase the intake of mycotoxin counteracting food components such as antioxidants (Galvano et al., 2001) could be feasible options.

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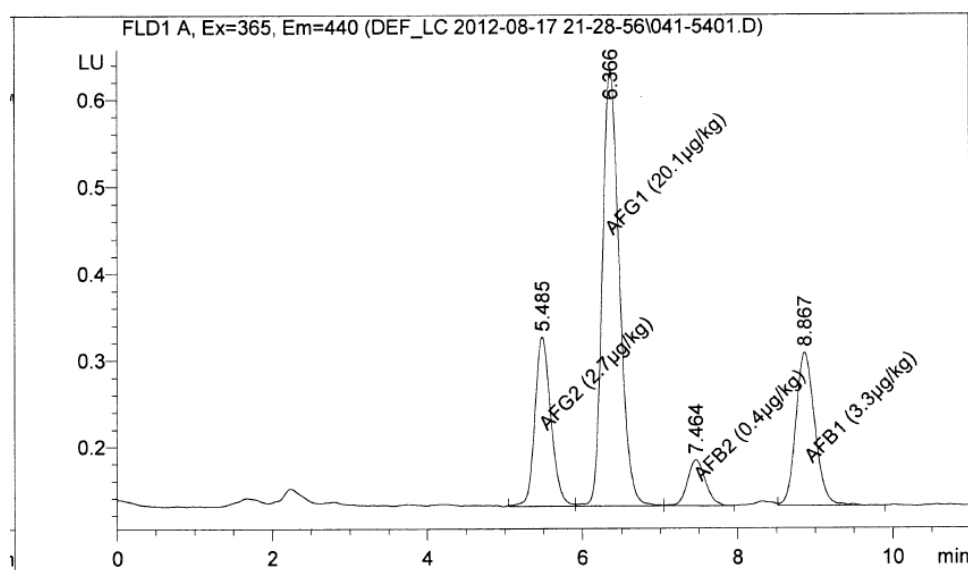
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It is generally accepted that Aflatoxin B1 (AFB1) dominates natural occurrence among the other aflatoxins. Moreover, it has been reported that AFB1 concentration generally exceeds half of the sum of the aflatoxins and that other aflatoxin analogs occur in lower concentrations. In the same regard several countries have set separate regulatory limits for AFB1 at half the regulatory limit of the sum of the four aflatoxins and likewise analytical methods for quantifying AFB1 alone in various matrices have been developed. Several sub-Saharan countries use this theory to regulate the aflatoxins. Building on the discussion from Chapter 1, the following chapter uses a case study of Malawi to verify the theory and analyze the feasibility of using such an approach in the regulation of aflatoxins. Chapter 3 is reproduced from the accepted manuscript ‘**Uncommon occurrence ratios of aflatoxin B1, B2, G1 and G2 in maize and groundnuts from Malawi**’ (Mycotoxin Research) written by Matumba, L., Sulyok, M., Njoroje, S.M.C., Ediage, E.N., Van Poucke, C., De Saeger, S. and Krska, R.



## **Chapter 3: Uncommon occurrence ratios of aflatoxin B1, B2, G1 and G2 in maize and groundnuts from Malawi.**

### **3.1. Introduction**

Aflatoxins are toxic and carcinogenic polyketide-derived secondary metabolites that are produced mainly by certain strains of the *Aspergillus* genus on a wide range of matrices. Most reports have indicated *Aspergillus flavus* and *A. parasiticus* as major aflatoxin producers but discovery of more novel aflatoxin producers continues (Horn, 1997; Ito et al., 2001; Peterson et al., 2001; Pildain et al., 2008; Varga et al., 2012). Four major naturally occurring aflatoxins include aflatoxin B1 (AFB1), AFG1, AFB2 and AFG2 (in order of decreasing toxicity) (IARC, 1993). *A. flavus* normally produces aflatoxins Bs, while *A. parasiticus* produces both aflatoxin Bs and Gs. Other important species that produce both aflatoxins B and G include *A. toxicarius*, *A. nomius*, *A. bombycis*, *A. parvisclerotigenus*, *A. minisclerotigenes* and *A. arachidicola* (Varga et al. 2009).

The biosynthetic pathway of aflatoxins has been extensively studied and elucidated (Yabe et al., 1988, 2003, 2004; Yu et al., 2004, 2012) and has been estimated to involve up to 27 enzymatic steps (Ehrlich, 2009). It has been found that the different forms of aflatoxin share a common pathway that later branches to form AFB1, AFB2, AFG1, and AFG2. It was established through feeding studies that AFB1 and AFG1 (both containing dihydrobisfuran rings) are produced from O-methylsterigmatocystin and that AFB2 and AFG2 (both containing tetrahydrobisfuran rings) are produced from dihydro-O-methylsterigmatocystin (see Annex III) (Bennett and Goldblatt, 1973; Bhatnagar et al., 1987; Yabe et al., 1988, Yu et al., 2004). Experimental results further demonstrated biosynthetic independence of AFB1 and AFB2 (Bhatnagar et al., 1987; Yabe et al., 1988) and AFG1 and AFG2 (Yabe et al., 1999).

Different generalized occurrence ratios of the four aflatoxins have been reported (Van Egmond and Jonker, 2004; Kensler et al., 2010; EC, 2012), but all agree that the AFB1 concentration generally exceeds half of the sum of the aflatoxins and that AFB2 and AFG2 occur in the lowest concentrations. In the same regard several countries have set separate regulatory limits for AFB1 at half the regulatory limit of the sum of the four aflatoxins (Van Egmond and Jonker, 2004). Likewise analytical methods for quantifying AFB1 alone in various matrices have been developed (Lee et al., 2004; Ardic et al., 2008; Yu et al., 2013).

Experimental findings indicate that the ratio of aflatoxin B and G concentrations are greatly influenced by temperature cycling (Lin et al., 1980; Schmidt-Heydt et al., 2010) and population ratios of fungal strains on given matrices (Wilson and King, 1995). Furthermore, gene cluster analysis of AFG1 dominant *A. parasiticus* (ratio AFG1/AFB1 > 5) revealed a history of mutation (Carbone et al., 2007). These findings imply that AFB and AFG concentration ratios could be regionally dependent, however there is hardly any occurrence data on this aspect. In this regard the present study was undertaken to investigate the occurrence ratios among aflatoxin B1, B2, G1 and G2 and get insights about the types of aflatoxigenic fungi present in Malawi.

### **3.2. Methodology**

A meta-analysis was done on aflatoxin (AFB1, AFB2, AFG1 and AFG2) positive results of raw maize and raw groundnuts samples from the lake shore middle and upper-Shire-, mid-elevation-, lower-Shire valley agro-ecological zones and unspecified locations within Malawi (Figure 2.1, Chapter 2). This included analytical results of 80 raw groundnuts and 125 raw maize samples measured by immunoaffinity column clean-up coupled with high performance liquid chromatography and on-line post-column photochemical derivatization-fluorescence detection (IAC-HPLC-PCD-FLD) and 31 raw maize samples measured by LC-MS/MS (Aflatoxin positive samples discussed in Chapter 2). The IAC-HPLC-PCD-FLD and LC-MS/MS methodology used for aflatoxin analysis were similar to those described by Matumba et al. (2014) and Warth et al. (2012) respectively. In all cases the aflatoxin analysis involved a sub-sample drawn from a milled aggregated sample of at least 1 kg mass. In terms of proportions, the dataset comprised of 39.0% of samples from upper-Shire agro-ecological zone, 22.5% from mid elevation agro-ecological zone, 21.6% from lower-Shire agro-ecological zone and 16.9 % from unspecified locations.

Limits of quantification (LOQ) of the analytical method included in this meta-analysis were 0.2 µg/kg for AFB1 and AFG1, and 0.1 µg/kg for AFB2 and AFG2 (IAC-HPLC-PCD-FLD). For the LC-MS/MS method LOQs for each of the four AFs were 1.3 µg/kg. Quality control in the aflatoxin IAC-HPLC-PCD-FLD analyses was achieved by the use of naturally contaminated reference materials (Product #: TR-A100, Batch #: A-C-268, R-Biopharm AG, Darmstadt, Germany). Further, randomly selected samples previously analysed by IAC-

HPLC-PCD-FLD shown to have concentrations of AFG1>AFB1 were re-analyzed by LC-MS/MS and results were comparable.

Aflatoxin data were not normally distributed, and were log transformed for statistical analysis (log AFB1+1, log AFG1 +1). The difference between the means was assessed by analysis of variance (ANOVA) or t-test. All the analyses were performed using SPSS® (version 16) software (SPSS Inc., Chicago, Illinois, USA). The level of confidence required for significance was set at  $P \leq 0.05$ .

### **3.3. Results and discussion**

Although a significant proportion of the aflatoxin positive samples included in the study originated from the mid elevation agro-ecological zone (22.5%), it is worth noting that this does not reflect the aflatoxin prevalence in the zone as at all times the majority of the samples that were tested were collected from the mid-altitude which is the main maize and groundnuts producing area in Malawi. In fact, the aflatoxin problem is more prominent in the lower Shire and the lake shore and upper-Shire agro-ecological zones than in the mid-elevation and highlands (Figure 2.1, Chapter 2).

All samples that tested positive for aflatoxin contained AFB1. This AFB1 co-occurred with AFG1, AFB2 and AFG2 in 95.3, 87.7, 78.8% of the samples respectively (Table 3.1). With exception of 3 samples (1.3%) where AFG2 co-occurred only with AFB1 and AFG1, in all samples AFG2 co-occurred with the three other toxins.

Table 3.1: Co-occurrence matrix for aflatoxin in raw-maize and groundnut samples from Malawi

	AFB1	AFB1+ AFB2
No AFGs	4.7%	1.7%
AFG1	95.3%	86.0%
AFG2	78.8%	77.5%
AFG1+AFG2	78.8%	77.5%

The levels of AFB1, AFB2, AFG1 and AFG2 ranged to 592 µg/kg, 54 µg/kg, 412 µg/kg, and 65 µg/kg, respectively (Annex IV). The mean levels of AFB1, AFB2, AFG1, and AFG2 levels for the samples were 44.7±79.7 (mean±SD); 5.7±8.5; 42.0±68.0 and 6.6±11.2 µg/kg. It is however interesting to note that due to the spread of the aflatoxin data, the Mean AFG1/AFB1 for all samples (groundnuts and maize) was found to be 2.0±3.2 (mean±SD) while as the mean AFB1/AFG1 was 2.7±8.8. There were no significant differences between mean AFG1/AFB1 or AFB1/AFG1 for maize and groundnuts and between products sampled in 2011 and 2012. Interestingly, in 110 samples (47%), the AFG1 concentration exceeded AFB1 (Figure 3.1 and 3.2) and in 42.8% of the samples AFG1 contributed to over half of total AF concentration. AFB1 and AFG1 concentration always exceeded AFB2 and AFG2, respectively. The mean relative percentages to which AFB1, AFB2, AFG1, and AFG2 contributed to the total aflatoxin content (100%) were 47%, 5%, 43%, and 5%, respectively (Figure 3.3).



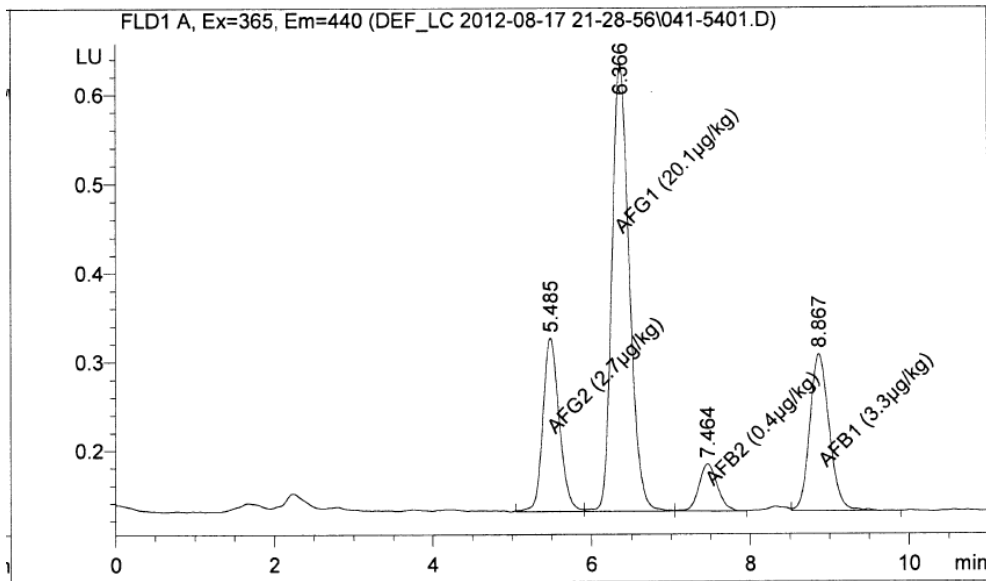


Figure 3.1: Example chromatogram (HPLC-FLD) of maize sample extract revealing a much higher concentration of AFG1 than AFB1.

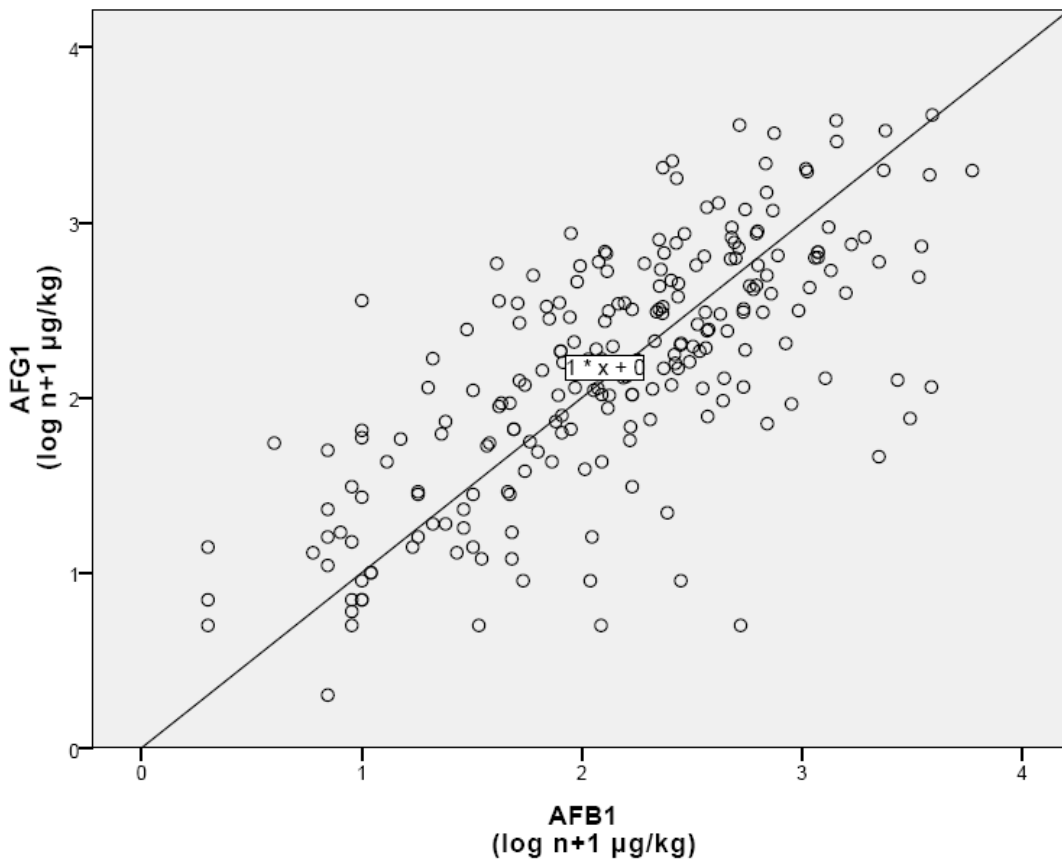


Figure 3.2. Levels and relationship between aflatoxin B1 and aflatoxin G1 in maize and groundnut samples from Malawi (n=236). Linear regression line ( $Y=1*X$ ) indicates equal levels of aflatoxin B1 and aflatoxin G1. Points above the regression line indicate aflatoxin G1 > aflatoxin B1. The regression line ( $Y=1*X$ ) is not a fit of the data points but rather a separator of points aflatoxin G1 > aflatoxin B1 and aflatoxin G1 < aflatoxin B1.

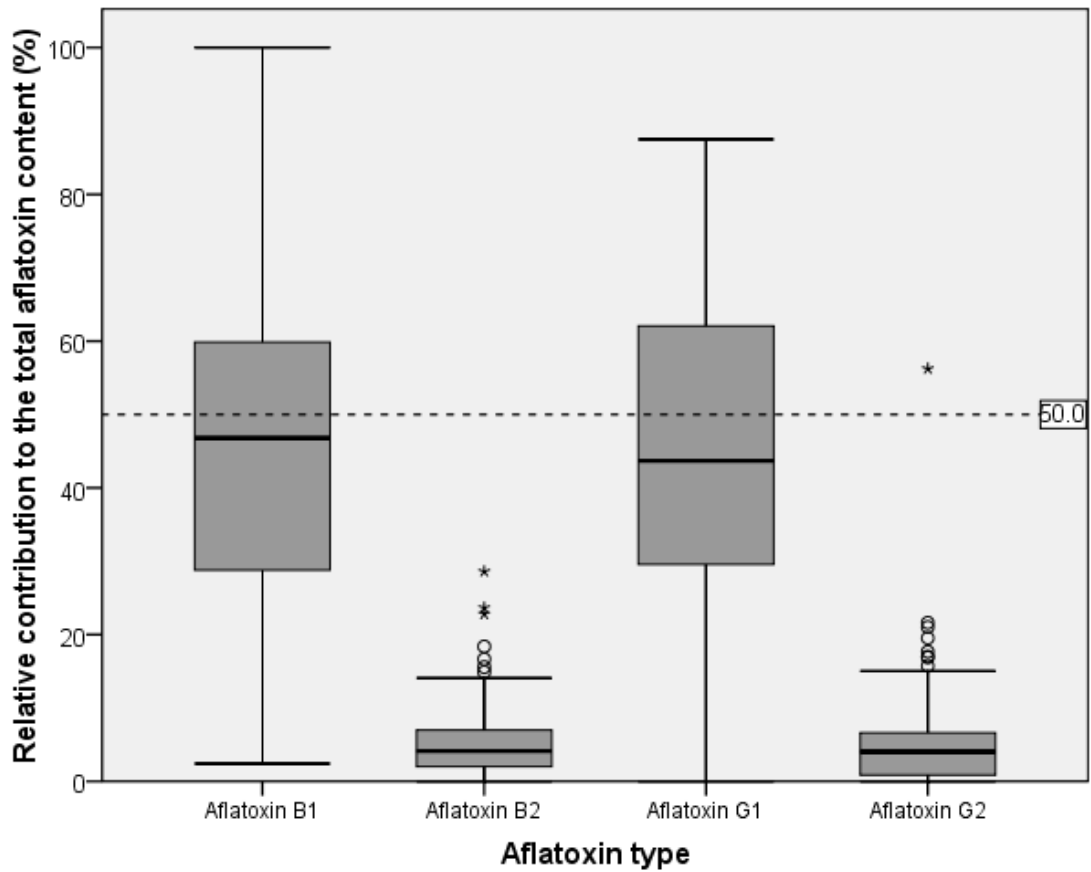


Figure 3.3: Relative contribution of AFB1, AFB2, AFG1 and AFG2 to the total aflatoxin content in raw maize and groundnut samples from Malawi. The horizontal line within the box represents the median. The bottom and upper ends of the box represent first and third quartiles, respectively. The bottom and upper whiskers extend from the box to the smallest or largest non-outliers in the data set (relevant quartile  $\pm 1.5 \times$  (interquartile range, IQR)). Circles depict mild outliers ( $1.5 \times$  IQR), asterisks depict extreme outliers ( $3 \times$  IQR). The dotted line represents a 50% contribution to the total aflatoxin content.

Out of the 236 AFs positive samples considered in this study, 182 had an AFs content higher than the Common Market for Eastern and Southern Africa's (COMESA) proposed regulatory limit for groundnuts and maize (10 µg/kg total AFs). If the AFB1 measurement was used to check compliance with the COMESA limit with an assumption that AFB1 ≥50% of the total AFs content, 14 samples with a total AF >10 µg/kg and AFB1 ≤5 µg/kg would have passed control. This would represent a 7.7% false negative rate. Similarly if the United States Food and Drug Administration's (U.S.FDA) limits for human food (20 µg/kg, total AF), grain intended for breeding livestock (100 µg/kg, total AFs), and grain intended for finishing swine of 45.4kg (100 pounds) or greater (200 µg/kg, total AF) (FAO, 2004) were to be estimated by AFB1 measurement, 13.4%, 24.2% and 25.5% false negative rates would have occurred respectively (Table 3.2). These results indicate that measurement of AFB1 alone may not satisfactorily be used to control the total AFs concentration in Malawi. In fact the European Commission (Decision 2002/657/EC) calls for a ≤5 % false negative rate for a screening technique to be acceptable (European Commission, 2002). Previously, Matumba et al, (2013) reported to have successfully screened shelled maize using the presence ≥4 bright greenish-yellow fluorescence (BGYF) grains per 2.5 kg maize sample as an indicator for total AF >10 µg/kg with a 4.4% false negative rate.

As shown in Table 3.2, the false negative rate increased as AFs limits increased from 10 to 200 µg/kg total AFs. This result indicates that AFG1>AFB1 phenomenon occurred more frequently at high AFs levels than at low levels (Table 3.2) which may signify that the AFG dominant producers in Malawi are also high AFs producers. *Aspergillus nomius* and *A. parasiticus* are among species that are known to generally produce high amounts of AFs however the former is considered to be rare in some geographical regions (Horn et al., 1996; Horn and Dorner, 1998; Tran-Dinh et al., 1999; Doster et al., 2009).

Table 3.2: Number of samples with total aflatoxin (AFB1+AFB2+AFG1+AFG2) level greater than the regulatory limit when aflatoxin B1 concentration was equal or less than half the regulatory limit and associated false negative rates.

Total AFs regulatory limit (AFB1+AFB2+AFG1+AFG2)	Number of samples with total AFs> regulatory limit	Number of samples with total AFs> regulatory limit and AFB1 ≤ ½ regulatory limit	False negative rate (%)
10 µg/kg <sup>a</sup>	182	14	7.7
20 µg/kg <sup>b</sup>	158	21	13.3
100 µg/kg <sup>c</sup>	62	15	24.2
200 µg/kg <sup>d</sup>	31	8	25.8

<sup>a</sup>Total aflatoxin (AFs) limit for human consumption as proposed by Common Market for Eastern and Southern Africa (COMESA); <sup>b</sup>Total aflatoxin limit for human consumption enforced by U.S.FDA; <sup>c</sup>Total AF limit for grain intended for breeding livestock enforced by U.S.FDA; <sup>d</sup>Total AF limit for grain intended for finishing swine of 45.4kg (100 pounds) or greater enforced by U.S.FDA.

The present findings differ with aflatoxin occurrence ratios reported in many surveys conducted in the world where aflatoxin B dominance is observed (Younis and Malik, 2003; Oliveira et al., 2009; Ghiasian et al., 2011; Adetunji et al., 2014; Haryadi and Setiastuty, 1994). However, similar patterns were reported in nuts of Brazilian origin (Olsen et al., 2008; Oliveira et al., 2009) where the concentration of AFB1 and AFG1 were comparable. In particular, Olsen et al. (2008) found the concentration of AFB1 and AFG1 to be 50/50 and through fungal isolation they concluded that *A. nomius* was responsible for the aflatoxin contamination of the Brazil nuts. This pattern is also apparent in a publication made on samples from neighboring Mozambique where average AFB1 and AFG1 concentrations were comparable (Warth et al., 2012).

Until now, aflatoxigenic fungal strains have not been fully characterized in Malawi. Monyo et al. (2012) only presented characterization information on aflatoxigenic *Aspergilli* by counting colony forming units on *Aspergillus flavus* and *parasiticus* Agar (AFPA). However from the current data, it seems likely that in addition to *A. flavus*, there are other *Aspergilli* strain(s) responsible for the high concentration of the G- aflatoxins. It could be further assumed that such strains are distributed across Malawi since no significant mean AFG1/AFB1 differences

were observed among the agro-ecologies of Malawi. One is tempted to speculate that the aflatoxigenic strains may be shared with neighboring Mozambique, hence the similarity of co-occurrence pattern of the aflatoxin analogs.

