College of Natural Science

Center for Food Science and Nutrition

Level of Aflatoxin in Sorghum Injera from Eastern Ethiopia

BY:

Samuel Marie

Advisors:

Emana Getu (Professor)

Nigussie Reta (Professor)

A THESIS SUBMITTED TO THE CENTER OF FOOD SCIENCE AND NUTRITION IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTRES IN FOOD SCIENCE AND HUMAN NUTRITION

ADDIS ABABA, ETHIOPIA

Nov, 2017

Addis Ababa University

School of Graduate Studies Center for Food Science and Nutrition

This is to certify that the thesis prepared by Samuel Marie Engida, entitled: “Level of Aflatoxin in Sorghum Injera from Eastern Ethiopia” and submitted in partial fulfillment of the requirements for the Degree of Masters in Food Science and Nutrition according to the regulation and standards of the University and originality of the thesis is acknowledge.

Examiners

Dr. Dawit Abate (External examiner) Signature:_____Date_____

Dr. Ashagerie Zewudu (Internal examiner) Signature:_____Date_____

Advisors

1. Prof. Emana Getu Signature:_____ Date_____
2. Prof. NIgussie Reta Signature_____ Date_____

Declaration

I, the undersigned, declare that this research thesis is my original work and that all sources of materials used for the thesis have been correctly acknowledged.

.

Name: Samuel Marie Engida

Signature: _____ Date: _____

ACKNOWLEDGMENT

First and foremost, I would like to thank God for his unconditional guidance and wisdom as I make my research. I would like to express my profound gratitude to my advisors: Prof. Emana Getu and Prof. Nigussie Reta. The work presented in this thesis is a testament of their invaluable advice, foresight and dedication. I am hugely indepted and thoroughly grateful to Dr. Ashagrie Zewedu for his exemplary professional guidance in chromatographic techniques, made available Immunoaffinity column, standard aflatoxins for the research and technical support regarding the topic of my research.

I would like sincerely thank Food Science and Nutrition Center staff; Mr. Tilahun, Debebe, Yemanhe, Woinshet and others who enabled this study to be done. Sincere gratitude is hereby extended to BLESS and my organization EFMHACA for their chemical, equipment and technical support. I also forward my gratitude to all participants on the KAP (Knowledge, Attitude and Practice) assessment for willingness and cooperativeness in condition of language barriers.

Finally, I would like to reflect my gratitude to my Colleagues, teachers, friends and My Diredawa's friend