### 3.4. Conclusions

The present study has demonstrated that aflatoxin proportions in maize and groundnuts in Malawi generally differ from ratios reported globally. Aflatoxin Gs particularly AFG1 do occur in significant proportion comparable to that of AFB1. Given the great variability of AFG1/AFB1 ratios found in the present study it indicates that the quantification of AFB1 alone cannot be used to effectively estimate the concentration of total aflatoxins in Malawi. This study has further (from a discussion in Chapter 1) demonstrated the need for regions to develop mycotoxin regulations that fit their respective conditions rather than just borrowing. In this case a regulatory limit for AFB1 set at half the regulatory limit of the sum of the four aflatoxins may not be appropriate. Likewise aflatoxin quantitation should consider the total AFs and not only AFB1.

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## CHAPTER FOUR

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As introduced in Chapter 1, sub Saharan Africa economies are predominantly agricultural-based. Sub Saharan Africa exports a variety of agricultural commodities including foodstuffs to high value markets even when the countries themselves have deficits of the commodities. This is a survival strategy. Considering that importers set some minimum safety standards, products are sorted at farmer and industry level before the exportation. Through a meta-analysis conducted on mycotoxin data generated between June, 2012 and January, 2013 Chapter 4 tries to evaluate the impact the sorting has on the export commodities and local food. Chapter 4 is built from analytical data generated by Chitedze Mycotoxin Laboratory, Lilongwe, Malawi. This chapter is adopted from a submitted manuscript **‘Concentrating aflatoxins on the domestic market through groundnut export: A focus on Malawian groundnut value and supply chain’(Food Control)** written by Matumba, L., Van Poucke, C., Monjerezi, M., Ediage, E.N. and De Saeger, S.



## **Chapter 4: Concentrating aflatoxins on the domestic market through groundnut export: A meta-analysis of aflatoxin data.**

### **4.1. Introduction**

Groundnuts play an integral role in the livelihoods of the majority of the population in Africa through the provision of dietary nutrients and income (Diop et al., 2004; Gowda et al., 2009). In Malawi, smallholders account for over 90% of groundnuts production. The groundnuts are widely cultivated throughout the country but over 70% of the production comes from central Malawi (which predominantly falls under mid-elevation agro-ecological zone) (Figure 2.1, Chapter 2) (Nguluwe et al., 2001). Groundnuts account for 25 percent of household's agricultural income (Diop et al., 2004) and the local demand for the groundnuts exceeds the domestic production (Sintowe et al., 2010). In spite of this, the country formally exports more than 15% of its total production (Derlagen and Phiri, 2012). In fact, with the declining demand of tobacco (Malawi's main foreign exchange earner) on the international market, groundnuts rank top on the list of tobacco alternatives (Mataya and Tsonga, 2001) and are featured in the Malawi's National Export Strategy 2013-2018 (Government of Malawi, 2012). However, just like any other commodity groundnuts are prone to pre- and post-harvest toxigenic fungal colonization and mycotoxin contamination. Among the currently most significant mycotoxins (aflatoxins (AFs) ochratoxin A, patulin, fumonisins, zearalenone and some trichothecenes including deoxynivalenol), AFs are most frequently found in groundnuts (Siame et al., 1998; Bankole et al., 2006; Gonçalez et al., 2008; Ezekiel et al., 2012) and indeed aflatoxins were reported to be widespread in groundnuts across Malawi (Monyo et al., 2012 and also discussed in chapter 3).

As already highlighted in chapter 1, it is not possible to completely avoid aflatoxin contamination in food crops. In that respect, ways of removing of AFs contaminated nuts particularly through manual sorting have been extensively explored and methods for achieving low levels were optimized (Galvez et al., 2002, 2003). By following such methods exporters have been able to penetrate high value markets with very stringent aflatoxin regulatory limits (Derlagen and Phiri, 2012).

The present study was aimed at comparing levels of aflatoxins in groundnut samples found on the Malawian local markets against those that were intended for export to high value markets.

## 4.2. Methodology

A meta-analysis was performed on data of AF (AFB1, AFB2, AFG1 and AFG2) contamination levels of raw groundnuts and groundnut-based products analysed in Chitedze Mycotoxin laboratory (Lilongwe) between July 2012 and January, 2013 generated by the use of immunoaffinity column clean-up coupled with high performance liquid chromatography and on-line post-column photochemical derivatization-fluorescence detection (IAC-HPLC-PCD-FLD) (Method described by Matumba et al., 2014).

The samples included 69 raw groundnuts samples randomly purchased from informal local markets across Malawi (between July and August, 2012), 27 samples ( $\geq 1$  kg mass) intended for exports brought to Chitedze Mycotoxin laboratory between July 2012 and January, 2013 for quality control, and groundnut based-products purchased from the supermarkets in Lilongwe City, Malawi (in December 2012). The groundnut based-products included 14 cans of peanut (groundnut) butter manufactured in Malawi; 11 cans of peanut butter imported from South Africa; 15 packs of de-skinned roasted groundnuts and 9 packs of un-skinned roasted groundnuts (the latter was labelled on the packaging as 'export quality'). With exception to the peanut butters which were already fine, other samples were ground using a laboratory blender in whole before a sub-sample was drawn.

AFs data were not normally distributed, and were log transformed for statistical analysis. The difference between the means were assessed by analysis of variance (ANOVA) or t-test. All the analyses were performed using SPSS<sup>®</sup> (version 16) statistical software (SPSS Inc., Chicago, Illinois, USA). The level of confidence required for significance was set at  $P \leq 0.05$ .

## 4.3. Results and discussion

### 4.3.1. AFs contamination in raw groundnuts samples from informal markets

The incidence of AF (frequency, 64/69; maximum, 501  $\mu\text{g}/\text{kg}$ ; mean, 122  $\mu\text{g}/\text{kg}$  (Figure 4.1)) in the raw groundnuts from the informal markets were comparatively higher than the levels found in maize sampled in the same year (May, 2012) (Matumba et al., 2013). Surprisingly the high aflatoxin levels were also frequently observed in samples from the mid-altitude agro-ecological zone, unlike with the maize surveys where comparatively higher AFs incidences were observed in the lake shore middle and upper-Shire and lower-Shire valley agro-

ecological zones (Matumba et al., 2013 and Chapter 2). In fact there were no statistical differences in the mean AFs concentrations across these zones. This could be due to the fact that most of the groundnuts in Malawi are produced in the mid-altitude and since there are no mechanical groundnut shellers available to farmers. It is likely that farmers in the region are overwhelmed by the labour of hand shelling and thus engage in the risky practice of sprinkling groundnuts with water prior to hand shelling to soften the shells, an act that may increase the risk of AFs contamination in the groundnuts (Emmott and Stephens, 2012).

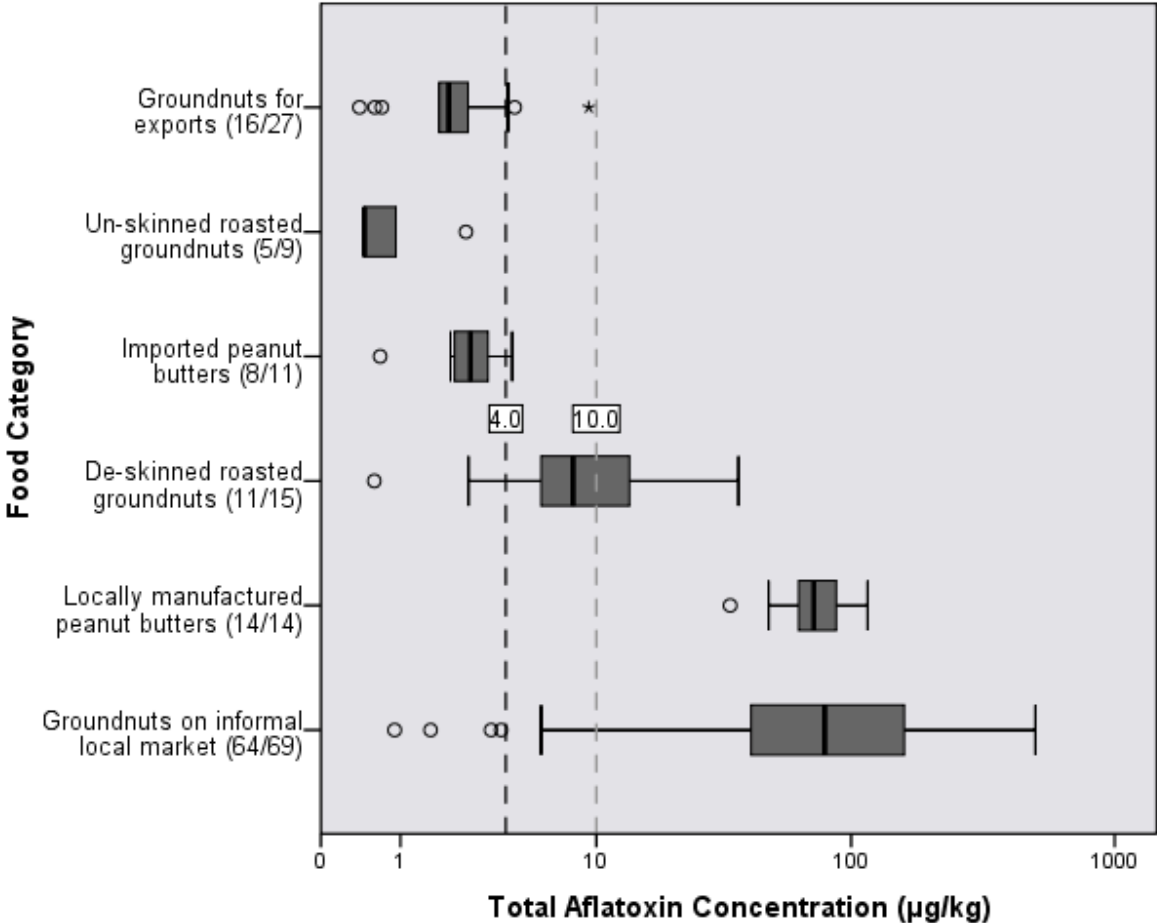


Figure 4.1: Distribution of total AFs (AFB1 + AFB2 + AFG1 + AFG2) in raw groundnuts samples found on informal local markets across Malawi, industrial processed groundnuts-based products marketed in Lilongwe City and samples of groundnuts intended for exports. A vertical line within the box represents the median. The front and back ends of the box represent first and third quartiles, respectively. The front and back whiskers extend from the box to the smallest or largest non-outliers in the data set (relevant quartile  $\pm 1.5 \times$  (interquartile range, IQR)). Circles depict mild outliers ( $1.5 \times$  IQR) and asterisks depict extreme outliers ( $3 \times$  IQR). Figures in parenthesis indicate the fraction of AF positive samples. Reference lines (dotted) indicate the following maximum level set for: total AFs in groundnuts ( $4\mu\text{g}/\text{kg}$ ) (EC, 2010) and AFs in ready-to-eat groundnuts set by Codex Alimentarius Commission ( $10\mu\text{g}/\text{kg}$ ) (CAC, 2004).

#### *4.3.2. AF contamination in groundnut-based products sampled from supermarkets and samples brought by groundnut exporters.*

Peanut butter production involves dry-roasting of raw shelled groundnuts at 140-160°C blanching, de-skinning, grinding and addition of other ingredients such as sugar, salt and stabilizers. Cumulatively, such processes have been reported to reduce AF concentration by as much as 89% (Siwela et al., 2011). However, in the present study, AF concentration in locally processed peanut butter ranged from 34.2 to 115.6 µg/kg and therefore does not fit for human consumption according to any existing regulation globally (FAO, 2004). Considering the level of AFs reduction during processing stated above, the present results indicate that peanut butter processors in Malawi use raw material with very high AFs contamination. It is also interesting to note that despite the fact that de-skinning of roasted groundnuts significantly reduces AFs content (Siwela et al., 2011), AFs levels in de-skinned groundnuts were comparatively higher than in un-skinned roasted groundnuts in this study (Figure 4.1). These results may be attributed to the grading of the raw groundnuts as raw material for processing different products. The AFs incidence suggests that heavily affected nuts were processed into the peanut butter, the intermediate into de-skinned nuts and the better quality nuts were sold as whole without de-skinning. In this case, the consumer would not visually detect mouldy nuts in the latter two products. On the other hand, 8 of the 11 imported peanut butter samples had significantly ( $P < 0.05$ ) lower total AFs content (maximum, 4.3 µg/kg; mean, 2.7 µg/kg) than locally produced ones. Ironically it is claimed that Malawi supplies 65% of South Africa's groundnut market (Government of Malawi, 2012). These results indicate that exported nuts are of better quality than what is sold on the local market. In fact this is corroborated by the fact that AFs levels in samples destined for exports had significantly lower levels than samples from the local informal market (frequency, 16/27; maximum 9.3µg/kg; mean, 2.6µg/kg) (Figure 4.1). Moreover the exporters admitted that they had graded their groundnuts by hand sorting to remove mouldy fractions from the lot before sample collection for quality check at Chitedze laboratory. If EU aflatoxin regulatory limits (EC, 2010) were to be considered, 24/27 (89%) of the lots for export would be accepted into EU.

#### **4.4. Conclusions**

The present findings demonstrated that through grading farmers could potentially access high value markets with stringent AFs regulatory limits. However, while this might be an exciting observation on a national economic point of view, it is worrisome that until now there are no



channels for diversion of the grade-outs. Therefore sorting out of the groundnuts might be concentrating AFs on the local table.

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Concerned with the likely negative impacts of sorting of food commodities for export purposes (Chapter 4), it has been proposed that sort-out groundnuts should be diverted to oil production in order to pull mycotoxins (contaminated nuts) away from the human food chain. Chapter 5 seeks to assess the feasibility of this option through a mycotoxin survey conducted in small-scale processed edible groundnut oils collected from the main groundnut growing region of Malawi. Chapter 5 is part of an on-going wider physical decontamination project in the Laboratory of Food Analysis, (UGent).



## **Chapter 5: Carry-over of mycotoxins into mechanically pressed groundnut oils and thermal degradation profile of aflatoxins in unrefined groundnut oil.**

### **5.1. Introduction**

The per capita consumption of edible vegetable oil has steadily risen over the years, particularly in the developing world (Popkin and Gordon-Larsen, 2004; O'brien, 2010; OECD-FAO 2011). On a global level, the major sources of the edible vegetable oil include palm, soybean, rapeseed, sunflower seed, palm kernel, groundnut (peanut), cottonseed, coconut and olive (in order of decreasing importance in terms of volume) (USDA FAS, 2014). Following the emergence of soybean oil in the 1970s, groundnut share in the total production of vegetable oil has continued to decline steadily and currently there is virtually no international trade of the product (Revoredo and Fletcher, 2002; Gunstone, 2011). This is due to the fact that groundnuts receive a higher premium price as confectionery than oil (as high as 100 times) (Diop et al., 2004). To date China and India account for about 75% of the total world groundnut oil production and consumption. A significant fraction of the remainder pertains to west Africa (Popkin and Gordon-Larsen, 2004; Gunstone, 2011).

The groundnut oil production and utilization status in Malawi is not different from the global picture described above. Between 2007 and 2010, all main local commercial vegetable oil processors in Malawi had abandoned groundnut oil production due to the strong competition from vegetable oil (mostly soybean oil) imports from neighboring countries (Derlagen and Phiri 2012). In spite of this, the production of confectionery groundnuts and exports continue to rise steadily (Derlagen and Phiri 2012; Government of Malawi, 2012). This is a result of efforts by Malawi Government and developing partners in an attempt to diversify the country's economy. As previously discussed in Chapter 4 the groundnuts are thoroughly hand-sorted at farmer and exporter level to eliminate contaminated kernels in order to meet the importers' stringent acceptable levels of aflatoxins (AFs) and unfortunately, the sort-outs are consumed locally. Realizing the likely negative health impact of such a practice, some developing partners have proposed diversion of the sort-outs to edible oil production (Emmott, 2013, Emmott and Stephens, 2012). Perhaps this stems from the fact that many believe AFs are not sequestered in vegetable oils due to their high polarity (Mahoney and Molyneux, 2010).

Coincidentally there are some parallel efforts to capacitate farmers to extract oil from oil seed including groundnuts and sunflower at small scale (OVOP, 2014). In this light, diesel and electricity powered oil expellers have been donated to farmer cooperatives within the main groundnut growing districts of Lilongwe and Mchinji in central Malawi. The introduction of the expellers by the donors in the area, has induced a rise of privately owned expellers which are open to the public at a fee (farmers bring their own seed for oil expulsion). In any case, oil is expelled from shelled or unshelled seeds by hot or cold pressing and sold or utilized as crude oil after only cloth filtration. It is noteworthy that groundnut oil processing is mostly done during the first 4 to 5 months after harvest. After this period there is virtually no groundnut oil production due to supply/demand dynamics (price for the nuts as a confectionery becomes very high).

Elsewhere, AFs have been detected in vegetable oils with comparatively higher levels reported in non-refined oil than refined ones. An extensive review on this aspect has recently been published by Bordin et al. (2014). Different transfer rates of AFs from seeds into oil following pressing have been reported in literature. In particular, Basappa and Sreenivasamurthy (1974); and Mahjoub and Bullerman (1990) reported 15 % and 18-47 % transfer rates, respectively. Although AFs have received much attention, other mycotoxins have also been reported in vegetable oils (Chulze et al., 1995; Papachristou and Markaki, 2004; Ferracane et al., 2007; Siegel et al., 2010).

This paper reports the findings of the first exploratory investigation on presence of mycotoxins in small-scale processed edible groundnut oils collected from the main groundnut growing region of Malawi.

## **5.2. Materials and methods**

### *5.2.1. Study 1: Survey of mechanically pressed groundnut oils*

Seven small-scale processed groundnut oil samples (approximately 30g each) were collected from the main groundnut producing district in Malawi (Mchinji district) in the month of February 2014. It is opportune to underline that at the time of sampling the small-scale groundnut oil had become scarce due to fact that the groundnuts were off-season. At this time of the year, the price of the groundnuts is 3-5 times higher than during harvest months and oil production becomes almost limited to sunflower seed (which has a constant low price). Two



of the samples were collected from the local market within the district, while 4 samples were sampled directly from farmer-cooperative processors and the other sample was collected from a private expeller (freshly produced from a groundnut lot brought by a farmer). Two of the samples that were collected from the farmer-cooperative processors were reported to have been produced from raw shelled nuts while the other two were prepared from raw in shell groundnuts (not removed from shell). Apart from the fresh sample highlighted above all samples had been reported to have been produced at least one month prior to the sampling. The samples were collected in 50 mL extraction tubes completely covered by Aluminium foil to limit light exposure and transported to Belgium for the mycotoxin analysis. Refined (bought from a supermarket in Belgium) groundnut oil was used for spiking experiments and also to make the matrix-matched calibration curve.

### *5.2.2. Reagents and laboratory materials*

Acetic acid (LC–MS/MS grade) was supplied by Merck (Darmstadt, Germany). Grace Pure aminopropyl (NH<sub>2</sub>) solid phase extraction (SPE) cartridges were obtained from Grace Discovery Sciences (Lokeren, Belgium). LC–MS grade methanol, HPLC grade methanol and n–hexane were purchased from VWR International (Zaventem, Belgium). Ammonium acetate was supplied by Grauwmeer (Leuven, Belgium). Ultrafree–MC centrifugal filter devices (0.22 µm) of Millipore (Millipore, Brussels, Belgium) were used. Water was purified on a Milli–Q Plus apparatus (Millipore, Brussels, Belgium).

### *5.2.3. Mycotoxin standards*

Standards namely deoxynivalenol (DON), deepoxy–deoxynivalenol (DOM), zearalenone (ZEN), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), T2-toxin (T2), aflatoxin G2 (AFG2), alternariol (AOH), ochratoxin A (OTA), zearalanone (ZAN), fumonisin B1 (FB1), fumonisin B2 (FB2) and sterigmatocystin (STERIG), were purchased from Oskar Tropitzsch (Marktredwitz, Germany). 3–acetyldeoxynivalenol (3–AcDON), 15–acetyldeoxynivalenol (15–AcDON), neosolaniol (NEO), alternariol methyl ether (AME), altenuene (ALT) and roquefortine C (ROQ C) were purchased from Biopure (Tulln, Austria). Fumonisin B3 (FB3) was obtained from Promec Unit (Tygerberg, South Africa). Nivalenol (NIV), HT2 toxin (HT2) and fusarenon–X (FUS–X) were purchased from Fermentek (Jerusalem, Israel). Diacetoxyscirpenol (DAS) and internal standard zearalanone (ZAN) were obtained from Sigma Aldrich (Bornem, Belgium). NIV and NEO were obtained as solutions

(100 µg/mL) in acetonitrile. FB2 and FB3 standards (1 mg) were prepared in 1 mL acetonitrile/water (50:50, v/v). Stock solutions of DON, 3-AcDON, 15-AcDON, FUS-X, AFB1, AFB1, AFG1, AFG2, HT2, T2, ALT, OTA, ZEA, FB1, STERIG, FB2, AOH, AME, DAS, ZAN and ROQ C were prepared in methanol at a concentration of 1 mg/mL. All stock solutions were stored at -18°C except FB2 and FB3, which were stored at 4°C. Following dilution of the individual stock standard solutions (1 mg/mL), a standard mixture was prepared at the following concentrations: AFG1, AFB2, DAS, OTA and STERIG (1 ng/µL); AFG2, AFB1 and T2 (2 ng/µL); AME, NEO, ROQ C, HT2, ZEN (2.5 ng/µL); ALT and FB1 (2.5 ng/µL); DON, FB2 and FB3 (10 ng/µL); 3-AcDON (12.5 ng/µL); FUS-X, 15-AcDON (25 ng/µL); NIV (40 ng/µL). The standard mixtures were prepared in methanol, stored at -18°C and renewed every 3 months.

#### *5.2.4. Sample preparation protocol*

Sample preparation was based on the method for vegetable oil described by Ediage et al (manuscript in preparation). Briefly, an oil sample ( $3.00 \pm 0.01$  g) (pre-spiked with internal standard ZAN and DOM at a concentration of 50 and 333 µg/kg respectively) was mixed with 15 mL acetonitrile/water/acetic acid (79:20:1, v/v/v) in 50 mL extraction tube and agitated vigorously (93 rpm) using an overhead shaker (Exacta, Delhi, India) for 40 min. Hexane (10 mL) was added to the mixture and further agitated for 15 min under the same conditions. The mixture was then centrifuged for 15 min at 3170 g after which the hexane-oil upper layer was aspirated and discarded. A 3 mL portion of the defatted extract was transferred into a clean 50 mL extraction tube while the rest of the extract (approximately 11.0 mL) was passed through a pre-equilibrated amino SPE (GracePure, 1000 mg/6 mL) column. The eluate from the SPE was collected into a clean test tube, added to the 3 mL portion in the 50 mL extraction tube and evaporated at 60°C to dryness under a gentle stream of nitrogen. The dried residue was re-dissolved in 100 µL injection solvent consisting of methanol/water/acetic acid (57.2:41.8:1, v/v/v) and 5 mM ammonium acetate to which 200 µL hexane was added and vortexed. The reconstituted extract was further filtered using Ultrafree®PVDF centrifuge filters (0.5 mL, pore 0.22 µm, Millipore Bedford, MA, USA) after which 80 µL bottom phase was transferred into an HPLC vial fitted with a glass insert ready for LC-MS/MS measurement. Matrix-matched calibrants were treated as described above.

### 5.2.5. LC-MS/MS parameters

For the analysis, a Waters Acquity UPLC<sup>®</sup> coupled to a Waters Quattro Premier XE tandem mass spectrometer (both Waters, Milford, MA, USA) was used. The chromatographic conditions were as previously described by Ediage et al. (2011). Briefly, analytes (10 µL injection) were separated on a Symmetry RP-18 column, (5 µm, 150 mm x 2.1 mm i.d.) protected by a Sentry guard column (10 mm x 2.1 mm i.d.) (both Waters, Zellik, Belgium) which was kept at ambient temperature. A binary gradient elution (flow rate of 0.3 mL/min) was performed with methanol/water/acetic acid 5:94:1, (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B) respectively. Both mobile phases contained 5 mM ammonium acetate. The following gradient program was applied: 0–7 min, 95–35% A; 7–11 min, 35–25% A, 11–13 min, 25–0% A; 13–14 min, 0% A; 14–16 min, 0–40% A; 16–26 min, 40–60% A; 26–28 min, 60–95% A. The total analytical run time was 28 min.

The mass spectrometric conditions described by Ediage et al (2011) were re-optimized by re-tuning the different analytes (Ediage et al. manuscript in preparation). Positive electrospray ionization (ESI) mode was used and data acquisition was carried out in the multiple reaction monitoring (MRM) mode. ESI-MS/MS parameters were as follows: source and desolvation temperatures 130°C and 350°C, respectively; capillary voltage 3.2 kV; cone and desolvation gas flows of 20 and 800 L/h, respectively. For positive identification of the target mycotoxins, the following criteria were met: (1) the deviations of the relative ion intensities of the MRM transitions were not greater than the maximum permitted tolerances (EC, 2002), (2) the relative retention times with regard to the internal standard were below the maximum permitted deviation of 2.5%, (3) a peak with a signal–noise ratio of at least 3 was identified for each MRM transition. Matrix-matched calibration curves were used for target analyte quantification. For both the samples and matrix-matched calibrants ratios of the peak areas of the target analytes and the internal standard were used. The limit of quantification (LOQ), limit of detection (LOD) and apparent recoveries for the detected analytes are presented in Table 5.1. Further mass spectrometric parameters are provided in Annex V.

Table 5.1: Apparent recoveries and limit of quantification (LOQs) of the mycotoxins as obtained in the LC-MS analysis of groundnut oil

Analyte	LOQ*	Apparent recovery (%)
AFG2	1.0	101
AFG1	1.0	102
AFB2	1.2	105
AFB1	1.0	105
DAS	1.4	103
OTA	1.3	102
ZEN	3.1	93
STERIG	1.2	91

\*LOD are half the LOQs

### 5.3. Results and discussion

#### 5.3.1. The incidence and levels of mycotoxins in the edible oil

The incidence and levels of mycotoxins in the edible oils are presented in Table 5.2. Of the investigated mycotoxins AFs, DAS, OTA, ZEN and STERIG were the only mycotoxins detected in the samples. All the 7 oil samples were positive for all of the following; AFB1, AFB2, AFG1, AFG2, DAS and STERIG (Table 5.2). OTA and ZEN were detected in 1 and 3 of the 7 samples respectively (Table 5.2). An extracted ion chromatogram of sample 6 showing co-occurrence of the mycotoxins has been provided in Annex VI. Interestingly 4 of the samples contained AFG1 higher than AFB1, a trend previously reported by Matumba et al. (manuscript in press; chapter 3). Both Malawi and the Common Market for Eastern and Southern Africa (COMESA) have no specific regulatory limits for AFs in oils. However, for 5 of the 7 oil samples, the total AF (AFB1, AFB2 AFG1, AFG2) exceeded 10 µg/kg, i.e., the maximum level proposed by COMESA for groundnuts and maize for human consumption.

The other four mycotoxins (OTA, ZEN, DAS and STERIG) were generally detected at much lower levels compared with the AFs. It is noteworthy that apart from ZEN (maximum level 200 µg/kg) no specific regulatory limits exist for these toxins in vegetable oil elsewhere (FAO, 2004). Nonetheless EUs regulatory limits for OTA in human food range only up to 10 µg/kg (EU, 2006). None of these (OTA and ZEN) maximum were exceeded in the present samples. Higher prevalence rates and higher levels of OTA have been reported in

Mediterranean virgin olive oils (Papachristou and Markaki, 2004; Ferracane et al., 2007). As for ZEN, STERIG and DAS, while these toxins have been reported in groundnut samples (Mehan et al., 1985; El-Magraby and El-Maraghy, 1988; Youssef et al., 2008; Ezekiel et al., 2012), there are hardly data on the occurrence of these toxins in oils. The presence of *Fusarium* toxins ZEN and DAS in groundnuts and groundnut oil is interesting considering that these toxins are known not to contaminate this commodity. Their presence in groundnuts and product thereof may therefore suggest possible cross contamination from cereal dust. In this case it is likely that the contaminated dust came from bags that were previously used for maize storage as it is common practice to Malawi to reuse bags without cleaning them. On the otherhand, the paucity of surveillance data on mycotoxins in edible oils and the general non-existence of related regulations may further underline the fact that the majority believe that mycotoxins are not carried into oils (Mahoney and Molyneux, 2010) or perhaps it is due to the fact that in countries where mycotoxins regulatory systems are advanced the edible oils are usually refined. Refining of crude edible oil is known to lead to a considerable reduction in the mycotoxin levels (Bordin et al., 2014).

Samples 6 and 7 had generally higher AFs levels than the rest (Table 5.2). Coincidentally these two oil samples were collected from two separate containers (light-tight) in a farmer-cooperative processing unit and were reported to have been expelled from in-shell groundnuts (still with the shell). It is tempting to speculate that the high AFs levels came from the groundnuts shells (or mouldy nuts inside the shells) however there is no statistical evidence to support that due to sample limitation size. Comparatively samples 1 to 5 had lower mycotoxin levels which would suggest that these oils were prepared from relatively lower contaminated nuts. However it is worth mentioning that samples (1-4) were sourced from transparent plastic bottles (which allow a certain degree of UV radiation to pass through), therefore photodegradation of the mycotoxins cannot be ruled out (Lillard and Lantin, 1970; Liu et al., 2010; Samarajeewa, 1985). Although numerous researchers have reported loss of mutagenicity following photodegradation (Andrellos et al., 1967; Lillard and Lantin 1970; Kleinwächter and Koukalova, 1979; Liu et al., 2011), contrary reports do exist (Feuell, 1966; Stark et al., 1990). Moreover, UV treatment as a decontamination strategy has a limitation in the sense that it induces oxidation of the oil (Choe and Min, 2006).

Table 5.2: Mycotoxin contamination in mechanically pressed groundnut oils from Malawi.

Sample	Mycotoxin ( $\mu\text{g}/\text{kg}$ )								
	AFB1	AFB2	AFG1	AFG2	Total AF	DAS	OTA	ZEN	STERIG
1	2	2	2	1	7	6	nd	11	3
2	21	2	30	10	63	5	3	nd	6
3	1	2	1	1	5	5	nd	nd	4
4	10	2	7	2	21	10	nd	nd	3
5	7	3	6	1	17	5	nd	nd	1
6	102	33	234	82	451	20	nd	6	11
7	15	6	42	12	75	10	nd	1	3
Average	22	7	46	16	91	9	3	6	5

Samples 1 and 2: Collected from farmer-cooperative processors, produced from raw shelled nuts and packaged in clear plastic containers;

Samples 3 and 4: Collected from the local market packaged in clear plastic containers;

Sample 5: Collected from a private expeller (freshly produced from a groundnut lot brought by a farmer),

Samples 6 and 7: Collected from farmer-cooperative processors, produced from raw in shell groundnuts and packaged in light-tight containers.

#### 5.4. Conclusions

This is the first report of the mycotoxin incidence in mechanically pressed unrefined groundnut oil produced by small scale processors in Malawi. Although the study was limited in terms of number of samples analysed, it provides a rough picture of the mycotoxin problem in small scale processed oils in Malawi. The study provides further evidence that significant amounts of mycotoxins may be carried over into pressed oils. The results indicate the necessity of a mycotoxin decontamination step in the process design otherwise only clean groundnuts should be used for oil pressing. Considering that mycotoxin testing may not be readily available at small scale level, perhaps an alternative option would be to let farmers continue to process oil and supply the crude oil to advanced refineries for decontamination and refining. On the other hand, it is also needed to rethink if diverting contaminated oilseeds to oil production is a feasible option. The proposal is built on the premise that oils are non-polar and therefore do not dissolve polar mycotoxins. Practically, oil may get oxidized due to fungal activity already within oilseeds, making it polar, thereby increasing its potential to dissolve high amounts of mycotoxins (Mahoney and Molyneux, 2010). Conversely, high levels of mycotoxins in an oil sample may already indicate that the oil is oxidized and perhaps

not even worth decontaminating as highly oxidized oil in itself is a health hazard (Esterbauer, 1993). In this light there is need to draw a 'cut-off' when contaminated oilseeds can be used for oil production. With the new insights about the existence of thermally and chemically modified mycotoxins (Rychlik et al., 2014), it is also high time that the current advanced oil technology gets scrutinized. There are possibilities that the technology would be modifying some mycotoxins thereby escaping analysis of their parent forms.

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In search of ways of mycotoxin decontamination strategies for grains contaminated by multi-mycotoxins as reported in Chapter 2, Chapter 6 explores the fate of mycotoxins during sodium hydrogen carbonate,-maize cake preparation. The role of salt and sucrose on the decomposition is also investigated. Chapter 6 is reproduced from a submitted manuscript **‘Practical limitations of baking as a mycotoxin decontamination strategy: Lessons drawn from a maize cake baking procedure (Food and Chemical Toxicology)** written by Matumba, L., Van Poucke, C., Ediage, E.N., Monjerezi, M. and De Saeger, S.



## **Chapter 6: Influence of sodium hydrogen carbonate, sodium chloride and sucrose on the thermal degradation of aflatoxins, fumonisins, deoxynivalenol, nivalenol, zearalenone and alternariol during maize cake baking.**

### **6.1. Introduction**

Mycotoxins are generally thermally stable and are not destroyed during most normal cooking processes (Bullerman and Bianchini, 2007). However, varying degrees of thermal degradation of different mycotoxins have been reported depending on the cooking conditions which include degree of heat penetration, temperature, time, moisture content, pH of food, and the concentration of the mycotoxins in the food matrix (Seefelder et al., 2003; Bretz et al., 2005; Castells et al., 2005; Bretz et al., 2006; Raters and Matissek, 2008; Kabak, 2009). In particular, fumonisin B1 is chemically modified during nixtamalization (alkaline cooking) (Dombrink-Kurtzman et al., 2000) and also reacts with reducing sugars during cooking (Howard et al., 1998). Bakery processing has been reported to reduce the concentration of aflatoxins (El-Tawila et al., 2003; Gumus et al., 2009); fumonisins (Avantaggiato et al., 2003); deoxynivalenol (Abbas et al., 1985; Boyacioglu et al., 1993; Neira et al., 1997); nivalenol (Kamimura et al., 1979) and zearalenone (Matsuura et al., 1981). Evaluation of some degradation products have indicated reduced or loss of toxicity (Bretz et al., 2006; Park and Kim, 2006; Voss and Snook, 2010) giving the impression that elevated heat treatment could be used as a decontamination strategy.

In contrast, several workers have reported high stability of mycotoxins during baking or an increase in mycotoxin levels after baking (the latter is attributable to the release of mycotoxins from conjugated forms (Berthiller et al., 2013)) (El-Banna et al., 1983; Scott et al., 1983; Young et al., 1984; Sugiyama et al., 2009; De Angelis et al., 2013). Moreover, some thermal processes have been reported to yield products that are as toxic as their parent mycotoxins (Dombrink-Kurtzman et al., 2000; Voss et al., 2001) or reversible under simulated gastrointestinal tract conditions e.g. the case of aflatoxin and nixtamalization of maize (Méndez-Albores et al., 2004). Nevertheless, the EU Regulation No. 1881/2006 outlaws mycotoxin detoxification in foodstuffs by chemical treatments (EC, 2006).

All the studies cited above focused on only one mycotoxin or a class of related mycotoxins. However, the co-occurrence of more than one mycotoxin or class of mycotoxins in a foodstuff

is more practical, particularly in the case of tropical environments (Warth et al., 2012; Mohale et al., 2013; Shephard et al., 2013; Adetunji et al., 2014; Ediage et al., 2014; Matumba et al., in press). In that regard, the present study was performed to evaluate the effect of baking on a wider range of mycotoxins through the use of a real life maize cake recipe popularly used in the southern Africa involving NaHCO<sub>3</sub>, NaCl and sucrose (the cake is known as chikondamoyo in Malawi, Zambia and Zimbabwe with additional names including chigumu (Malawi), tobomotwe (Zambia) and chimodho (Zimbabwe)). The results from this study could be useful in the dietary exposure management of mycotoxins of bakery products.

## 6.2. Material and methods

### 6.2.1. Maize flour containing mycotoxins

Mouldy maize (~5kg) naturally contaminated with mycotoxins, was purchased from a vendor in Chikwawa district, Malawi. The whole maize sample was ground using a laboratory mill (Christy and Norris Ltd, Suffolk, UK) and the flour was mixed thoroughly after which the sample was ready for mycotoxin extraction (analysis) and cake preparation (Experiment 1).

### 6.2.2. Experiment 1: Influence of sodium hydrogen carbonate, NaCl and sucrose during baking of maize cake

NaHCO<sub>3</sub>, NaCl and sucrose are a prerequisite in traditional cake baking. However to unravel the role of these ingredients in the thermal reduction of the mycotoxins, eight different baking recipes were tried out in a 2<sup>3</sup> factorial design (Table 6.1).

Table 6.1: Test conditions of the 2<sup>3</sup> factorial design.

Treatment number	Coded factors		
	sucrose (ingredient 1)	NaCl (ingredient 2)	NaHCO <sub>3</sub> (ingredient 3)
1	0	0	0
2	1	0	0
3	0	1	0
4	1	1	0
5	0	1	1
6	1	1	1
7	0	0	1
8	1	0	1

Codes (1) and (0) indicate inclusion and exclusion of the ingredient respectively.

As there were no available documented maize cake recipes, the amount of NaHCO<sub>3</sub>, NaCl and sucrose used in the current study was pre-optimized in a sensory evaluation exercise involving 7 panelists.

Each recipe involved maize flour (40g), distilled water (40 mL) and where applicable NaHCO<sub>3</sub> (1g), NaCl (1g) and sucrose (5g). Each suspension was thoroughly homogenized, poured and completely filled a mini cupcake liner and baked in a Memmert oven (Fiers, Kuurne, Belgium) at a constant temperature of 190°C for 50 min. It is noteworthy that 12 min elapsed for a preheated oven to attain a constant temperature of 190°C from 142°C when the cakes were put in the preheated oven. All recipes were prepared in triplicate. After baking the whole cake was ground (M20 Universal Mill, IKA®, Werke Staufen, Germany) to between 0.5 and 1 mm sieve size after which the sample was ready for mycotoxin extraction.

The moisture content of the cakes and maize flour used to prepare the cake was determined by forced-air oven drying (Gallenkamp, Model OV-160, England) at 105°C for 24 h in duplicate.

#### *6.2.3. Experiment 2: Further assessment on the fate of Ac-DON during alkaline baking*

Informed by the alkaline thermal degradation results of DON, 3-AcDON and 15-AcDON obtained from experiment 1, blank maize flour was separately spiked with 3-AcDON and 15-AcDON at a 1000 µg/kg level and baked with NaHCO<sub>3</sub>, as described above.

#### *6.2.4. Experiment 3: Acidification of the cakes*

The stability of alkaline catalyzed thermal degradation products of the considered mycotoxins was assessed by adjusting 5g of ground NaHCO<sub>3</sub>-maize cake in 50 mL extraction tubes in triplicate to pH of around 1.9 (pH similar to the human stomach) by means of 0.1 M HCL (19 mL). The pH was determined by means of a pH test strip (pH indicator strip, pH 0—2.5, Merck, Darmstadt, Germany). The mixture was shaken for 12 hrs, dried off under a gentle stream of nitrogen at 60°C and thereafter mycotoxins were extracted following the protocol described by Monbaliu et al., (2010). The effect of the hydrolysis was leveled off by adding the same volume of de-ionized water to another set of ground maize cake (NaHCO<sub>3</sub>)—and a pH of 10.0—10.5 was recorded (using pH indicator strip, pH 7.5—14. Merck, Darmstadt, Germany).

### 6.2.5. Mycotoxin analysis

#### 6.2.5.1. Reagents and chemicals

##### *Reagents and chemicals for mycotoxin analysis*

The sources of reagents and chemicals and their preparations are the same as described in chapter 5.

#### 6.2.5.2. Sample preparation and cleanup

Sample preparation (maize cake and maize flour) was based on the procedure described by Monbaliu et al. (2010). Briefly, ground samples (5 g) (pre-spiked with internal standard ZAN and DOM at a concentration of 250 and 150 mg/kg respectively) were mixed with 20 mL acetonitrile/water/acetic acid (79:20:1, v/v/v) in 50 mL extraction tubes and agitated vigorously using an overhead shaker (AG 6A, Exacta, Mery sur Oise, France) at 93 rpm for 1 h. The mixture was then centrifuged (IEC Centra MP4, VWR, Zaventem, Belgium) at 4000g for 15 min. The supernatant was pipetted onto a pre-conditioned C18 column (500 mg/6 mL) (Grace, Lokeren, Belgium), passed through the column with gravitational force and the eluent was collected into a 25 mL volumetric flask. The extraction was repeated by adding 5 mL of acetonitrile/water/acetic acid (79:20:1, v/v/v), vortexed and agitated again for 10 min, centrifuged at 4000g for 15 min and the supernatant was passed through the same C18 column and the eluent collected into the same 25 mL flask. Acetonitrile/water/acetic acid (79:20:1, v/v/v) was added to the flask up to the grade mark. The sample was transferred into an extraction tube and defatted by agitation after addition of 10 mL of hexane. After phase separation (centrifuged at 4000g for 15 min) the lower phase was split into two parts. One part (10 mL) was filtered through a glass filter (GF/A, Whatmann, VWR, Zaventem, Belgium) and the other part (12.5 mL) was purified further by means of a MultiSep®226-column (Romer Labs, Coring System Diagnostics, Gernsheim, Germany). After a conditioning step, 30 mL of a mixture comprising of the 12.5 mL of the defatted extract and 27.5 mL of acetonitrile/acetic acid (99:1, v/v) was applied on the MultiSep®226-column and washed with 5 mL of acetonitrile/acetic acid (99:1, v/v). The total eluent was collected and combined with 2 mL of the glass filtered portion. The mixture was dried under a gentle nitrogen flow in a thermostatic water bath heated at 40 °C. The dried residue was re-dissolved in 150 µL mobile phase (water/methanol/acetic acid (94:5:1, v/v/v) 5 mM ammonium acetate and water/methanol/acetic acid (2:97:1, v/v/v) 5 mM ammonium acetate mixed in the ratio of 3:2, v/v). Prior to injection onto the LC-MS/MS, samples were further filtered using



Ultrafree®PVDF centrifuge filters (0.5 µL, pore 0.22 µm, Millipore Bedford, MA, USA). The different matrix-matched calibrants were prepared as described above.

#### 6.2.5.3. *Liquid chromatography–tandem mass spectrometry.*

Liquid chromatography and tandem mass spectrometry parameters were as described in chapter 5.

#### 6.2.6. *Statistical analysis*

The influence of heating on the mycotoxin degradation in plain maize cakes was assessed by analysis of variance (ANOVA) of the maize flour and the plain cake mycotoxin contents. Influence of the ingredients (NaHCO<sub>3</sub>, NaCl, sucrose) and of the interaction between the ingredients on thermal degradation of the mycotoxins was determined by means of regression analysis of mycotoxin contents of the raw maize and cakes. All statistical analyses were performed using SPSS® (version 16) software (SPSS Inc., Chicago, Illinois, USA). The level of confidence required for significance was set at  $p \leq 0.05$ . However  $p$ -value of 0.1 was used as the criterion for including interaction terms.

### **6.3. Results and discussion**

To compensate for matrix effects and to allow accurate quantification of the mycotoxin content, calibration curves were separately prepared in I) raw maize flour, II) in cake baked with and III) without NaHCO<sub>3</sub>. The calibration curves for cake baked with and without NaHCO<sub>3</sub> were superimposable. This could be attributable to the extensive sample cleanup described above.

The naturally contaminated maize flour used for baking the cakes had  $9.1 \pm 0.2\%$  moisture and was found to contain on dry matter basis:  $796 \pm 27$  µg/kg (mean  $\pm$  SE) AFB1,  $81 \pm 2$  µg/kg AFB2,  $1505 \pm 118$  µg/kg AFG1,  $167 \pm 15$  µg/kg AFG2,  $24620 \pm 1211$  µg/kg FB1,  $16558 \pm 1090$  µg/kg FB2,  $5528 \pm 153$  µg/kg FB3,  $3239 \pm 238$  µg/kg NIV,  $3492 \pm 40$  µg/kg DON,  $192 \pm 14$  µg/kg AOH and  $52 \pm 3$  µg/kg ZEN. The chromatographic method (Monbaliu et al., 2010) used herein could not provide satisfactory separation of 3-AcDON and 15-AcDON and therefore they were reported as a sum of the two analytes (3-AcDON + 15-AcDON ( $\Sigma$ Ac-DON)) and was found to be  $91 \pm 2$  µg/kg. The study was therefore limited to these mycotoxins.

### *6.3.1. Thermal degradation of mycotoxins in plain maize cakes (no sodium hydrogen carbonate, NaCl or sucrose added)*

The cakes contained  $14.6 \pm 0.6\%$  moisture and mycotoxin concentration results were adjusted accordingly. The percentage of mycotoxin degradation resulting from baking alone was determined by comparing the mycotoxin contents of the cakes that excluded ingredients ( $\text{NaHCO}_3$ , NaCl, sucrose) (plain cakes) against the concentration in the raw flour. Concentrations of all the mycotoxins in the plain cake were significantly lowered by  $14 \pm 4\%$  for ZEN to as high as  $70 \pm 6\%$  for FB1 (Figures 6.1, 6.2, 6.3 and 6.4).

The relative stability of ZEN reported herein supports findings by Yumbe-Guevara et al. (2003) who reported that the ZEN decomposition time at  $220^\circ\text{C}$  was about 8 times longer compared to DON and NIV. These authors also observed that heat treatments at  $140$  and  $160^\circ\text{C}$  did not cause a significant ZEN decomposition. Heating maize flour contaminated with ZEN at  $110^\circ\text{C}$  for 12 days resulted in only 13% ZEN decomposition when water was added (Lauren and Smith, 2001). Similar results were also reported by Ryu et al. (2003) and Numanoglu et al. (2013) but no explanation was found.

The degradation percentages observed for fumonisins (FBs) corroborate findings by Scott and Lawrence (1994) who reported 60–80% FBs (FB1 and FB2) degradation following heating of dry and moist corn flour at  $190^\circ\text{C}$  for 60 mins. Comparatively, lower or no degradation at all of FB1 was reported following baking of muffins spiked with 5 mg/kg FB1 at temperatures of  $200$ ,  $204$  and  $232^\circ\text{C}$  (Jackson et al., 1997; Castelo et al., 1998). This discrepancy in results could be attributed to differences in the baking time employed, the type of matrices (corn mix vs. pure corn flour) and the thickness of the cakes.

As for aflatoxins (AFs), the concentration of each aflatoxin (AFB1, AFB2, AFG1 and AFG2) was reduced by  $\sim 50\%$  in the plain cakes. Raters and Matissek (2008) reported 70% and 100% degradation of AFB1 in its pure form after heat treatment at temperatures of  $150$  and  $180^\circ\text{C}$  for 60 min respectively. However, they found AFB1 to be relatively more stable in starch than in protein matrix. Thermal degradation of AFs is said to be influenced by the availability of  $\text{H}_2\text{O}$  which hydrolyses the lactone ring of the AF molecule, consequently leading to the formation of a terminal carboxylic acid, which further undergoes a heat-induced decarboxylation (Samarajewa et al., 1990; Mann et al., 1997; Buser and Abbas, 2002).

As regards to DON and  $\Sigma$ AcDON, about 70% of the initial contents were recovered in the plain cakes. It has previously been demonstrated that appreciable DON degradation is realized at elevated temperatures. Baking of 1 mm thick maize bread at 150°C for 180 mins and 250°C for 30 mins resulted in only 37 and 32% loss of DON respectively (Numanoglu et al., 2012). However, there is paucity of literature on thermal degradation of 3-AcDON and 15-AcDON.

The degradation percentage for NIV in the plain cake observed in the present study was two times greater than that for DON. These findings conflicts findings by Yumbe-Guevara et al., (2003) who reported similar decomposition rates for these toxins in naturally contaminated barley heated at 140, 160, 180, 200, or 220°C. The difference in the two findings could be due to differences in the matrices. The same reason may hold for the relatively higher thermal degradation in plain cakes observed for AOH in the present study compared to the results obtained by Siegel et al. (2010).

### 6.3.2. Main and interactive effects of sodium hydrogen carbonate, NaCl and sucrose

Data on the influence of the ingredients (NaHCO<sub>3</sub>, NaCl, sucrose) and of the interaction between these ingredients on the thermal degradation of the toxins are presented in Table 6.2. The regression equation that applies to the present factorial design is described by Eq. 6.1.

$$E[Y] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (\text{Eq 6.1})$$

where Y is the measured response with conditional expected value  $E[Y]$ ;  $\beta_0$  is the intercept;  $X_1$ ,  $X_2$  and  $X_3$  are sucrose factor, NaCl factor and NaHCO<sub>3</sub> factor respectively;  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  are coefficients for the main factors  $X_1$ ,  $X_2$  and  $X_3$  respectively;  $\beta_{12}$ ,  $\beta_{13}$ ,  $\beta_{23}$ , and  $\beta_{123}$  are coefficients for factors (interactions):  $X_1 X_2$ ,  $X_1 X_3$ ,  $X_2 X_3$ ,  $X_1 X_2 X_3$  respectively.

Table 6.2: Regression analysis coefficients for main and interactive factors (NaHCO<sub>3</sub> NaCl and sucrose) influencing thermal degradation of mycotoxins during baking of maize cake based on the % mycotoxin remaining (calculated based on mycotoxin content of the raw flour)

	AFG2	AFB1	AFB2	AFG1	FB1	FB2	FB3	NIV	DON	AcDON	AOH	ZEN
Constant (baking a plain cake)	61.2***	45.3***	61.1***	51.2***	33.7***	36.4***	42.0***	39.6***	74.6***	82.5***	70.0***	85.9***
Sucrose only	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	-17.0***	-17.8**	n.s	-7.8*
NaCl only	n.s	n.s	n.s	n.s	34.8***	32.2***	42.4***	13.4*	-19.4***	-14.1**	n.s	n.s
NaHCO <sub>3</sub> only	-58.6***	-42.8***	-53.8***	-50.3***	-16.0***	-15.6***	-16.6**	-17.6***	-26.0***	-76.6***	-37.6***	-27.4***
Sucrose/NaCl	n.s	n.s	n.s	n.s	-21.5***	-17.9***	-26.1***	-12.4‡	20.0***	17.1**	n.s	11.1*
Sucrose/ NaHCO <sub>3</sub>	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	13.9***	20.8**	n.s	8.8*
NaCl/ NaHCO <sub>3</sub>	n.s	n.s	n.s	n.s	-28.6***	-23.1***	-32.3***	n.s	19.7***	n.s	n.s	n.s
Sucrose/ NaCl/ NaHCO <sub>3</sub>	n.s	n.s	n.s	n.s	n.s	n.s	20.7*	n.s	-24.2***	-20.8*	n.s	-10.9‡

‡, \*, \*\* and \*\*\* represents p-value of < 0.1, <0.05, <0.01 and <0.001 respectively without eliminating non-significant (n.s) factors.

NaHCO<sub>3</sub> was the only ingredient that significantly influenced thermal degradation of all of the considered mycotoxins. The inclusion of sucrose and NaCl affected only some the mycotoxins. Data on the mycotoxin percentage remaining in the cake following degradation resulting from the interaction of the heating and the inclusion of the additional ingredients (NaHCO<sub>3</sub>, NaCl and sucrose) are also presented in Figures 6.1, 6.2, 6.3 and 6.4.

Addition of NaHCO<sub>3</sub> resulted in  $\geq 94\%$  degradation of all the four AFs (Figure 6.1). Previously Karaca and Nas (2009) evaluated the thermal degradation of AFs in figs under a pH range (3.1—10) and reported maximum degradation at pH 10.0. The influence of alkalinization on the thermal degradation has also been reported by other research groups (Torres et al., 2001; Pèrez-Flores et al., 2011).

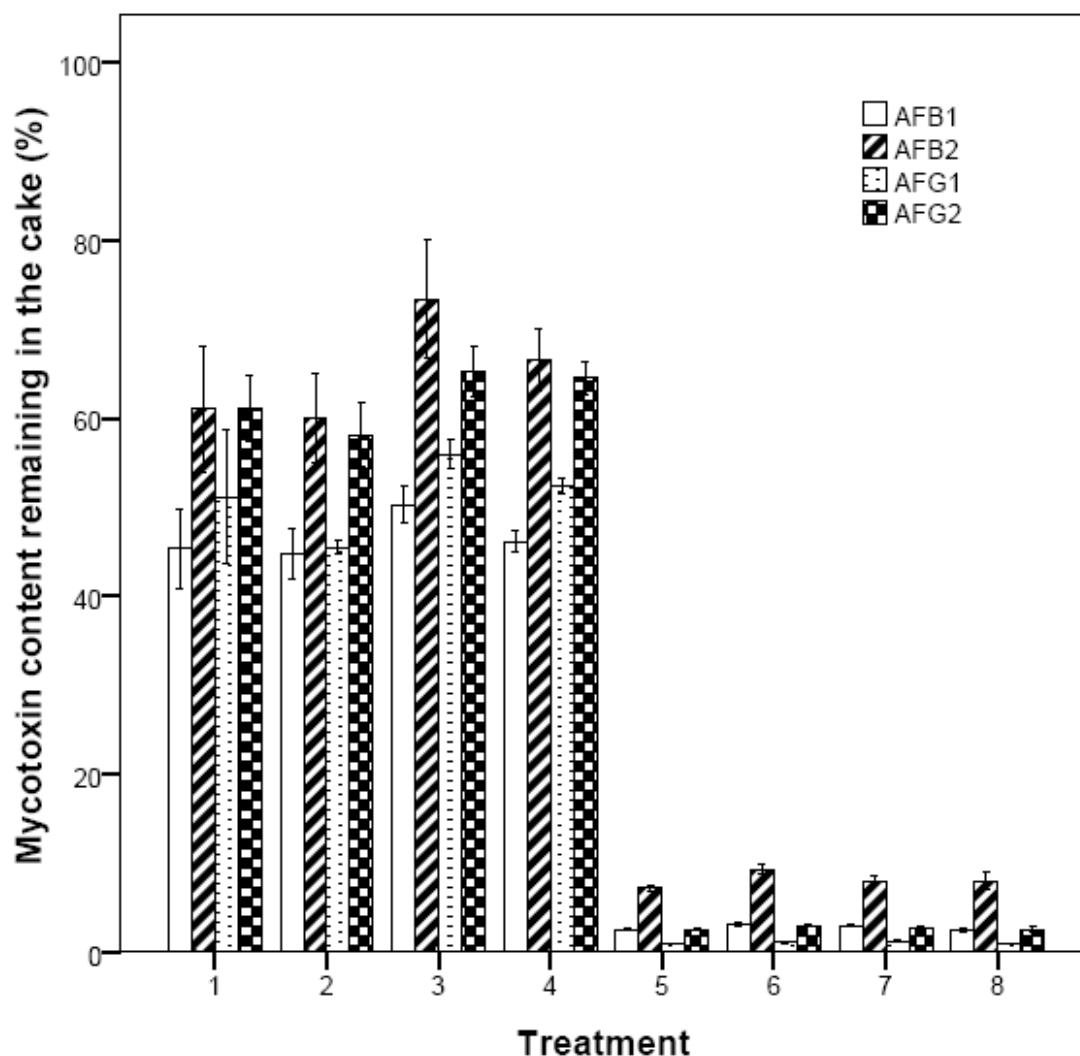


Figure 6.1: Effect of NaHCO<sub>3</sub>, NaCl and sucrose on the degradation of AFs during baking of maize cake at 190°C for 50 mins. Treatments: 1- plain cake; 2- sucrose only; 3- NaCl only; 4- sucrose and NaCl; 5- NaHCO<sub>3</sub> only; 6- sucrose and NaHCO<sub>3</sub>; 7- NaCl and NaHCO<sub>3</sub>; 8- sucrose, NaCl and NaHCO<sub>3</sub>.

The observed influence of  $\text{NaHCO}_3$  on the thermal degradation of FBs (Figure 6.2) agrees with Jackson et al. (1996a, 1996b) who observed greater FBs reduction under alkaline conditions than at neutral pH. Heating of FB under alkaline conditions results in the hydrolysis of the tricarballylic esters at C-14 and C-15 yielding partially or totally hydrolyzed derivatives which are as toxic as their parent mycotoxin (Dombrink-Kurtzman et al., 2000; Voss et al., 2001). Notably, in the present study a similar percentage in reduction of FB1, FB2 and FB3 was observed in the cakes suggesting a common point of reactivity in the three types of FB molecules.

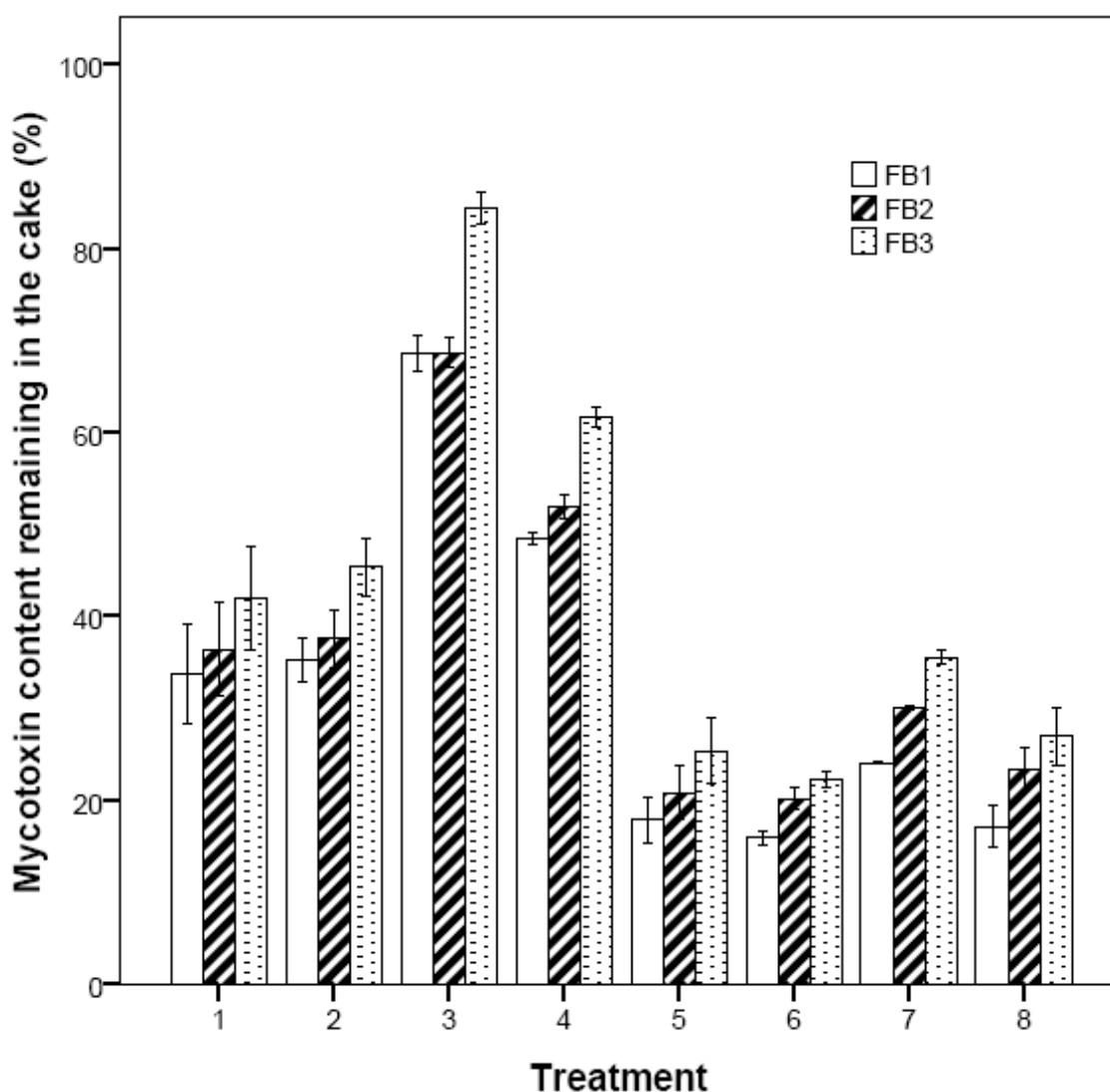


Figure 6.2: Effect of  $\text{NaHCO}_3$ ,  $\text{NaCl}$  and sucrose on the degradation of FBs during baking of maize cake at  $190^\circ\text{C}$  for 50 mins. Treatments: 1- plain cake; 2- sucrose only; 3 - $\text{NaCl}$  only; 4-sucrose and  $\text{NaCl}$ ; 5-  $\text{NaHCO}_3$  only; 6- sucrose and  $\text{NaHCO}_3$ ; 7-  $\text{NaCl}$  and  $\text{NaHCO}_3$ ; 8- sucrose,  $\text{NaCl}$  and  $\text{NaHCO}_3$ .

The significant influence of  $\text{NaHCO}_3$  on the degradation of the *Fusarium* mycotoxins DON, Ac-DON and NIV (Figure 6.3), observed in the present study further demonstrates the positive influence of alkalization on thermal degradation. Previously Bretz et al. (2006) observed an alkaline catalyzed thermal degradation of NIV, DON and 3-ADON after treatment with 0.1 M NaOH. The degradation products showed less cytotoxicity compared to the parent mycotoxins and the degradation compounds were also detected in commercial thermally processed food by the same authors.

The significant reduction in mycotoxin content observed in cakes that included  $\text{NaHCO}_3$  may have resulted from an interaction between  $\text{NaHCO}_3$  and the heat treatment ( $190^\circ\text{C}$ ) and not on  $\text{NaHCO}_3$  alone since Yumbe-Guevara et al. (2003) previously recorded the stability of NIV, DON and ZEN in a mixture of ground maize and aqueous  $\text{NaHCO}_3$  under a temperature range of  $20\text{--}110^\circ\text{C}$  for up to 12 days.

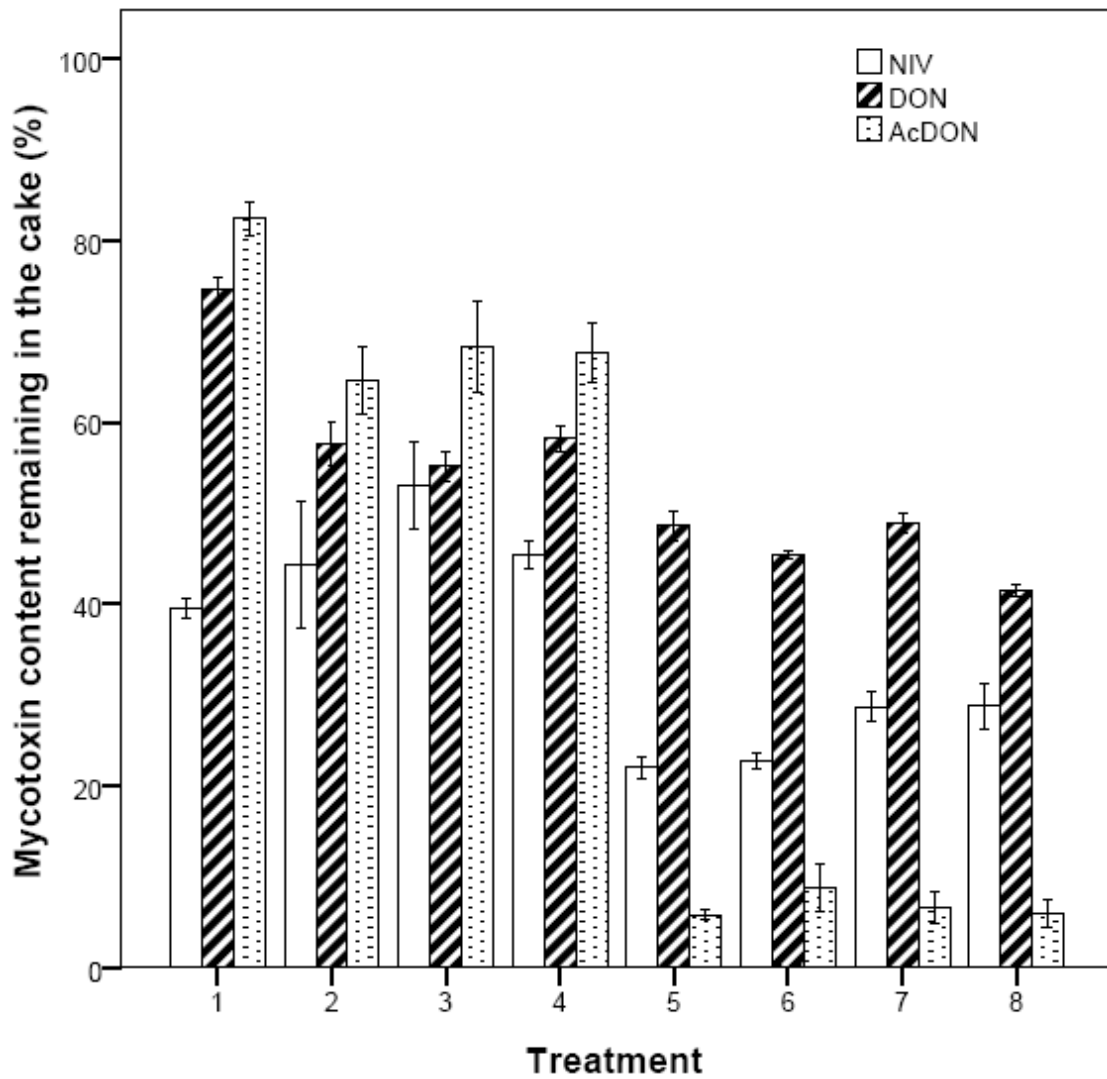


Figure 6.3: Effect of NaHCO<sub>3</sub> NaCl and sucrose on the degradation of NIV, DON, AcDON during baking of maize cake at 190°C for 50 mins. Treatments: 1- plain cake; 2- sucrose only; 3 -NaCl only; 4- sucrose and NaCl; 5- NaHCO<sub>3</sub> only; 6- sucrose and NaHCO<sub>3</sub>; 7- NaCl and NaHCO<sub>3</sub>; 8- sucrose, NaCl and NaHCO<sub>3</sub>.



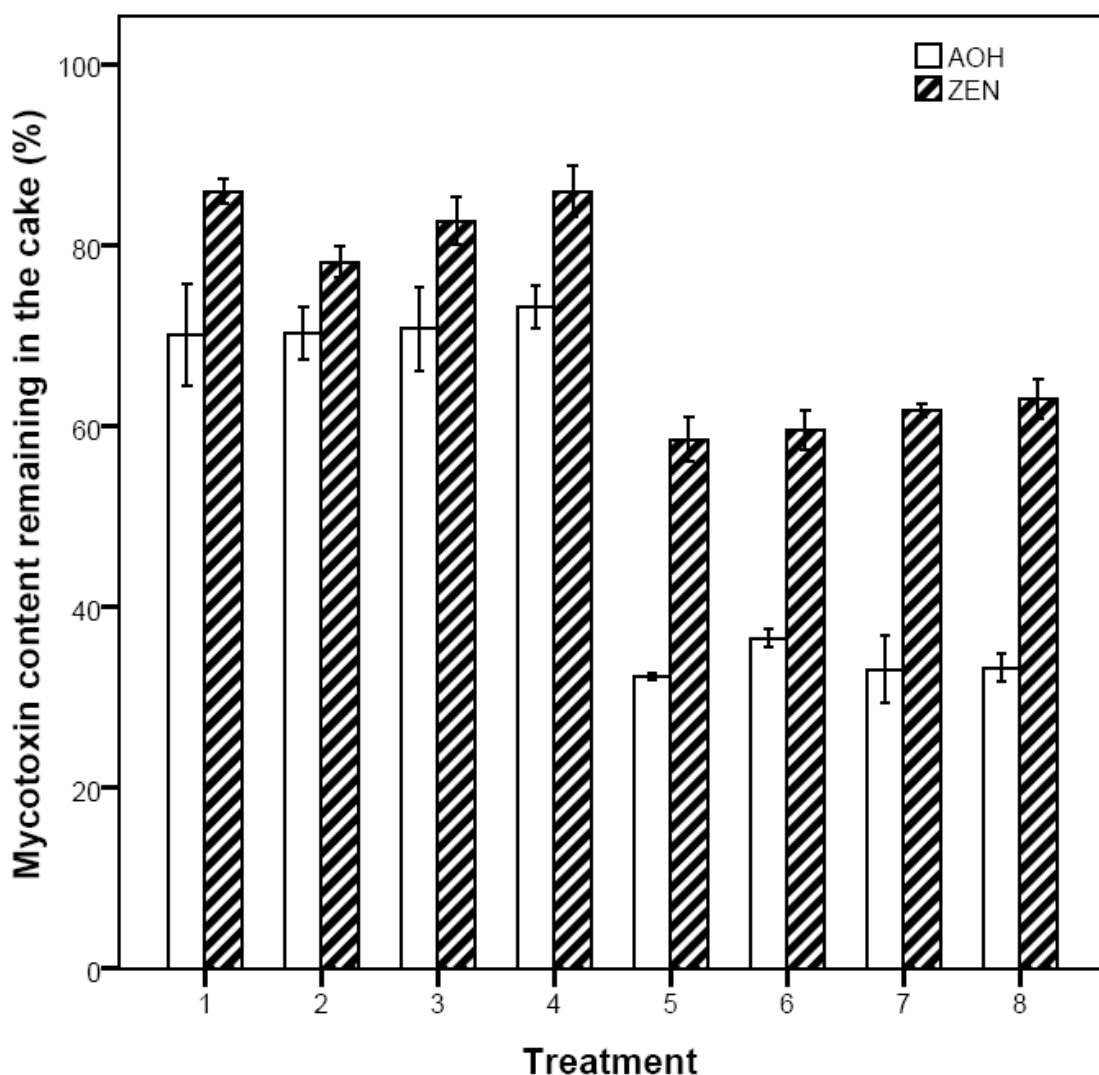


Figure 6.4: Effect of NaHCO<sub>3</sub>, NaCl and sucrose on the degradation of AOH and ZEN during baking of maize cake at 190°C for 50 mins. Treatments: 1- plain cake; 2- sucrose only; 3 -NaCl only; 4-sucrose and NaCl; 5- NaHCO<sub>3</sub> only; 6- sucrose and NaHCO<sub>3</sub>; 7- NaCl and NaHCO<sub>3</sub>; 8- sucrose, NaCl and NaHCO<sub>3</sub>.

Regression analysis indicated that sucrose had no influence on the thermal degradation of FBs during maize cake baking. (Figure 6.2). Reducing sugars such as glucose and fructose are known to enhance thermal degradation of FBs (Liu et al., 2001; Lu et al., 2002; Poling et al., 2002). However, Castelo et al. (2001) observed that sucrose (a non-reducing sugar) also enhanced the degradation of FBs during extrusion process. The latter findings suggest the possibility of inversion of sucrose into the reducing sugars due to acidity of the food matrix or excessive pressure during an extrusion, environments which were not applicable in the present study. Sucrose only enhanced the thermal degradation of DON, AcDON and ZEN but for these mycotoxins the influence was rather limited.

The inclusion of NaCl enhanced DON and AcDON degradation but it increased the thermal stability of FBs. It could be assumed that NaCl reduced water activity thus slowing down thermal reduction of the FBs. This effect of counteracting thermal reduction of mycotoxins was also significant for NIV (Figure 6.3) and apparent in the case of AFs (Figure 6.1) although in the latter the effect was not statistically significant. Unlike in the plain cake where similar, thermal degradation rates (about 70%) for DON and acetylated versions of DON ( $\Sigma$ AcDON) were observed, the inclusion of NaHCO<sub>3</sub> resulted in a greater degradation rate of  $\Sigma$ AcDON than DON. These results suggest that the degradation of the AcDON is related to the cleavage of the acetyl group as this is the only difference with DON. Earlier, Bretz et al. (2006) isolated novel norDON A, norDON B, and norDON C after heating 3-AcDON in 0.1 M aqueous NaOH for 60 min at 75 °C. In the present study, additional alkaline (NaHCO<sub>3</sub>) bakery experiments of maize flour separately spiked with 3-AcDON and 15-AcDON to 1000 µg/kg resulted in an almost complete loss of metabolites and yielded 6±1% and 7±1 % (SD) of DON respectively. This result indicates that thermal degradation of 3-AcDON and 15-AcDON yielded other degradation products which were not considered in the present study. Considering that the maize flour used for baking in Experiment 1, contained only 91±2 µg/kg  $\Sigma$ AcDON, the 6-7% formation of DON from  $\Sigma$ AcDON could not have influenced the high recovery of DON, if anything, this could have resulted from the release of DON from other conjugated forms (Voss and Snook, 2010).

The interactive effects of NaCl, sucrose, and NaHCO<sub>3</sub> on the thermal degradation of the different mycotoxins varied considerably. Sucrose\*NaHCO<sub>3</sub> increased the stability of DON,  $\Sigma$ AcDON and ZEN; NaCl\*sucrose enhanced thermal degradation of FBs but increased stability of DON,  $\Sigma$ AcDON and ZEN; NaCl\*NaHCO<sub>3</sub> enhanced thermal degradation of FBs; the presence of the three ingredients together enhanced the degradation of DON and  $\Sigma$ AcDON and increased the stability of FB3 and to some extent ZEN. However these interactive effects were far lower than the single effect of NaHCO<sub>3</sub> during baking.

Acidification of alkaline (NaHCO<sub>3</sub>) -baked maize cakes to a pH 1.9 (pH within range of the human stomach), had no significant effect on any of the considered mycotoxins (AFB1, AFB2, AFG1, AFG2, FB1, FB2, FB3, NIV, DON,  $\Sigma$ AcDON, AOH and ZEN) as there were no statistical differences in mycotoxin concentrations between acidified and non-acidified cakes (data not shown). Previously, reconversion of 57% and 34% aflatoxins from

degradation compounds was reported in *masa* and *tortillas* (both Mexican traditional nixtamalization process maize products) respectively following acidification (Méndez-Albores et al., 2004). The difference in the results could be due to the differences in the cooking methods. The authors reported to have prepared *masa* from ground maize which had previously been cooked for 35 min at 85 °C under alkaline conditions (calcium hydroxide). The *tortilla* was prepared by heating 1.2 mm thick *masa* on a heated griddle at  $270 \pm 5$  °C for 17 sec each side. Comparatively the present treatment involved higher heat energy compared to the Mexican traditional nixtamalization process and therefore may have resulted into different degradation products.

#### **6.4. Conclusions**

The present study evaluated the effect of baking a plain maize cake and the influence of NaHCO<sub>3</sub>, NaCl, sucrose and combinations thereof on the thermal degradation of AFB1, AFB2, AFG1, AFG2, FB1, FB2, FB3, AOH, ZEN, NIV, DON and the the combination of 3-AcDON and 15-AcDON. Although the study only focused on the degradation of the toxins at a fixed temperature, time, cake volume and ratio of the ingredients it has provided general tendencies of compound thermal stabilities under practical baking conditions. Increasing baking temperature, prolonging baking time and increasing concentrations of NaCl, sucrose, and NaHCO<sub>3</sub> may have resulted in much higher mycotoxin degradation but could have obviously resulted in undesirable sensory parameters. Thicker cakes on the other hand could have resulted in less degradation as mycotoxin decomposition decreases with increased thickness of a food matrix (Jackson et al., 1997). Whatever the case, the present study has provided practical evidence that thermal degradation rates of mycotoxins vary considerably at a practical baking temperature and time. While some mycotoxins will almost completely disappear, others will potentially survive the harsh alkaline thermal decomposition providing further evidence of the limitations of using a thermal-chemical decomposition as mycotoxin decontamination strategies, consequently baking should not be considered as feasible mycotoxin decontamination method. Moreover, based on existing literature the reduction in the mycotoxin levels observed herein may have resulted from chemical modifications or matrix binding induced by the heat treatment or both (Rychlik et al., 2014). Both situations would not necessarily mean that there is no more (or less) toxicity as the modified mycotoxins would be harmful and the bound mycotoxins would potentially be freed following ingestion.

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Based on the limitations of chemically assisted thermal decontamination (reduction of only selected toxins and the uncertainty of fate of the formed compounds), Chapter 7 seeks effective and feasible ‘physical’ decontamination ways suitable for an agrarian setting that would remove simultaneously all mycotoxins making the food undoubtedly safer. To a certain extent it also builds on the successes of the physical decontamination strategy discussed in Chapter 4. Chapter 7 is reproduced from a manuscript in preparation ‘**Effectiveness of hand sorting, flotation/washing, dehulling and combinations thereof on the decontamination of mycotoxin contaminated white maize**’ written by Matumba, L., Van Poucke, C., Ediage, E.N., Jacobs, B. and De Saeger, S.



## **Chapter 7: Effectiveness of hand sorting, flotation/washing, dehulling and combinations thereof on the decontamination of mycotoxin contaminated white maize.**

### **7.1. Introduction**

More than 90% of Africa's maize total production is white maize and it is predominantly intended for human consumption (McCann, 2005). However, as already highlighted in the previous chapters maize just like many other cereals, is subjected to pre- and post-harvest contamination with mycotoxins. The best approach to prevent the effect of mycotoxins is to minimize the mycotoxin production itself both on the field and during storage (Bruns 2003; Chulze, 2010). However, it is impractical to totally preclude mycotoxin contamination. In Africa, maize production is mainly subsistent (McCann, 2005) and the consumers have limited or no access at all to mycotoxin screening and testing (Strosnider et al., 2006). Worse still, discarding mycotoxin contaminated food is not an easy thing in Africa due to persistent food shortage (Clover, 2003; Baro and Deubel, 2006; FAO, IFAD and WFP, 2013). There is therefore a need for efficient and cost-effective mycotoxin decontamination methods to salvage mycotoxin contaminated maize at household level.

Several mycotoxin decontamination techniques for grains have been reported and these are classified as physical, chemical or microbiological (Park et al., 1996; El-Sharkawy, 1991; Samarajeewa et al., 1990). From a food safety point of view, physical decontamination methods are considered to be superior due to the fact that they do not produce toxin degradation products which may also be toxic (Park et al., 2004). For maize, the first decontamination step is the removal of defective maize cobs at harvest and before storage (Hell et al., 2003; Kimanya et al., 2009). But even after performing this step significant amount of mouldy grains remain and are ineluctably carried over into shelled lots. Moreover under poor storage conditions moulds growth and mycotoxin contamination do occur in shelled grains. There are several reports on mycotoxins reduction through hand removal of visibly mouldy grains, removal of floating fraction /washing and dehulling (Fandohan et al., 2005; Njapau et al., 1998; Huff, 1980; Van der Westhuizen et al., 2011; Shetty and Bhat, 1999; Huff and Hagler 1985), however it remains unclear which of these methods is more efficient. Furthermore, there is hardly data on the efficacy of combinations of these physical methods.

The present study was therefore initiated to compare efficiencies of hand removal of visibly mouldy grains, removal of the floating fraction and subsequent washing of grains, dehulling and combinations thereof in removing mycotoxins from contaminated white maize with the aim of developing an efficient decontamination strategy.

## 7.2. Methodology

### 7.2.1. Origin of maize and treatments

Visually mouldy maize was purchased from a local market in the Chikwawa district, Malawi. As is a tradition in many parts of Africa, maize was first winnowed to remove chaff and dust particles. The maize was then mixed thoroughly after which 72 kg of maize was divided by quartering it into sixteen equal portions each weighing  $4.5 \pm 0.05$  kg (Campbell et al., 1986). The maize portions were used in a  $2^3$  factorial designed experiment involving three variables (hand removal of visibly mouldy grains; removal of the floating fraction and washing; and dehulling) at two levels (absent or present) (Table 7.1). Each treatment was performed in duplicate.

Table 7.1: Test conditions of the  $2^3$  factorial scheme.

Treatment number	Treatment code	Treatment		
		Flotation/washing	Hand sorting	Dehulling
1	–	0	0	0
2	$X_1$ (FL)	1	0	0
3	$X_2$ (SO)	0	1	0
4	$X_3$ (DH)	0	0	1
5	$X_1X_2$ (FLSO)	1	1	0
6	$X_1X_3$ (FLDH)	1	0	1
7	$X_2X_3$ (SODH)	0	1	1
8	$X_1X_2X_3$ (FLSODH)	1	1	1

Codes (1) and (0) indicate inclusion and exclusion of the processing step respectively.

The present experimental design can be described by the following regression equation (Cohran and Cox, 1957):

$$E[Y] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (7.1)$$

where Y is the measured response with conditional expected value  $E[Y]$ ;  $\beta_0$  is the intercept;  $\beta_1, \beta_2$  and  $\beta_3$  are coefficients for the main factors  $X_1, X_2$  and  $X_3$  respectively;  $\beta_{12}, \beta_{13}, \beta_{23}$ , and  $\beta_{123}$  are coefficients for factors (interactions):  $X_1 X_2, X_1 X_3, X_2 X_3, X_1 X_2 X_3$  respectively.

#### *7.2.1.1. Removal of mouldy grains by flotation and the washing of the non-floating fraction*

A 4.5 kg maize sub-sample was divided into 2 halves and each half was added to 9 litres of ambient (25 °C) water in a conical water bath (36 cm basal diameter; 48 cm top diameter and 28 cm high). The maize-water mixture was stirred by hand and allowed to stand for 5-10 seconds after which the top floating fraction was scooped out using a colander and pooled. This process was repeated until all floating grains and particles were removed as much as possible. The bottom non-floating fraction was washed twice with fresh water (9 L/ wash for 1 min) and at the end of each flotation/washing cycle the water was removed. Floating and non-floating fractions of the 4.5 kg maize samples were separately pooled, blotted, dried first under the sun and further by a blow-air oven (at 50°C) overnight and weighed. The edible fraction was then grounded in totality using a laboratory blender (Waring Products, New Hartford, CT, USA), further fine-milled using a laboratory mill (Christy and Norris Ltd, Suffolk, UK) and stored at 4°C. For simplicity the treatment flotation/washing will henceforth be referred to as flotation.

#### *7.2.1.2. Hand removal of visibly mouldy grains*

Maize sub-samples (4.5 kg or 4.5 kg minus the floating fraction) were hand-sorted on a plastic sheet under shade but with sufficient daylight by physically pushing each grain to either the 'good grains' or 'bad grains' side. The good grains were the unstained clean intact grains, while shriveled, immature, broken and discoloured grains were categorized as 'bad grains'. Where applicable, flotation preceded hand sorting, and the sort-out fractions were kept apart and treated as two separate samples and prepared as described above. It is also worth noting

that after the flotation step the maize was immediately hand sorted without first drying the maize grains. The time taken to hand sort 4.5 kg maize was recorded.

#### *7.2.1.3. Dehulling*

Maize dehulling is a common practice in Africa and it is done by traditional mortar-pestle techniques or by mechanized dehullers. In this experiment, maize sub-samples (4.5 kg or 4.5 kg minus fraction removed through flotation or hand sorting) were dehulled by using a wooden mortar and pestle after addition of 200 mL of water. The bran (mainly pericarp, germ tissues and residual endosperm tissue) and grits were separated by manual winnowing and the two fractions were dried, weighed, and the edible portion was ground and stored as described earlier.

#### *7.2.1.4. Reference untreated grain*

A 4.5 kg maize sub-sample was milled without removal of any fraction and stored as described above. This portion was used as a reference untreated grain (Treatment 1) (Table 7.1).

#### *7.2.2. Mycotoxin analysis*

Mycotoxin analyses of the maize flours were performed by LC-MS/MS as described in chapter 6. The limit of detection (LOD) and limit of quantification (LOQ) for the analytes found in the samples are presented in Table 7.2

#### *7.2.3. Statistical analysis*

Influence of hand sorting, flotation/washing, dehulling and combinations thereof on the decontamination of the mycotoxin contaminated maize was determined by means of regression analysis of the analyte response factor (peak area of analyte/peak area of internal standard (DOM or ZAN) for each mycotoxin (refer mycotoxin analysis, Chapter 6.).

Additionally, a factor analysis was performed to create one overall mycotoxin reduction measure and 3 class factors: B trichothecenes (NIV, DON and AcDON); aflatoxins (AFB1, AFB2, AFG1 and AFG2); and fumonisins (FB1, FB2 and FB3). The data was reduced using principal component analysis (in the case of a missing value, conditional mean imputation



was used) and an ANOVA was performed on the overall mycotoxin reduction, each of the 3 class factors and AOH separately.

All statistical analyses were done using SPSS<sup>®</sup> (version 16) software (SPSS Inc., Chicago, Illinois, USA) and R version 3.1.0 (The R Foundation for Statistical Computing). The level of confidence required for significance was set at  $P \leq 0.05$  unless otherwise stated.

Table 7.2: Limit of detection (LOD) and limit of quantification (LOQ) for the analytes found in the samples as analysed by LC-MS/MS

Mycotoxin	LOD ( $\mu\text{g}/\text{kg}$ )	LOQ ( $\mu\text{g}/\text{kg}$ )
NIV	66.2	132.5
DON	110.9	221.7
3-AcDON	9.0	11.2
15-AcDON	3.0	3.7
AFG2	4.4	8.7
AFG1	3.5	7.0
AFB2	2.8	5.5
AFB1	3.2	6.4
AOH	21.9	43.3
FB1	58.2	116.5
FB3	42.4	84.8
FB2	44.6	89.2

### 7.3. Results and discussion

#### 7.3.1. Mycotoxin content of untreated maize

The untreated maize used for these experiments contained  $105 \pm 8$   $\mu\text{g}/\text{kg}$  (mean  $\pm$  SE (mean of two replications of treatment 1)) AFB1,  $20 \pm 5$   $\mu\text{g}/\text{kg}$  AFB2,  $247 \pm 36$   $\mu\text{g}/\text{kg}$  AFG1,  $34 \pm 3$   $\mu\text{g}/\text{kg}$  AFG2,  $2270 \pm 71$   $\mu\text{g}/\text{kg}$  FB1,  $1577 \pm 247$   $\mu\text{g}/\text{kg}$  FB2,  $1034 \pm 208$   $\mu\text{g}/\text{kg}$  FB3,  $146 \pm 16$   $\mu\text{g}/\text{kg}$  NIV,  $755 \pm 52$   $\mu\text{g}/\text{kg}$  DON,  $54 \pm 1$   $\mu\text{g}/\text{kg}$  AOH. The chromatographic method (Monbaliu et al., (2010)) used herein could not provide satisfactory separation of 3-AcDON and 15-AcDON and therefore they were reported as a sum of the two analytes (3-AcDON + 15-AcDON, ( $\Sigma\text{Ac-DON}$ )) and was found to be  $29 \pm 1$   $\mu\text{g}/\text{kg}$ .

### 7.3.2. Regression analysis results

Since in several cases the experimental treatments resulted in mycotoxin concentration levels below the quantification limit (LOQ) of the analytical method (Table 7.2), regression analysis was performed on the analyte LC-MS/MS response calculated by dividing peak area of the analyte by the peak area of the internal standard. For simplicity the ‘analyte LC-MS/MS response calculated by dividing peak area of analyte by peak area of internal standard’ will henceforth be referred to as A/I-ratio in the text.

All comparisons were made based on these A/I-ratios. For all the mycotoxins, the relationship between A/I-ratio and predictor variables (flotation/washing, hand sorting and dehulling) was statistically significant at  $P < 0.001$  with a very high coefficient of determination ( $R^2$ ) (Table 7.3). Almost all effects were significant at the 5% level (all were significant at the 10% level) (Table 7.3).

Table 7.3: Regression analysis coefficients and measure of fit of the effect of flotation/washing, hand sorting, dehulling and combinations thereof on the reduction of different mycotoxins in maize grains as measured by A/I-ratio (the LC MS/MS response ratio (analyte/internal standard area)).

	NIV	DON	AcDON	AFG2	AFG1	AFB2	AFB1	AOH	FB1	FB2	FB3
(intercept)	100***	100***	100***	100***	100***	100***	100***	100***	100***	100***	100***
FL	-57.3***	-60.4***	-32.3***	-68.2***	-55.9***	-64.2**	-62.6***	-61.7***	-74.3***	-77.8***	-67.0**
SO	-95.4***	-96.3***	-95.7***	-97.7***	-99.2***	-96.0***	-98.7***	-93.1***	-92.1***	-95.7***	-91.6***
DH	-71.8***	-62.9***	-51.6***	-94.4***	-94.1***	-91.9***	-87.8***	-92.6***	-85.2***	-90.3***	-88.9***
FLSO	60.3***	58.5***	38.7***	74.2***	60.2**	68.9*	64.9***	61.1***	71.2***	76.5***	63.8*
FLDH	51.1***	36.4**	11.8†	70.2***	55.5**	60.4*	55.6***	58.9***	64.2***	70.4***	60.7*
SODH	68.7***	61.0***	48.4***	92.7***	93.9***	89.1**	87.3***	87.4***	79.2***	87.3***	82.4**
FLSODH	-53.4**	-35.5*	-16.1†	-76.3***	-60.2**	-65.1†	-58.2**	-58.9***	-61.9***	-69.8**	-58.6†
R-squared	0.980	0.982	0.994	0.988	0.966	0.895	0.980	0.989	0.995	0.973	0.902

FL, SO and DH represent of flotation/washing, hand sorting and dehulling respectively. With significance codes (p-values) 0 ‘\*\*\*’ 0.001 ‘\*\*’ 0.01 ‘\*’ 0.05 ‘†’ 0.1.

### 7.3.2.1. Main effects

As shown in Table 7.3 and Figure 7.1, hand sorting had the biggest effect among the single methods, followed by dehulling and flotation in that order. The flotation treatment resulted in a reduction of the A/I-ratio by 30% for the  $\Sigma$ AcDON to 70-80% for the FBs. Dehulling led to a reduction of between 50-70% for the type B trichothecenes (NIV, DON,  $\Sigma$ AcDON) to 85-95% for the rest of the mycotoxins (Figure 7.1). On the other hand, hand sorting of 4.5 kg of maize caused a 92-99% drop of the A/I-ratio for all the toxins (Figure 7.1). Treatments caused the following mass losses: flotation  $290\pm 11$  g; hand sorting,  $645\pm 17$  g; and dehulling,  $1290\pm 68$  g which translate to 6%, 14% and 29% respectively (Figure 7.3). The flotation and dehulling results are discussed in this section, while as hand sorting results are discussed in subsequent sections.

Considering the reduction in the A/I-ratios associated with the treatments, it can be seen here that although flotation removed only a smaller fraction of the mouldy grains, it removed the most heavily contaminated ones (Figure 7.1). Decontamination of aflatoxin contaminated grains by flotation is a patented technology (Hagen et al., 1989). However just like the present findings, several authors have previously reported carryover of a significant proportion of mycotoxins following removal of the floating fraction even after addition of sodium chloride and sucrose (Huff, 1980; Huff and Hagler 1985; Shetty and Bhat, 1999; Van der Westhuizen et al., 2011;). The fact that significant fractions of different mycotoxins remained in the maize following the washing step indicate that the remaining mycotoxins might have been located in the inner parts of the pericarp and/or the endosperm. In spite of the high solubility of FBs in aqueous solutions (Canela et al., 1996), a reduction of only 11-17% FB was observed following a 10 min wash of contaminated maize grains using water at ambient temperature (Van der Westhuizen et al., 2011). The authors (Van der Westhuizen et al., 2011) did not observe further significant reduction even after prolonging the ambient wash for 24 h. However they observed appreciable reduction when warm (40 °C) water was used for washing the grains.

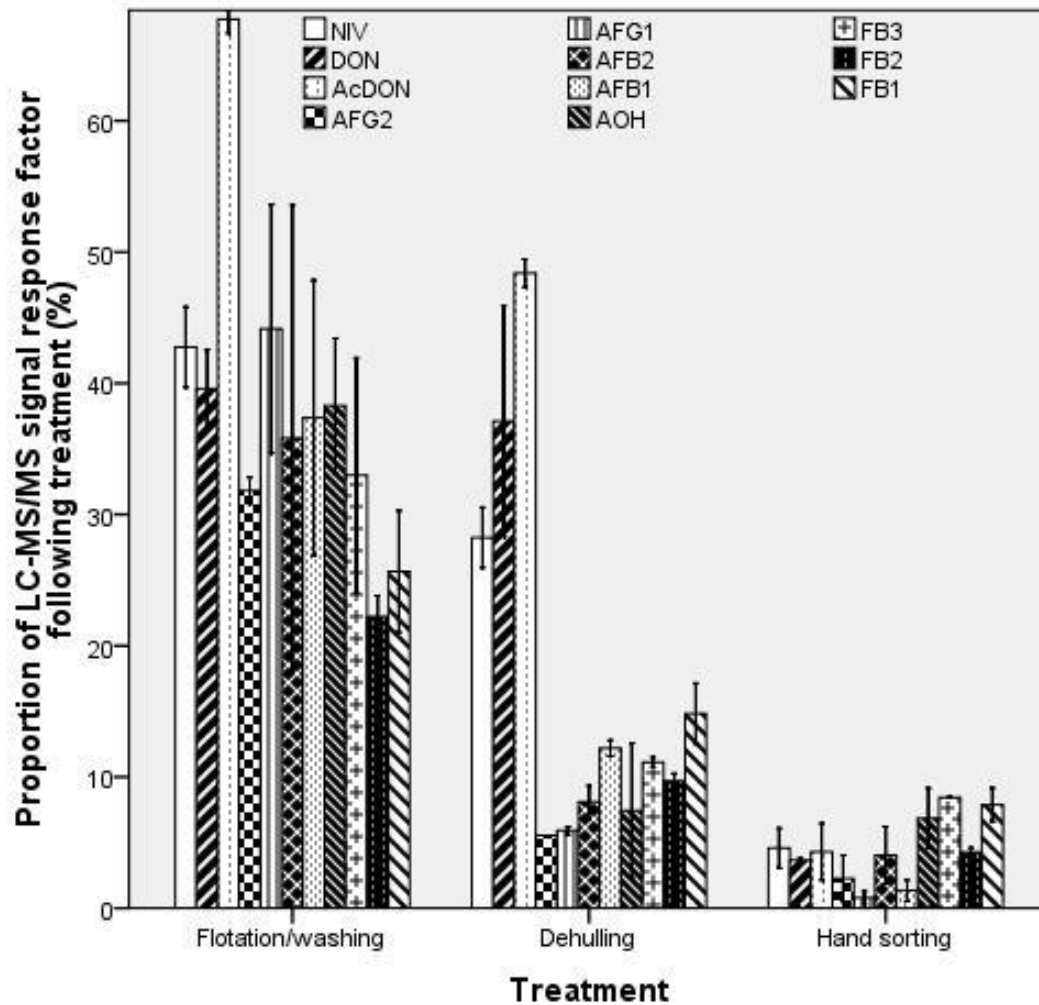


Figure 7.1: The proportions (%) of the A/I-ratio for the different mycotoxins in the maize flours prepared following flotation/washing, dehulling and hand sorting procedures with respect to the A/I-ratio induced by untreated maize flour as measured by LC-MS/MS.

Dehulling of maize has been reported to have induced varying mycotoxin reduction depending on the level of fungal and mycotoxin penetration into the grains (Fandohan et al., 2005; Njapau et al., 1998; Fandohan et al., 2006; Siwela et al., 2005). Reportedly mycotoxins are concentrated onto the outer parts of the cereal grains and therefore dehulling leaves the endosperm with lower mycotoxin levels (Duncan and Howard, 2010). However, as shown in Figure 7.1, maize dehulling left higher proportions of the type B trichothecenes (NIV, DON,  $\Sigma$ AcDON) in the endosperm compared to other mycotoxins. Specifically DON and  $\Sigma$ AcDON proportions left in the endosperm were significantly ( $p < 0.05$ ) higher than non trichothecenes

mycotoxin. As for NIV, the proportion left in the endosperm was also significantly higher compared with the non trichothecenes with the exception of AFB1 and FB1 (according to Tukey's multiple testing). This result suggested that the type B trichothecenes had penetrated deeper into the endosperm fraction of the grains. Thus, it can be speculated that during biosynthesis (when moisture levels are significantly higher) the toxins are dissolved and diffuse to all parts of the grains. Similar results were recently reported for wheat milling fractions (Schwake-Anduschus et al., 2014). The authors observed a uniform distribution of DON and its glucoside (DON-3-glucoside) between the endosperm and the fiber-rich outer layers of wheat whereas ZEN and its conjugates were highly concentrated in the outer layers. The uniform distribution of the type B trichothecenes in the grain fractions could probably be due to its high solubility in water (Lauren and Ringrose, 1997).

The removal of contaminated grains and the dehulling of maize grains present a serious potential risk to livestock health considering that the by-products are mostly used as animal feed. In Malawi for instance, maize bran is extensively used as feed, worst still the bran is usually inadequately dried or not dried at all after the wet dehulling process thereby increasing the chance of mycotoxin contamination. It is well established that mycotoxins negatively affect livestock production (Fink-Grenmels, 1999; D'mello et al., 2009; Akande et al. 2006; Goossens et al., 2012). Moreover mycotoxins are carried over into animal food products such as meat, eggs and milk (Ferrufino-Guardia et al., 2000; Matrella et al., 2006; Tangni et al., 2009; Duarte et al., 2012).

#### *7.3.2.2. Factor interactions*

The somehow opposite interaction effects of the different treatment combinations could be explained as follows: For the interaction 'hand sorting \* dehulling', as already indicated above hand sorting induced  $\geq 92\%$  reduction of A/I-ratios for all analytes. Therefore the impact of subsequent dehulling treatment could not be substantial as there were few remaining bad (mycotoxin containing) grains. The magnitude of the interaction effect thus reflected the difference between the expected efficiency of dehulling on an untreated sample and the efficiency it actually had on a cleaned sample. Similarly for the interaction 'flotation \* dehulling' as shown in Figure 7.2, substantial proportions of mycotoxins were already removed by the flotation process. The same reason holds for the interaction 'flotation \* hand sorting' which seemed slightly inferior to a complete hand sorting of the contaminated maize

(from start to finish). It is worth mentioning here that a complete hand sorting process removed a slightly larger mass proportion (not statistically significant) of bad maize compared to the flotation/hand sorting process. This result may suggest that visibility of the bad grains became compromised following moistening of the grains. Perhaps the ‘flotation\* hand sorting’ process could have yielded better results if the maize was first dried before proceeding with hand sorting . Unfortunately this treatment sequence was not tried out.

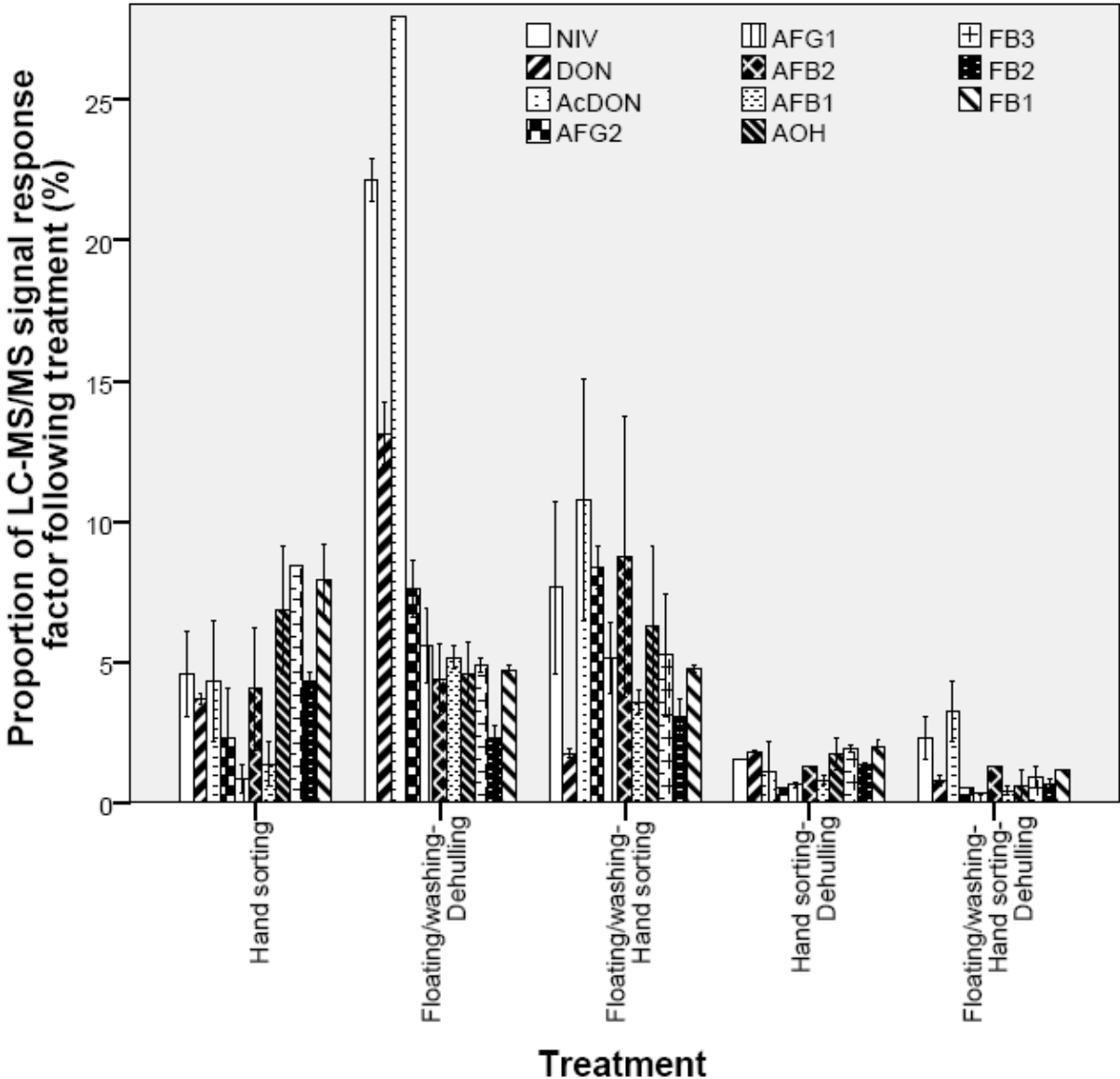


Figure 7.2: Proportions (%) of A/I-ratios for the different mycotoxins induced by maize flours prepared following hand sorting and different combination procedures with respect to the A/I-ratio induced by untreated maize flour as measured by LC-MS/MS.

Mass losses associated with factor interaction are presented in Figure 7.3. As opposed to the untreated maize where 29% mass loss was incurred due to dehulling, a lower (23%) mass loss in terms of bran (calculated with respect to maize being dehulled) was obtained when hand sorting preceded dehulling. The difference could certainly be due to the fact that the shriveled immature, broken and mouldy grains have low grain integrity and therefore during the dehulling process the grains readily disintegrated and accumulated into the bran fraction explaining the high and low relative bran percentages before and after hand sorting respectively.

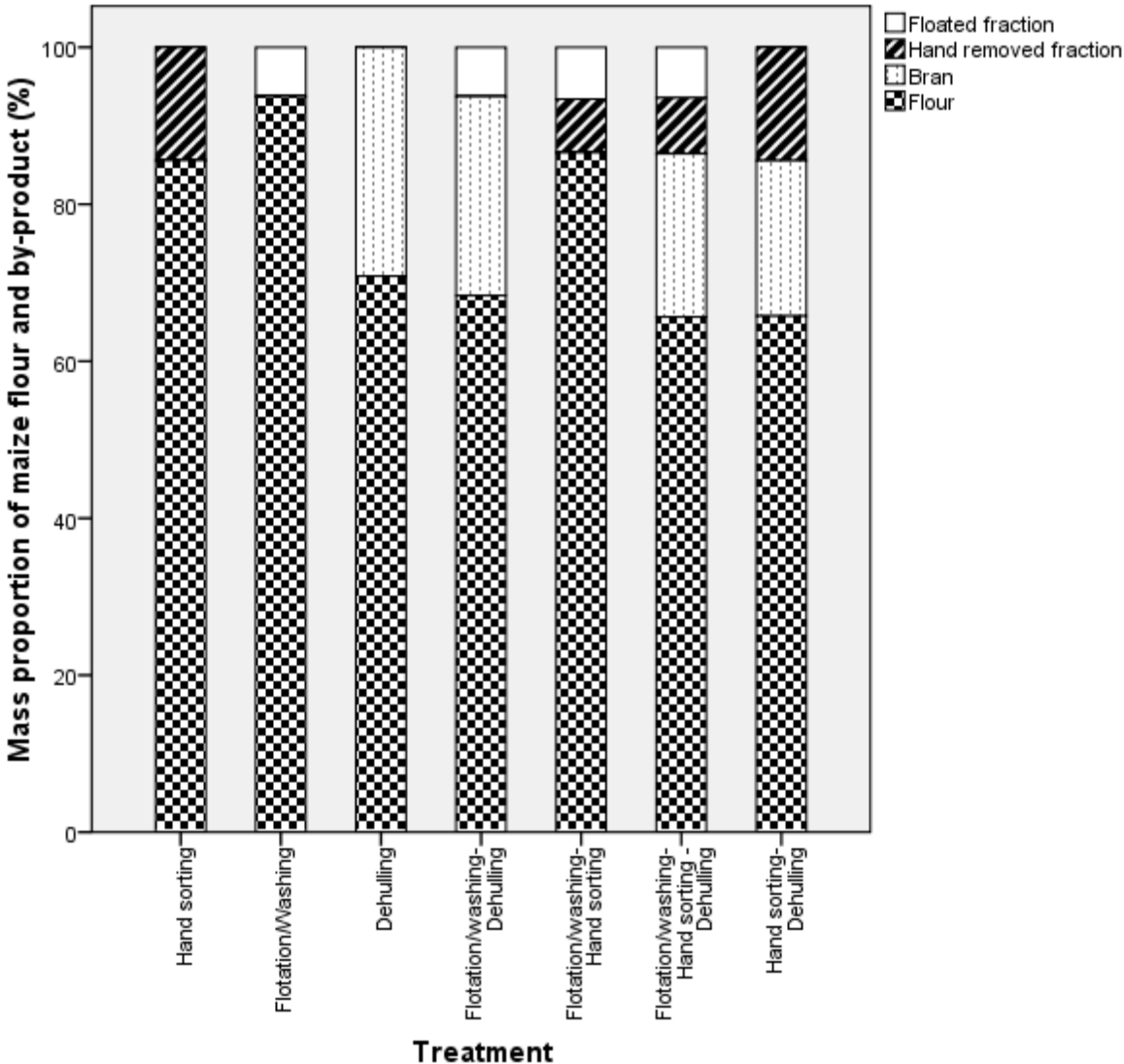


Figure 7.3: Mass proportion (percentage) of maize flour and by-products following different maize treatment procedures.



### *7.3.3. The supremacy of the hand sorting process based on ANOVA*

In light of the differences in trends among mycotoxins observed above, mycotoxins were grouped into their respective classes (as classified by Bennett and Klich, 2003). Factor analysis was subsequently performed to summarize the majority of the variance (information) into a single factor for each group. Based on mycotoxin classification three factors (mycotoxin classes) were specified and these were: B trichothecenes (NIV, DON and AcDON); aflatoxins (AFB1, AFB2, AFG1 and AFG2); and fumonisins (FB1, FB2 and FB3). AOH was analysed as a stand-alone variable as it had no 'siblings'. A fourth factor was the grand factor created by combining all the mycotoxins into one factor (Table 7.4). As can be seen in Table 7.4,  $\geq 97.5\%$  of the variance could be explained for the three sub-factors (mycotoxin classes) while as for the grand factor 'all mycotoxins' a lower percentage of variance was explained (Table 7.4). These results were not surprising as similarities and differences in treatment performances (mycotoxin reduction) were already observed among the class members and mycotoxin classes respectively (Table 7.3; Figures 7.1 and 7.2 displayed earlier). Results of ANOVA and pairwise comparisons (Tukey's test multiple testing) for responses comparing the performances of the 7 treatments measured against the reference (untreated maize) are presented in Table 7.4.

Table 7.4: ANOVA and pairwise comparisons (Tukey's test multiple testing) of A/I-ratio percentage reductions induced by different treatments (floating/washing, hand sorting, dehulling and combinations thereof). Also displayed is the variance explained by factor created.

Treatment/Parameter	Factor created by factor analysis**				
	B trichothecenes (%)	Aflatoxins (%)	Fumonisin (%)	AOH	All mycotoxins
FLSODH	97.9a	99.4a	99.1a	98.9a	98.9a
SODH	98.5a	99.2a	98.3a	98.3a	98.7a
SO	95.8ab	97.9a	93.1a	93.1a	95.6ab
FLSO	93.4ab	93.6a	95.7a	93.7a	94.1ab
FLDH	79.0bc	94.3a	96.0a	95.4a	91.8ab
DH	63.4cd	92.1a	88.1a	92.6a	83.0b
FL	51.4d	63.8b	73.1a	61.7b	63.3c
Untreated maize (reference)	0e	0c	0b	0c	0d
Variance explained factor*	97.6	97.5	98	100**	94.3

Same letters in a column indicate that means are not significantly different from each other.

\*Variance of the variables included in the respective factor analyses that is explained by the first factor of said class. Groups were based on mycotoxin class (B trichothecenes (NIV, DON and AcDON), Aflatoxins (AFB1, AFB2, AFG1 and AFG2); Fumonisin (FB1, FB2 and FB3)). Additionally 'all mycotoxins were summarized into one grand factor as well. \*\* No factor analysis was performed on AOH as it is already a single factor. FL, SO and DH represent of flotation/washing, hand sorting and dehulling respectively.

From the results displayed in Table 7.4, it can be seen (among other things) that the best four treatments (which are never pairwise significantly different after correcting for pairwise testing) in terms of mycotoxin reduction involved at least a hand sorting step. Floating and dehulling was a viable alternative (not significantly different from the top 4) for AFs and FBs, but failed to perform as well as procedures including hand sorting when it came to the removal of the B trichothecenes. Regarding this phenomenon, it has already been hypothesized above that the B trichothecenes might have penetrated deeper into the grains

thereby requiring complete removal of the contaminated grains and not merely removing the hull (dehulling).

In a ranking, a combination of all steps ‘flotation \* hand sorting \* dehulling’ outperformed ‘hand sorting \* dehulling’ although not statistically significant. This result was interesting considering that the reverse was true for ‘flotation \* hand sorting’ and hand sorting (also not significantly different) (Table 7.4). The somehow superior performance of ‘flotation \* hand sorting’ when dehulling may have resulted from the fact that the grains were moistened during the flotation/washing step thereby increasing the dehulling efficiency. Tempering (moisten the grains prior to dehulling) increases the dehulling efficiency (Moeser et al., 2002). On the other hand considering that these differences were not statistically different it might also have resulted from random errors (heterogeneity of the experimental samples or human error (dehulling efficiencies)).

As indicated earlier, the percentage mycotoxin reductions discussed so far were based on a one point calibration (mean of response factors induced by mycotoxins in untreated maize) and therefore might not necessarily reflect actual concentrations of mycotoxins following treatment. Nonetheless, in terms of actual mycotoxin concentrations a complete hand sorting process, a flotation, hand sorting-dehulling process and a hand sorting-dehulling process reduced all the considered mycotoxins to below the LOQs provided in Table 2. Considering the LOQs this represents the following percentage of mycotoxin reductions: >94% for AFB1 (Figure 7.4), >97% for AFG1 (Figure 7.4), >92% for FB1, >95% for FB2 and >93% for FB3. Since for the other mycotoxins the concentrations in the untreated maize grains were not far from LOQs, estimation of mycotoxins percentage reductions in terms of concentration might be misleading.

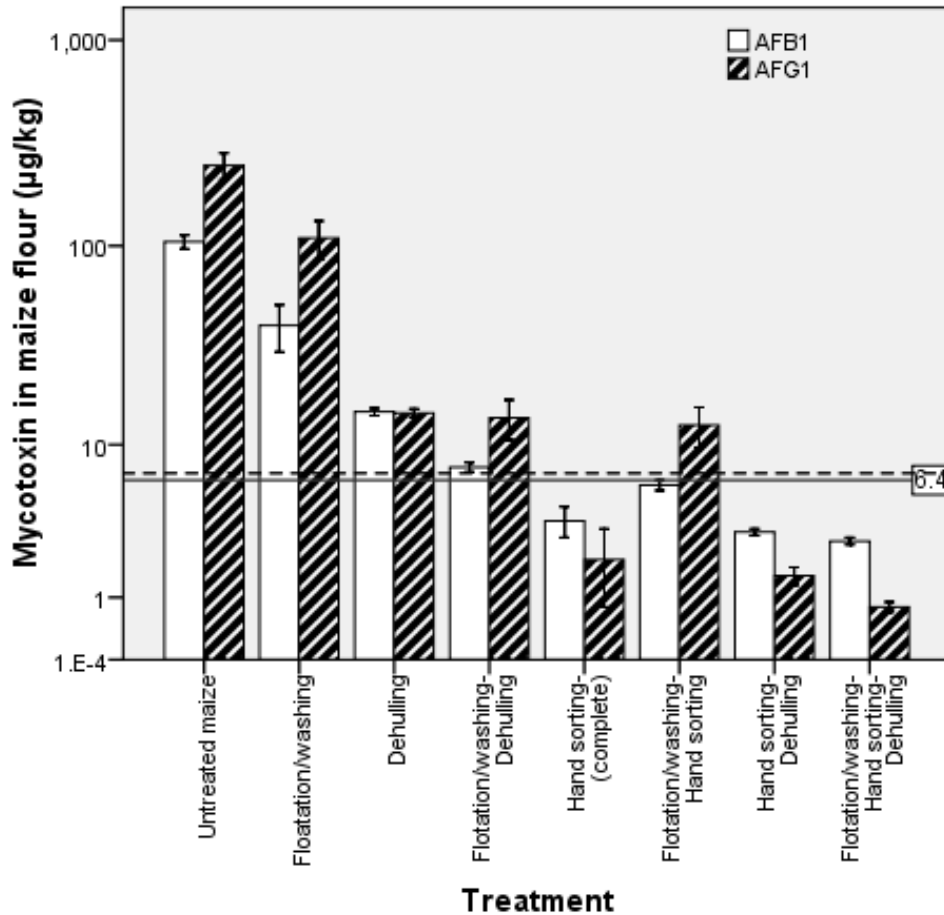


Figure 7.4: AFB1 and AFG1 levels (mean±SE) in untreated maize and treated maize. The continuous line indicates the LOQ for AFB1(6.4µg/kg) and the dotted line indicates the LOQ for AFG1(7.0 µg/kg)

Given the mycotoxin removal efficiencies provided above, hand sorting could be a more feasible last line of defense against high mycotoxin exposure among subsistent maize consumers. Although a tedious task (it took  $54 \pm 4$  (mean±SE) min to hand sort 4.5kg maize), a thorough hand sorting of the maize resulted in much lower mass loss compared to the dehulling process, a practice that is commonly accepted in sub-Saharan Africa (not necessary as a mycotoxin decontamination strategy but to improve the texture of the *Nsima* (thick staple porridge)). Owing to the excellent mycotoxin removal efficiencies of hand sorting realized in the current study, the dehulling step might not be necessary especially when the maize is already fairly clean. In addition to the huge mass loss and poor mycotoxin removal efficiencies (particularly for the type B trichothecenes), maize dehulling leads to high loss of vitamins and minerals (calcium, phosphorus zinc and iron) (Bauernfeind and DeRitter, 1991).

In Africa, these nutrient losses could even be much greater considering that both the mortar–pestle technique and most dehullers in use are not specialized to strip only the hull from the grain; they also remove the germ (embryo).

‘Flotation\*hand sorting’ was tried in this experiment with the thinking that it would save time of hand sorting. Indeed the time taken to remove the remaining bad grains was somewhat shorter  $43\pm 3$  min. But considering that only about half the amount of grains remained it can be seen that the flotation step was not a great time saver. This could be due to the fact that the hand sorting technique used in the present study involved ‘scanning’ (physically pushing the grains to their respective side (‘good side and bad side’)) thus taking almost the same time as with sorting the original sample. It is an opportune time to mention here that the application of the flotation step has a drawback in the sense that an additional drying step is required before storage or whole grain milling. However the flotation/washing step could complement the winnowing process by mechanically removing dust particles which contain high levels of mycotoxins (Ehrlich and Lee, 1983) and also remove pesticide residues (Norman and Panton, 2001). In that sense perhaps the reverse order would be logical (hand sorting and then washing). However a risk analysis would be necessary to determine whether washing step could not increase the chances of fungal growth and mycotoxin production under practical rural setting particular if the grains are to be stored thereafter.

Hand sorting of food products using the human naked eye to remove mouldy foodstuffs has probably existed since the origin of mankind. However, hand sorting has been optimized only for high value crops and is being used successfully by exporters from the developing world taking advantage of the availability of relatively cheap labour (Galvez et al., 2002, 2003; Derlagen and Phiri, 2012). In fact previous research conducted in the 1970/90s demonstrated the supremacy of hand sorting over electronic colour sorting technology in reducing aflatoxin levels in peanut and almonds (Dickens and Whitaker, 1975; Schade et al., 1975; Pelletier and Reizner, 1992). In spite of the legendary history of hand sorting and its successes in mycotoxin management in export commodities the technology remains underutilized in the management of mycotoxins in subsistent food commodities. This could be due to the fact that in general negative effects of mycotoxins are still not known or recognized by most consumers.

#### **7.4. Conclusions**

The present study has provided further evidence that mycotoxins are concentrated in shriveled immature, broken and discoloured grains. A thorough manual removal of these 'bad grains', although a tedious task, can significantly remove mycotoxins with an efficiency of 95% or higher in contaminated white maize. Integrating hand sorting into the maize production and utilization chain can therefore potentially reduce mycotoxin dietary exposure among agrarian consumers. In that regard, governments and relevant developing partners in agrarian communities should endeavor to popularize the technique among the substituent consumers. Realizing the fact that adoption of such a tasking technique would demand huge incentives, advocates of such need to begin with augmenting the public awareness on the health risk associated with consuming mycotoxin contaminated food. Future research efforts should be directed towards finding a sustainable alternative use(s) of the contaminated grains in order to pull them away from the human food chain.

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## **CONCLUSIONS AND PERSPECTIVES**

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## CONCLUSIONS AND PERSPECTIVES

“The problem with most problem-solving is that the problem itself is seldom clearly defined and understood” (Anonymous). Although the multifaceted issue of mycotoxins in food in sub-Saharan Africa might have been covered by numerous studies performed before, the problem remains complex.

Through an in-depth analysis of key issues including food security, socio-economics, mycotoxin testing capacity and the agrarian nature of sub-Saharan Africa, it has become clearer that the institutionalization of mycotoxin regulations would have very little positive impact on the majority of the local consumers. It was further seen that for those countries that have borrowed mycotoxin regulation limits from elsewhere, the regulations do not serve the purpose as the consumption patterns in the sub-Saharan Africa differ from the ‘lenders’. Moreover it is apparent from the analysis that in most countries these ‘borrowed regulations’ are merely ‘white elephants’ as the countries have no regulatory framework in place.

Using Malawi as a case study, the present study demonstrated that mycotoxin diversity in food is greatly influenced by micro-climatic conditions. Small as Malawi is (94,079 sq km land size), significant metabolite quality and quantity differences existed across the four investigated micro-climatic zones with a spectrum of up to 65 metabolites detected in human food samples. More alarming is the co-occurrence of up to 41 metabolites in the same food sample. These results are not really surprising, considering that Malawi and sub-Saharan Africa in general are situated in the tropics where the climate favours fungal growth and mycotoxin formation, particularly now that droughts have become a recurrent phenomenon. However, this is disturbing considering the existence of mycotoxin synergism. Moreover, the mycotoxin co-occurrences observed herein clearly indicate that the sole focus on aflatoxins is ‘just the tip of the iceberg’. Inasmuch as aflatoxin is one of the most dangerous toxins among the known mycotoxins, the synergistic effect of two or more ‘mediocre’ mycotoxins might be as worse. These results underline the need of the multi-mycotoxin analysis technology which unfortunately in the current socio-economic state of sub-Saharan Africa is unattainable. Perhaps a feasible alternative is to periodically (with the help of an advanced laboratory) conduct comprehensive mycotoxin surveys and identify risky zones and consequently recommend crops such as cassava that are somewhat less susceptible to mycotoxins.

The uncommon ratios of aflatoxin B1, B2, G1 and G2 observed in food samples from Malawi and the negative influence these ratios have on the use of Aflatoxin B1 measurements in estimating the total aflatoxin content is a perfect example of the limitations of borrowed solutions adopted without feasibility assessments. The uncommon ratios of aflatoxins reported herein might also have a bearing on the efficacy of a bio-control strategy as it mainly focuses on bio-competitive exclusions of *Aspergillus flavus* which in this case seems not to be the only culprit. It is recommendable to first fully characterise the *Aspergillus strains* before implementation of a bio-control. On the other hand, considering the broad spectrum of mycotoxins (65 metabolites) detected in the current study, it remains questionable if elimination of the *Aspergillus flavus* alone is really worth anyway. Perhaps embracing a total good agricultural practices (GAP) followed by good manufacturing practices (GMP) would pay-off much more. In that sense, rigorous adaptive local agronomic and breeding researches are required.

The current study has further provided evidence that the exportation of food products somehow concentrates mycotoxins on a local market. Inasmuch as many sub-Saharan African countries need to export food to high value markets for their economic survival, there is need for more sustainable strategies. As seen from the present findings, emphasis is placed on making the export commodities safer and little or no attention is paid on the safety of the locals. This is not surprising as these are efforts of private or trading partners whose goal is primarily to maximize profits. It remains the responsibility of the government to ensure the safety of the local consumers. However, as uncovered by the literature search, governments opt for silence on issues concerning mycotoxins in local food because of fear of affecting trade by scaring importers and causing consumer alarm. Obviously this is the greatest drawback. Unless the agrarian communities of sub-Saharan Africa are not equipped with adequate information on the health problems associated with mycotoxins, their prevention and management options, subsistent consumers will continue to carry the mouldy foodstuffs from fields onto their tables, continue to 'manufacture' their own poisons due to poor storage and continue to buy mouldy food stuff from the local market just for the sake of low prices.

To date there are scores of papers discussing the thermal decomposition of mycotoxins, most of which deal and monitor with the disappearance of one or a class of metabolites. Often the disappearance is misconceived as detoxification even in the absence of toxicological data. In

this study, without even performing the toxicological assessments it became evident that mycotoxins degradation rates are not uniform for different mycotoxins. Some mycotoxins potentially survived the harsh alkaline thermal decomposition providing further evidence of limitations of using chemical and thermal decomposition means as mycotoxin decontamination strategies. After all with current technological advancements it is increasingly becoming evident that most treatments result in conjugation or transformations of toxins thereby making them escape analysis of its parent forms. In light of this information and further evidence generated by present study, it is clear that thermal degradation is not a feasible option. It is further recommendable that future toxicological assessments on degradation products should thoroughly stretch beyond the horizons of expected health impacts of parent mycotoxins as breakdown products could manifest completely different health problems.

The literature is full of sweeping statements that indicate that mycotoxin contaminated foodstuffs cannot be identified by naked eyes. However a thorough hand sorting of white maize naturally contaminated with 11 different mycotoxins proved otherwise. Unlike the thermal decontamination which was found to decompose selected mycotoxins, hand sorting removed all the 11 mycotoxins almost completely. In fact it is surprising that while such sweeping statements continue to go on, the same hand sorting technique is constantly being used to clean food commodities destined for export. Although seemingly tedious, the benefits of hand sorting outweigh the costs as far as mycotoxin removal is concerned and is highly recommended as a sustainable decontamination strategy. However, the popularization of the hand sorting technique among the subsistence consumers would require some advocacy. In this regard governments and relevant developing partners in agrarian communities should therefore endeavour to augment public knowledge of the health risks associated with consuming mycotoxin contaminated food. It is only then that they would take the cost of hand sorting. Future research should also try to develop and explore the communal use of electronic sorters in decontamination of maize grains. If informed about the risks of mycotoxin contaminated food products, it is likely that consumers would be willing to pay for a sorting service just as they do with private grain dehuller and mills.

Future research efforts should also be directed toward finding a sustainable alternative use of the contaminated grains/foodstuffs in order to pull them away from the human food chain.

The present study just proved that diversion of contaminated groundnuts to oil production through small-scale pressing technology is not a feasible option as all the oil samples were found to contain mycotoxins. Perhaps a feasible option would be letting the farmer cooperative press the oil (in order to create jobs and allow the farmer to gain higher profit) and supply the crude oil to advanced industrial firms where appropriate 'physical mycotoxin decontamination' steps would be performed. In that light, research should be directed toward finding sustainable physical decontamination ways that physically 'remove' the toxin from the oil and not break them down. However, the mycotoxin decontamination of the oil would require to have some limits as it is already known that oils that are highly oxidized are more polar and therefore have a greater capability of dissolving the polar mycotoxins. In that sense high levels of mycotoxins may already indicate that the oil is oxidized not even requiring decontamination but rather discarding as highly oxidized oil in its self is a health hazard.

Given the enormous potential hand sorting has on the sustainable removal (without leaving some degradation products) of mycotoxins from grains (as demonstrated in the present study), it is evident that in spite of the hostile environmental conditions of sub-Saharan Africa, the agrarian consumer can potentially escape high mycotoxin dietary exposure. In fact, an agrarian consumer has an advantage over a consumer who relies on supermarkets as the former has control over the whole food chain from the field to the fork. An agrarian consumer has a chance of physically removing mouldy grains in their uncrushed (whole) state, which is not the case with a consumer relying on supermarkets as foods are presented in crushed and disguised form making it difficult if not impossible to detect the moulds. It should be emphasized that the present study explored the feasibility of decontamination strategies among agrarian communities as a last line of defense and a complementary effort of GAP and not an alternative.

In summary, the Government of Malawi should consider the following measures (not exhaustive list):

- Break the silence on the mycotoxin issue through a proper risk communication. The government needs to shift from prioritising exports toward a more holistic approach which ensures farmer and local consumer safety as well. Well informed farmers would ensure that their own food is safe and certainly improve the whole food production chain making the exportation of safer products much simpler. The Government



should rigorously raise awareness of the general public on the dangers of mycotoxins and its management through national campaigns. Considering that testing of mycotoxins is hardly accessible to many, emphasis should be placed on informing the public to refrain from consuming mouldy foodstuffs or giving them to animals.

- Through the Ministry of Agriculture, the government should thoroughly scrutinise and revise the existing agricultural production and utilisation guidelines in line with addressing the mycotoxin problem. A typical example is the current advocacy for minimum tillage, a practice that is known to increase the risk of mycotoxin contamination. It is therefore recommendable to perform risk analysis of all the existing agricultural recommended practices with respect to mycotoxin contamination and make necessary adjustments. In this regard, the government should promote and support agricultural research and the dissemination of findings.
- Through relevant ministries and agencies, the government should establish and enforce realistic mycotoxin regulations to control locally traded foods and related processes. A typical example of a process is the marketing of grade-outs of say groundnuts. In that regard, the government should strengthen institutional capacity. Ensuring that at least two laboratories are internationally accredited to carry out mycotoxin analysis would be helpful to not only local mycotoxin regulation but also for the facilitation of exports.

Lastly the present study has provided many insights, however from a researcher point of view it could be interesting to extend the study in Malawi in the following directions:

- Further define risky zones for different mycotoxins and consequently identify less susceptible crops or varieties for the respective zones
- Characterize the mycotoxin producing strains beginning with but not limited to aflatoxin producers
- Establish a causal relationship between mycotoxin exposure and certain illnesses through detailed epidemiological studies with particular emphasis on less known mycotoxins and synergistic interactions
- Explore effective communication tools/strategies for augmenting farmer/consumer knowledge of mycotoxin management suited to an agrarian setting

- Further explore simple and sustainable methods for mycotoxin decontamination in grains and other foodstuffs (applicable to an agrarian setting)
- Explore sustainable profitable alternative uses of mycotoxin contaminated high value foodstuffs (export commodities) in order to pull them away from the food chain (otherwise farmers would continue to consume contaminated commodities)
- Further scrutinize the fate of mycotoxins under elevated temperatures and acidic/alkaline conditions to get more insights about safety of associated food products
- Explore feasible dietary strategies to counteract the effects of relevant mycotoxins,
- Scrutinise the existing recommendations for baby and infant food recipes and develop more practical ones with respect to mycotoxin safety.

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## THESIS SUMMARY

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## **Thesis Summary (English)**

Inspired by the problem of mycotoxins in food facing the sub-Saharan Africa, this thesis aimed at gaining further understanding of the multifaceted issue and exploring feasible mycotoxin decontamination methods as a complementary strategy to prevention. The seven chapters could be summarized as follows:

Chapter 1: While mycotoxin regulations in foods are getting more detailed and extensive in most parts of the world, the situation in sub-Saharan Africa remains contrary. Up till now there are still many countries in sub-Saharan Africa which have no mycotoxin regulations in place. A study was carried out to critically analyse the challenges thwarting the establishment of mycotoxin regulations and their impacts on human dietary mycotoxin exposure in Africa. Through an in-depth analysis, the promulgation of mycotoxin regulations was projected to have little positive impact on locals considering that the majority are subsistent consumers. As regards to the existing regulations, it became evident that their establishment was not based on scientific evaluation but rather they were borrowed from industrialized trading partners and therefore do not serve the purpose as consumption patterns differ. Moreover, countries lack capacity to enforce the regulations. In conclusions, subsistent based mycotoxin exposure reduction strategies are favoured.

Chapter 2: Different fungal species have specific temperature and water activity ranges for optimal growth and mycotoxin production. Consequently different mycotoxins have been generally linked to macro-climatic regions; for instance aflatoxins and zearalenone are generally linked to tropical and temperate regions respectively. Using Malawi, a small sub-Saharan country (94,079 sq km land size) with four micro-climatic zones, the present study aimed at verifying if aflatoxins are really the major problem for sub-Saharan Africa and tried to assess if a country or sub-Saharan Africa as a whole could be treated as one block as regards to the mycotoxin occurrence pattern. A total of 65 metabolites were found in the samples. Seventy-five percentage of samples from the hottest agro-ecological zone contained either aflatoxins, fumonisins, deoxynivalenol, zearalenone, or a combination thereof in levels exceeding the European Union (EU) maximum levels whereas the related fraction in the cool temperature zone was only 17%. Aflatoxins, citrinin, 3-nitropropionic acid, monocerin and equisetin were most prevalent and in higher levels in samples from hot agro-ecological zones whereas deoxynivalenol, nivalenol, zearalenone and aurofusarin were most prevalent in cool

agro-ecologies. On the basis of the per-capita maize consumption, estimated daily intakes for all samples from the hot ecologies were well above the JECFA's provisional maximum tolerable daily intake (PMTDI) of 2.0 µg/kg body weight (bw)/day for fumonisins whereas for deoxynivalenol PMTDI of 1.0 µg/kg bw/day was exceeded in relatively more (90%) samples from the cool highlands than in the other zones. These results demonstrate the influence of the micro-climatic conditions on mycotoxin prevalence patterns and underscores the need for the development of agro-ecological specific mycotoxin dietary exposure management strategies.

Chapter 3: It is generally accepted that aflatoxin B1 levels in a naturally contaminated sample generally exceed half of the sum of the aflatoxins and that other aflatoxin analogs occur in lower levels. Likewise several countries have set separate regulatory limits for AFB1 at half the regulatory limit of the sum of the four aflatoxins and analytical methods for quantification of AFB1 alone have been developed. This study used a case study of Malawi to verify the theory and analyzed the feasibility of using such an approach in the regulation of aflatoxins. Results showed that the natural aflatoxin occurrence ratio in Malawi differed from those reported globally. In 47% of the samples the concentration of aflatoxin G1 was higher than aflatoxin B1. If the aflatoxin B1 measurement was used to check compliance with the regulatory limit of 10 µg/kg with an assumption that aflatoxin B1  $\geq$  50% of the total aflatoxin content, use of aflatoxin B1 quantification would have resulted into a 7.7% false negative rate. Similarly, if the regulatory limits for total aflatoxins were set at 20 µg/kg, 100 µg/kg, and 200 µg/kg, use of aflatoxin B1 measurement would have led to 13.4%, 24.2% and 25.5% false negative rates respectively. In this case a regulatory limit for aflatoxin B1 set at half the regulatory limit of the sum of the four aflatoxins may not be appropriate for Malawi.

Chapter 4: As a survival strategy sub-Saharan Africa, whose economies are predominantly agricultural-based, exports a variety of agricultural commodities including foodstuffs to high value markets even when the countries themselves have deficits of these commodities. Considering that importers set some minimum safety standards, products are sorted at farmer and industry level before the exportation. A meta-analysis was performed on mycotoxin data generated between June, 2012 and January, 2013 to evaluate the impact the sorting has on the export commodities (groundnuts) and the local groundnut based products. Local groundnut samples contained distinctly higher aflatoxin levels compared with samples of groundnuts destined for exports. The results demonstrated that through grading farmers

could potentially access high value markets with stringent aflatoxins regulatory limits. However, it was discovered that there were no channels for diversion of the grade-outs and therefore the exportation of the nuts was projected to be concentrating aflatoxins on the local table.

Chapter 5: Concerned with the likely negative impacts of sorting of food commodities for export purposes (discussed in Chapter 4), it has been proposed that sort-out groundnuts should be diverted to oil production in order to pull mycotoxins (contaminated nuts) away from the human food chain. A study was conducted to assess the feasibility of this option through a mycotoxin survey conducted in small-scale processed edible groundnut oils that were collected from the main groundnut growing region of Malawi. All samples were positive for aflatoxins, sterigmatocystin and diacetoxyscirpenol. Additionally ochratoxin A and zearalenone were also detected in some samples. On the basis of the current findings, it appears that the diversion of contaminated groundnuts to oil production through small-scale pressing technology is not a feasible option. An alternative strategy is required.

Chapter 6. Mycotoxins are generally thermally stable and are not destroyed during most normal cooking processes. However, varying degrees of thermal degradation of different mycotoxins have been reported under elevated temperatures depending on the degree of heat penetration, temperature, time, moisture content, pH of food, and the concentration of the mycotoxins in the food matrix. Evaluation of some degradation products have indicated reduced or loss of toxicity thus giving an impression that elevated heat treatment could be used as a decontamination strategy. Existing researches focused on only one mycotoxin or a class of related mycotoxins. However, as discussed in Chapter 2, the co-occurrence of more than one mycotoxin or class of mycotoxins in a foodstuff is more realistic particularly in a case of tropical environments (sub-Saharan Africa). In that regard, the present study evaluated the effect of baking on 12 different types of mycotoxins naturally contained in maize flour through use of a real life maize cake recipe popularly used in southern Africa involving  $\text{NaHCO}_3$ ,  $\text{NaCl}$  and sucrose (three-factorial design experiment). While some mycotoxins almost completely disappeared others survived the harsh alkaline thermal decomposition providing further evidence of the limitations of using a thermal-chemical decomposition means as feasible mycotoxin decontamination strategy. Moreover, based on existing literature the reduction in mycotoxin content may not necessarily mean that there is no more (or less)

toxicity as the mycotoxins could merely be modified thereby escaping analysis of its parent forms.

Chapter 7: Based on the limitations of chemical assisted thermal decontamination discussed in Chapter 6 a study was conducted to find an effective and feasible decontamination strategy suitable for an agrarian setting that would simultaneously remove all mycotoxins making the food undoubtedly safer. A three-factorial design experiment was conducted to examine and compare the efficacy of hand sorting, flotation, dehulling and combinations thereof in removing naturally occurring aflatoxins, fumonisins, nivalenol, deoxynivalenol, and alternariol in shelled white maize. Regression analysis was used to determine the significant ( $p < 0.1$ ) process variables on the removal of mycotoxins from the maize. Results from this experiment indicated that hand sorting had the greatest effect on mycotoxin removal while flotation yielded the least effect. In particular hand sorting left <6% of aflatoxin B1 and <5 % of fumonisin B1. Based on the obtained results, hand sorting of maize grains is being recommended as a complementary effort to good agricultural practices and consequently a last line of defense against mycotoxin exposure among subsistent consumers.



## **Thesis Samenvatting (Dutch)**

Geïnspireerd door de mycotoxine problematiek in de voeding waarmee sub-Sahara Afrika geconfronteerd wordt, is deze thesis gericht op het verwerven van verder inzicht in dit veelzijdige probleem en het verkennen van haalbare mycotoxine decontaminatiemethoden als een aanvullende strategie naast preventie. De zeven hoofdstukken kunnen als volgt samengevat worden:

Hoofdstuk 1: Terwijl in de meeste delen van de wereld de wetgeving omtrent mycotoxinen in voeding steeds uitgebreider en gedetailleerder wordt, blijft de situatie in sub-Sahara Afrika het tegenovergestelde. Tot op vandaag zijn er meerdere landen in sub-Sahara Afrika die geen wetgeving omtrent mycotoxinen hebben. Er werd een studie opgezet die de uitdagingen die het in voege treden van wetgeving omtrent mycotoxinen in de weg staan en de impact hiervan op de mycotoxine blootstelling in Afrika kritisch analyseert. Dankzij een diepgaande analyse kon besloten worden dat het creëren van regelgeving omtrent mycotoxinen een beperkte positieve impact zou hebben op de bevolking omdat de meerderheid van de consumenten voorzien in hun eigen behoeften. Met betrekking tot de bestaande regelgeving werd het duidelijk dat deze niet gebaseerd was op wetenschappelijke evaluatie maar eerder werd geleend van handelspartners uit geïndustrialiseerde landen en bijgevolg niet toepasbaar zijn gezien het verschil in consumptiepatroon. Bovendien beschikken de landen uit sub-Sahara Afrika niet over de capaciteit om de regelgeving af te dwingen. Als besluit kan er gesteld worden dat er bij voorkeur nood is aan strategieën die de mycotoxine blootstelling kunnen beperken voor consumenten die voorzien in hun eigen levensbehoeften.

Hoofdstuk 2: Verschillende schimmelsoorten hebben een specifieke bereik wat betreft temperatuur en water activiteit waarbinnen ze optimaal groeien en mycotoxinen produceren. Bijgevolg worden verschillende mycotoxinen over het algemeen gelinkt aan regio's met een welbepaald macroklimaat, zo worden aflatoxinen en zearalenone over het algemeen gelinkt aan respectievelijk tropische en gematigde regio's. In Malawi, een klein sub-Sahara land (94,079 km<sup>2</sup>) met vier microklimaat gebieden, werd onderzocht of aflatoxinen inderdaad het voornaamste probleem zijn voor sub-Sahara Afrika en werd getracht te evalueren of één land of sub-Sahara Afrika in zijn geheel met betrekking tot het mycotoxineprofiel werkelijk als een eenheid behandeld kunnen worden. In totaal werden 65 metabolieten gevonden in de onderzochte stalen. In 75% van de stalen uit de hete agro-ecologische zone werden

aflatoxinen, fumonisen, deoxynivalenol, zearalenone, of een combinatie hiervan gevonden in concentraties boven de EU maximum limiet. In de gerelateerde fractie uit de koele temperatuur zone daarentegen was slechts 17% gecontamineerd. Aflatoxine, citrinine, 3-nitropropionzuur, monocerine en equisetine werden overwegend in hogere concentraties gedetecteerd in stalen uit de hete agro-ecologische zones, terwijl deoxynivalenol, nivalenol, zearalenone en aurofusarin meer voorkwamen in de koele zones. Op basis van de maisconsumptie per persoon lagen de geschatte dagelijkse innames voor alle stalen uit de hete agro-ecologische zones duidelijk boven de JECFA's voorwaardelijke maximaal toelaatbare dagelijkse inname (VMTDI) van 2.0 µg/kg lichaamsgewicht (lw)/dag voor fumonisen, terwijl voor deoxynivalenol de VMTDI van 1.0 µg/kg lw/dag overschreden werd in relatief meer (90%) stalen afkomstig uit de koelere hooglanden dan uit andere zones. Deze resultaten tonen de invloed aan van de omstandigheden in het microklimaat op het mycotoxineprofiel en benadrukken de noodzaak om agro-ecologisch specifieke strategieën te ontwikkelen die de blootstelling aan mycotoxinen kunnen reduceren.

Hoofdstuk 3: Het is algemeen aanvaard dat aflatoxine B1 concentraties in natuurlijk gecontamineerde stalen meestal de helft van de som van de aanwezige aflatoxinen concentraties overschrijden. Bijgevolg hebben verschillende landen aparte wettelijke normen voor AFB1 geïmplementeerd, en op de helft van de waarde van de som van de vier aflatoxinen werden specifieke analytische methoden ontwikkeld voor de kwantificatie van AFB1 alleen. In deze studie werd Malawi gebruikt om deze theorie te verifiëren en nagegaan of deze benadering een haalbare kaart is in de regelgeving betreffende aflatoxinen. Resultaten toonden echter aan dat in Malawi de natuurlijke verhouding van de aflatoxinen verschillend is van deze die wereldwijd gerapporteerd wordt. In 47% van de stalen was de AFG1 concentratie hoger dan deze van AFB1. Indien enkel de AFB1 concentratie gebruikt werd om te controleren of de stalen conform waren met de 10 µg/kg wettelijke norm, aannemende dat het gehalte AFB1  $\geq$  50% is van de totale AF concentratie, zouden 7.7% vals negatieve resultaten bekomen worden. Gelijkwaardig zouden wettelijke normen van 20 µg/kg, 100 µg/kg en 200 µg/kg respectievelijk aanleiding geven tot 13.4%, 24.2% en 25.5% vals negatieve resultaten. Bijgevolg is het gebruik van een wettelijke norm voor AFB1 op de helft van de wettelijke norm voor de som van de aflatoxinen niet geschikt voor Malawi.

Hoofdstuk 4: sub-Sahara Afrika, waarvan de economie voornamelijk landbouwgebaseerd is, gebruikt de export van verschillende landbouwgrondstoffen, inclusief voedingsmiddelen, zelfs wanneer deze landen hiervan tekorten hebben, als een overlevingsstrategie. Gelet op het feit dat importeurs minimum veiligheidsnormen geïmplementeerd hebben, worden producten vooraleer ze geëxporteerd worden, gesorteerd door de landbouwers en de industrie. Een meta-analyse werd uitgevoerd op mycotoxine data die gegenereerd werd tussen juni 2012 en januari 2013 om zo de impact van deze sorteerstap op de geëxporteerde en lokale grondstoffen (pindanoten) te evalueren. Lokale pindanoten bleken duidelijk hogere concentratie aan aflatoxinen te bevatten dan de pindanoten die bedoeld waren voor export. Deze resultaten tonen aan dat door het sorteren van de grondstoffen landbouwers kunnen exporteren naar landen met strenge aflatoxine wetgeving. Er werd echter ook vastgesteld dat er geen kanalen beschikbaar zijn voor het veilig verwerken van de slechte grondstoffen en bijgevolg zorgt de export van de pindanoten voor een aanconcentrerings van aflatoxine in het lokale dieet.

Hoofdstuk 5: Bezorgd om de mogelijk negatieve impact van het sorteren van grondstoffen bedoeld voor export (zie hoofdstuk 4), werd gesuggereerd dat de slechte pindanoten gebruikt dienen te worden in de productie van olie, om zo de mycotoxinen te verwijderen uit de voedselketen. Er werd een studie uitgevoerd om de haalbaarheid van deze optie te onderzoeken, en dit door het mycotoxine gehalte in verschillende eetbare pindanoottolien te analyseren. Deze oliën werden op kleine schaal geproduceerd uitgaand van pindanoten afkomstig uit de belangrijkste pindanoot producerende regio in Malawi. Alle stalen waren positief voor aflatoxinen, sterigmatocystine en diacetoxyscirpenol. Additioneel werd soms ook ochratoxine A en zearalenone gedetecteerd. Op basis van deze vaststellingen lijkt het dat het afleiden van gecontamineerde pindanoten naar kleinschalige olieproductie geen haalbare optie is. Alternatieve strategieën zijn bijgevolg noodzakelijk.

Hoofdstuk 6: Mycotoxinen worden over het algemeen als thermostabiel beschouwd en worden bijgevolg tijdens de meeste kookprocessen niet vernietigd. Voor verschillende mycotoxinen zijn echter verschillende niveaus van thermische degradatie gerapporteerd in de literatuur, waarbij de invloed van hittepenetratie, temperatuur, tijd, vochtgehalte, pH en concentratie van het mycotoxine onderzocht zijn. Evaluatie van bepaalde afbraakproducten met verminderde of geen toxiciteit geven de indruk dat behandeling met verhoogde temperatuur gebruikt kan worden als een decontaminatie strategie. Bestaand onderzoek heeft

tot op heden altijd gefocust op één mycotoxine of één klasse van mycotoxinen. Het samen voorkomen van meer dan één (klasse van) mycotoxinen is echter, zoals in hoofdstuk 2 besproken, meer realistisch in tropische gebieden (sub Sahara Afrika). In dat opzicht heeft de huidige studie het effect van het bakken van een in het zuiden van Afrika populaire maiscake met een recept op basis van maismeel,  $\text{NaHCO}_3$ ,  $\text{NaCl}$  en suiker op 12 verschillende natuurlijk in het meel voorkomende mycotoxinen onderzocht aan de hand van een drie factor experimenteel design. Terwijl sommige mycotoxinen bijna volledig verdwenen, waren er andere die de thermische afbraak onder basische omstandigheden overleefden. Deze resultaten tonen nogmaals de beperkingen aan van een thermisch-chemische afbraak als bruikbare decontaminatie strategie. Bovendien kan op basis van bestaande literatuur besloten worden dat de daling in mycotoxine concentratie niet noodzakelijk aanleiding geeft tot afwezigheid van of verminderde toxiciteit omdat de mycotoxinen bijvoorbeeld gemodificeerd kunnen zijn waardoor ze niet langer gedetecteerd worden.

Hoofdstuk 7: Als gevolg van de beperking van de chemisch geassisteerde thermische decontaminatie zoals besproken in hoofdstuk 6, werd een studie uitgevoerd om een effectieve en haalbare decontaminatie strategie te vinden die enerzijds efficiënt is in een agrarische setting en die anderzijds toelaat om simultaan alle mycotoxinen te verwijderen. Een drie factor experimenteel design werd opgezet om het effect van handmatig sorteren, drijven, dorsen en hun onderlinge combinaties op het verwijderen van natuurlijk voorkomende aflatoxinen, fumonisinen, nivalenol, deoxynivalenol en alternariol in witte mais te onderzoeken. Regressie analyse werd gebruikt om de factoren te identificeren die een significant ( $p < 0,10$ ) effect hadden op het verwijderen van de mycotoxinen uit de mais. Resultaten van dit experiment tonen aan dat handmatig sorteren het grootste effect heeft op het verwijderen van de mycotoxinen en drijven het minste. Meer bepaald werd na handmatig sorteren  $<6\%$  aflatoxinen en  $<5\%$  fumonisine B1 teruggevonden. Gebaseerd op de bekomen resultaten wordt handmatig sorteren van maiskorrels aanbevolen als een complementaire techniek aan goede landbouw praktijken (GAP) en kan dit aldus beschouwd worden als de laatste verdedigingslijn tegen mycotoxine blootstelling van de lokale consument.

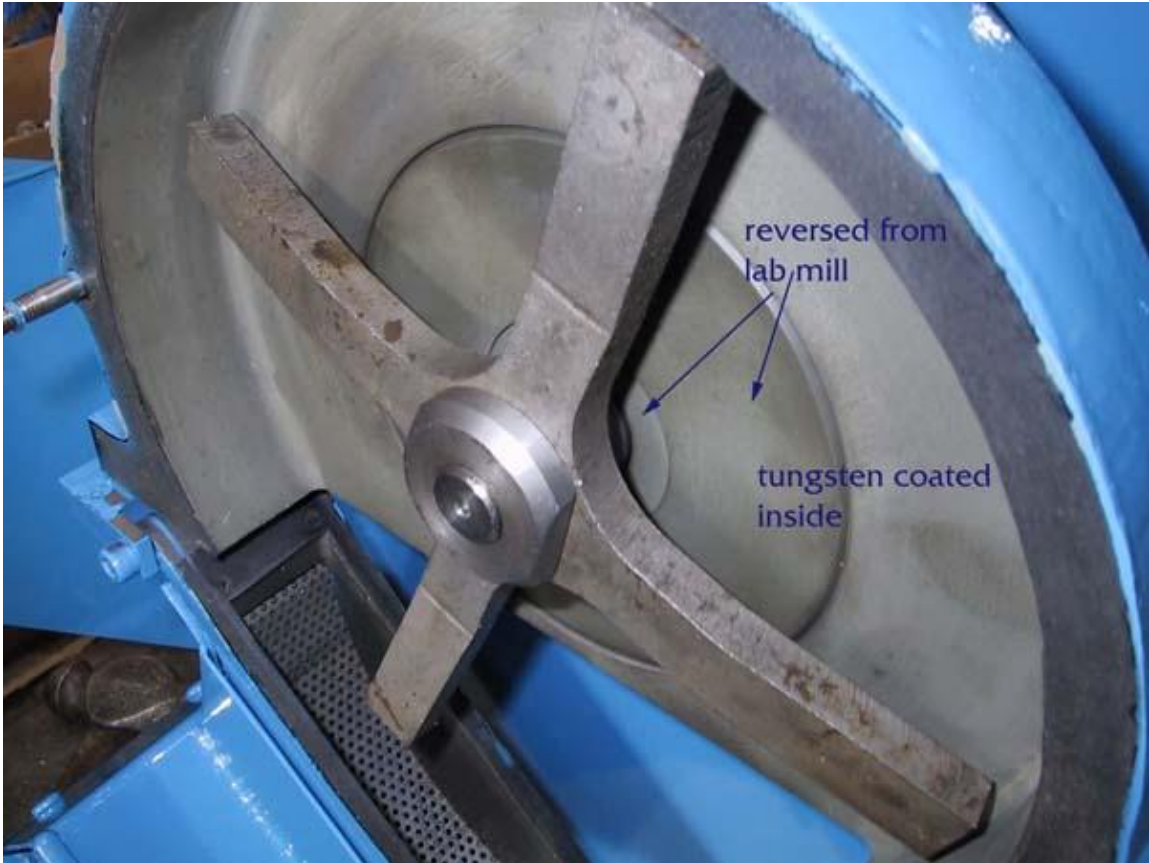
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## ANNEXES

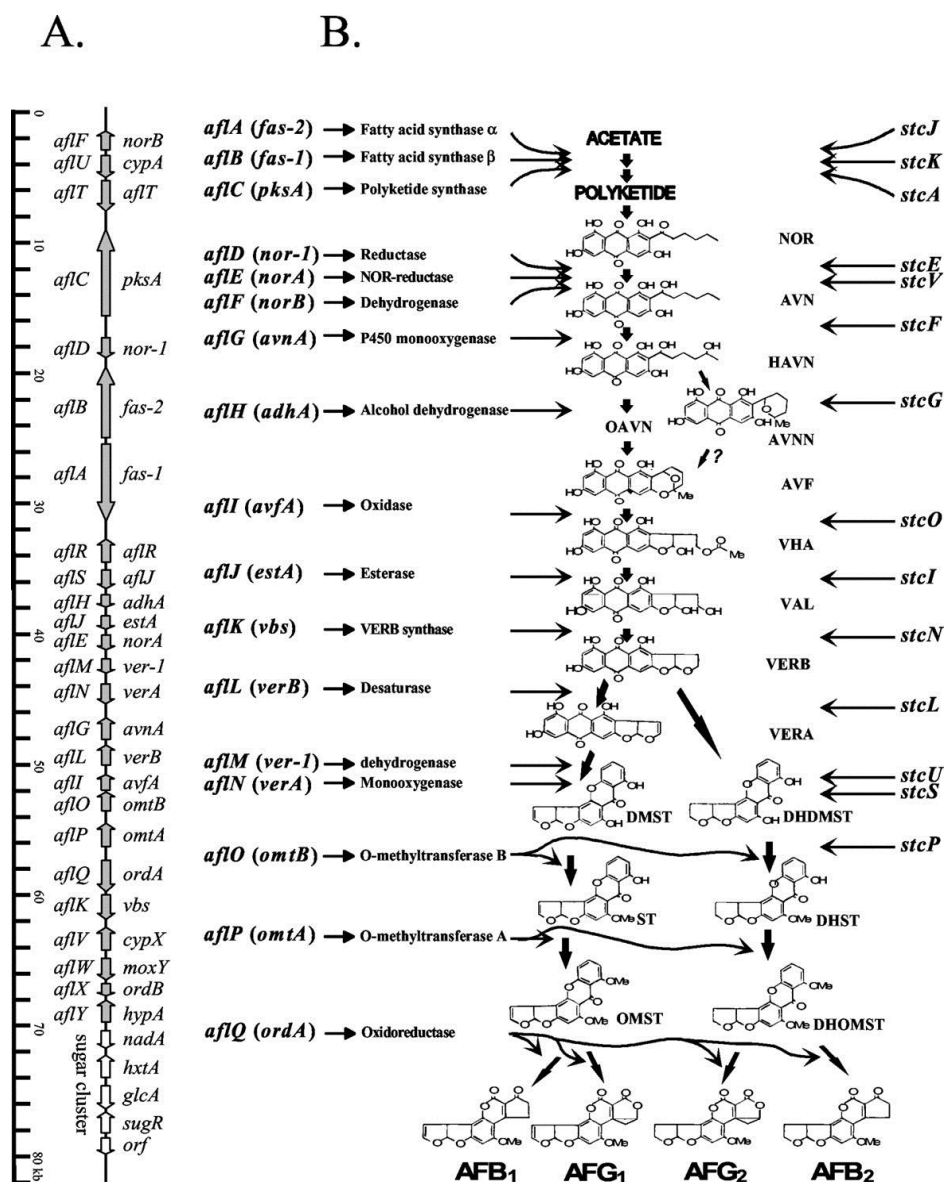
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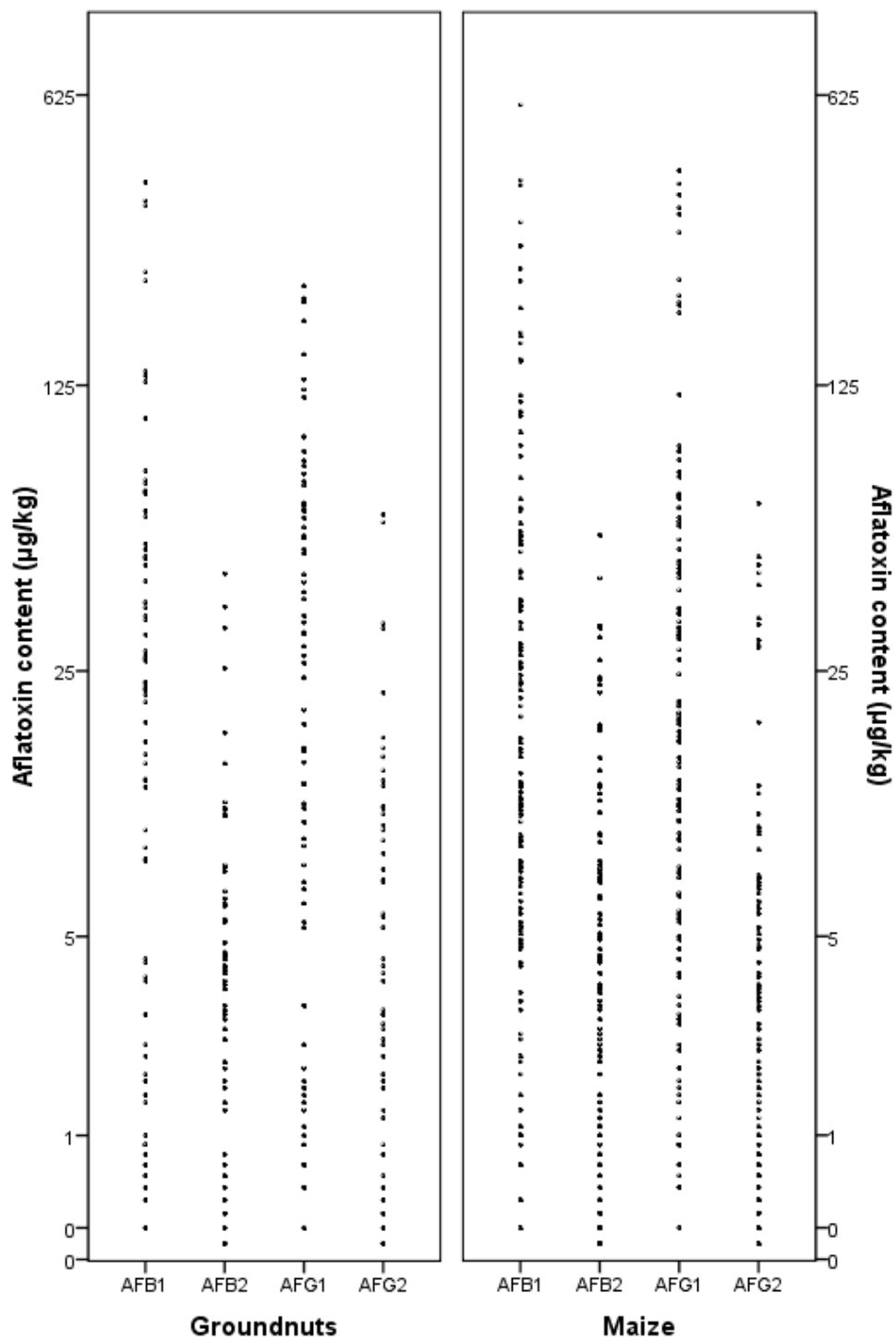
Annex I: An improvised sampling spear used to draw samples discussed in Chapter 2.



Annex II: Laboratory mill (Christy and Norris Ltd, Suffolk, UK) showing grinding chamber (used for sample preparation at Chitedze Mycotoxin Laboratory described in Chapter 2).



**Annex III: Clustered genes (A) and the aflatoxin biosynthetic pathway (B).** The generally accepted pathway for aflatoxin and ST biosynthesis is presented in panel B. The corresponding genes and their enzymes involved in each bioconversion step are shown in panel A. The vertical line represents the 82-kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in *A. parasiticus* and *A. flavus*. The new gene names are given on the left of the vertical line and the old gene names are given on the right. Arrows along the vertical line indicate the direction of gene transcription. The ruler at far left indicates the relative sizes of these genes in kilobases. The sterigmatocystin (ST) biosynthetic pathway genes in *A. nidulans* are indicated at the right of panel B. Arrows in panel B indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to the products in the aflatoxin bioconversion steps. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'-hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2. **Reproduced from Yu J et al. *Appl. Environ. Microbiol.* 2004;70:1253-1262.**



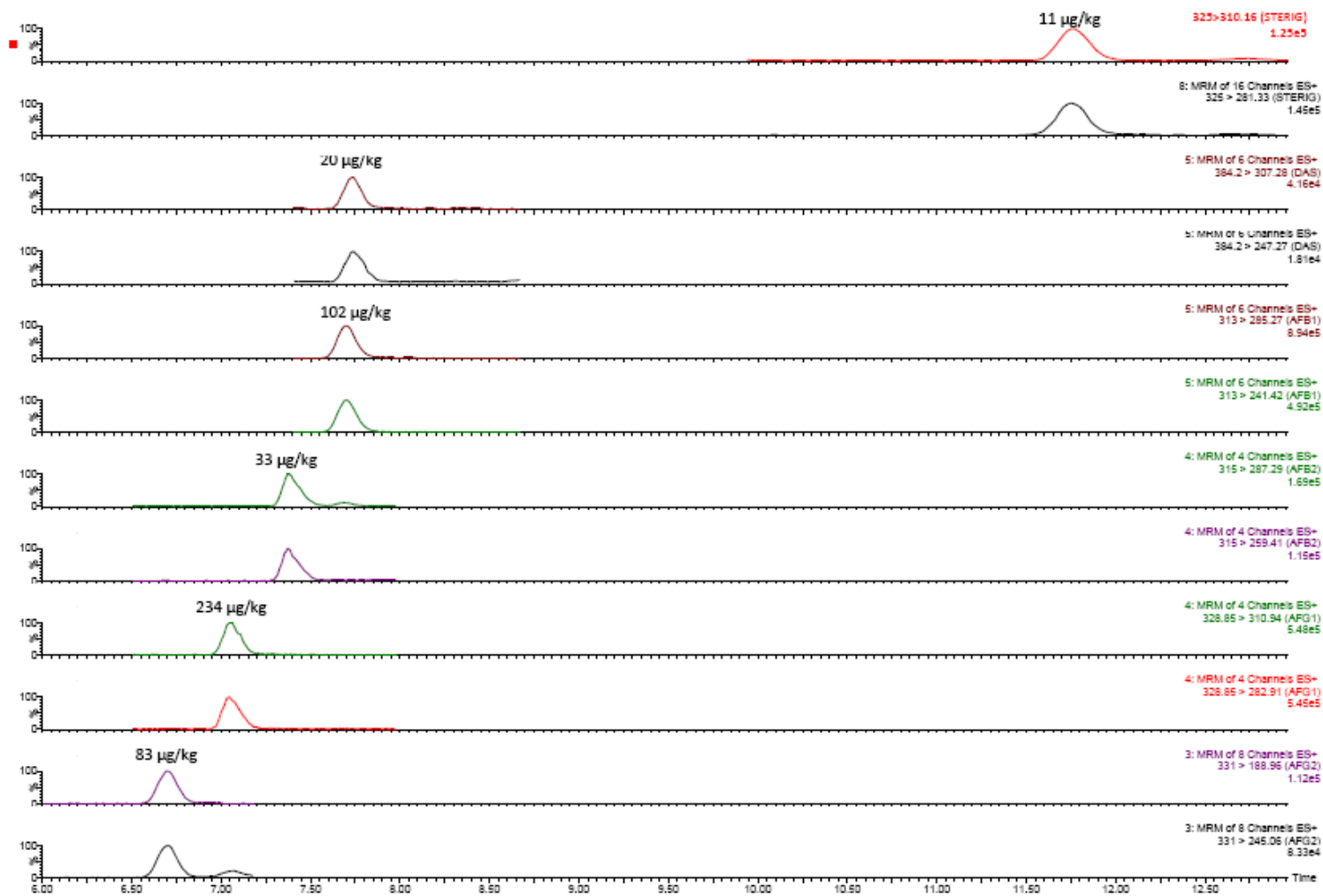
Annex IV: Distribution of aflatoxins levels in data used in the meta-analysis in Chapter 3



Annex V: Mass spectrometric parameters for the different target analytes related to analysis of groundnut oils (Chapter 5)

Component	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (eV)	Cone Voltage (V)	Expected retention time (min)
NIV	313.0	124.9*	10	23	3.1
		177.2	15		
DON	297.0	203.3*	12	26	4.3
		249.4	13		
FUS-X	355.1	175.2*	11	22	5.3
		247.2	9		
NEO	400.1	305.2*	13	20	5.4
		184.9	10		
DOM	281.2	109.2*	18	23	5.5
		137.1	25		
3-AcDON	339.2	231.2*	10	23	6.5
		203.1	10		
15-AcDON	339.1	291.3*	10	24	6.5
		297.4	8		
AFG2	331	188.9*	24	46	7.1
		245.0	30		
AFG1	328.8	310.9*	25	43	7.2
		282.9	20		
AFB2	315.0	287.3*	25	50	7.6
		259.4	29		
AFB1	313.0	285.2*	21	47	7.9
		241.4	34		
DAS	384.2	307.2*	11	19	7.9
		247.2	15		
ALT	293.1	257.1*	14	24	8.1
		275.1	10		
ROQC	390.2	193.3*	26	40	8.8
		322.2	21		
HT2	442.0	214.9*	10	16	9.3
		245.2	12		
FB1	722.5	334.3*	37	51	9.4
		352.2	36		
AOH	259.1	185.1*	30	53	10.0
		213.1	25		
T2	484.0	215.0*	12	12	10.1
		245.2	12		
FB3	706.1	336.5*	35	51	10.5
		688.5	29		
OTA	403.9	239.1*	22	24	11.1
		101.9	35		
ZAN	321.2	303.2*	14	27	11.2
		189.2	21		
ZEA	319.2	301.2*	12	22	11.5
		238.3	18		
STE	325	310.1*	35	44	11.7
		281.3	25		
FB2	706.4	336.2*	35	51	11.9
		318.0	38		
AME	272.9	258.2*	26	54	12.7
		199.2	25		

\*Most abundant fragment/product ion



Annex VI: Extracted ion chromatogram of sample 6 discussed in Chapter 5 showing co-occurrence of sterigmatocystin (STERIG) and diacetoxyscirpenol (DAS) and aflatoxins (AFG2, AFG1, AFB2 and AFB1).

## **Annex VII : Brief Curriculum Vitae**

### **Limbikani MATUMBA**

Born July 7th, 1978 in Lilongwe (Malawi)

#### **Educataion and Experience**

Qualification: MSc. Environmental Sciences (Chemistry bias) (2009), BSc. Environmental Science and Techology (Food Techology bias) (2002), The University of Malawi.

Employment: Research Scientist (Food and Feeds)- Department of Agricultural Research Services (Malawi Government) (2005-todate)

#### **Peer-reviewed publications**

Matumba, L., Monjerezi, M., Khonga, E. B., and Lakudzala, D.D. 2011. Aflatoxins in sorghum, sorghum malt and traditional opaque beer in southern Malawi. *Food Control* 22: 266-268

Matumba, L., Monjerezi, M., Van Poucke, C., Biswick, T., Mwatseteza, J. and De Saeger, S., 2013. Evaluation of the bright greenish yellow fluorescence test as a screening technique for aflatoxin-contaminated maize in Malawi. *World Mycotoxin Journal* 6: 367-373.

Matumba, L., Van Poucke, C., Biswick, T., Monjerezi, M., Mwatseteza, J., De Saeger, S. 2014. A limited survey of mycotoxins in traditional maize based opaque beers in Malawi. *Food Control* 36: 253-256.

Matumba, L., Monjerezi, M., Biswick, T., Mwatseteza, J., Makumba, W., Kamangira, D. and Mtukuso, A., 2014. A survey of the incidence and level of aflatoxin contamination in a range of locally and imported processed foods on Malawian retail market. *Food Control* 39: 87-91

Matumba, L., Sulyok, M., Monjerezi, M., Biswick., and Krska. R. 2014. Fungal metabolites diversity in maize and associated human dietary exposures relate to micro-climatic patterns in Malawi (*World Mycotoxin Journal*, in press (DOI: <http://dx.doi.org/10.3920/WMJ2014.1773>))

Matumba, L., Sulyok, M., Njoroje, S.M.C., Ediage, E.N., Van Poucke, C., De Saeger, S. and Krska R. 2014. Uncommon occurrence ratios of aflatoxin B1, B2, G1 and G2 in maize and groundnuts from Malawi (*Mycotoxin Research*, in press (DOI: <http://dx.doi.org/10.1007/s12550-014-0209-z> )

- Matumba, L., Van Poucke, C., Ediage, E.N., and De Saeger, S. 2014. Keeping mycotoxins away from the table: Does the existence of regulations have any impact in Africa? *Critical Reviews in Food Science and Nutrition* (submitted).
- Matumba, L., Van Poucke, C., Ediage, E.N., Monjerezi, M., and De Saeger, S. 2014. Practical limitations of baking as a mycotoxin decontamination strategy: Lessons drawn from a maize cake baking procedure. *Food and Chemical Toxicology* (submitted).
- Matumba, L., Van Poucke, C., Monjerezi, M., Ediage, E.N. and De Saeger, S. Concentrating aflatoxins on the domestic market through groundnut export: A focus on Malawian groundnut value and supply chain. *Food Control* (submitted).
- Matumba, L., Van Poucke, C., Ediage, E.N., Jacobs, B. and De Saeger, S. Effectiveness of hand sorting, flotation/washing, dehulling and combinations thereof on the decontamination of mycotoxin contaminated white maize (manuscript in preparation).

### **Oral presentations**

- Matumba, L., Van Poucke, C., Ediage, E.N. and De Saeger, S. Effectiveness of hand sorting, flotation/washing, dehulling and combinations thereof on the decontamination of mycotoxin contaminated white maize. Presented at the 36th Mycotoxin Workshop, 16–18 May 2014, Göttingen, Germany.
- Matumba, L., Van Poucke, C., Monjerezi, M., Biswick, T., Mwatseteza, J., De Saeger, S. 2012. Mycotoxin regulations: A white elephant in agrarian countries? A case study of Malawi. Presented at Gent Afrika Platform Symposium 7 (GAPSYM7) Ghent, 6 December 2013, Ghent, Belgium.

### **Posters**

- Matumba, L., Sulyok, M., Biswick, T., Monjerezi, M., Mwatseteza, J.F., Krska, R. Survey of fungal metabolites in 90 maize samples from four agro-ecological zones in Malawi by LC-MS/MS. A poster presented at 34th Mycotoxin Workshop, 14 - 16 May 2012, Braunschweig (Germany).
- Matumba, L., Van Poucke, C., Biswick, T., Monjerezi, M., Mwatseteza, J.F., De Saeger, S. A limited survey of mycotoxins in traditional maize based opaque beers in Malawi. A poster presented at 35th Mycotoxin Workshop, 22-24 May 2013, Ghent (Belgium).
- Matumba, L., Van Poucke, C., Biswick, T., Monjerezi, M., Mwatseteza, J.F., De Saeger, S. The influence of NaHCO<sub>3</sub> on thermal reduction of aflatoxins, fumonisins, deoxynivalenol,

nivalenol and zearalenone in maize flour during baking. A poster presented at 35th Mycotoxin Workshop, 22-24 May 2013, Ghent (Belgium).

Matumba, L, Van Poucke, C., Biswick, T., Monjerezi, M., Mwatseteza, J.F., De Saeger, S. A limited survey of mycotoxins in traditional maize based opaque beers in Malawi. A poster presented at Gent Afrika Platform Symposium 7 (GAPSYM7) Ghent, 6 December 2013, Ghent, Belgium.

### **Some mycotoxin related initiatives**

Pioneered the establishment of the Chitedze Mycotoxin Laboratory (Department of Agricultural Research Services), the major mycotoxin laboratory in Malawi which is rapidly transforming into a general food safety laboratory.

Developed the first aflatoxin extension booklet for frontline extension staff in Malawi: Matumba, Limbikani, Albert Chamango, and Wills Munthali 2012. Guidelines for management of aflatoxin contamination in groundnuts and maize in Malawi. Department of Agricultural Research Services, Lilongwe, Malawi.  
<http://www.ndr.mw:8080/xmlui/handle/123456789/518>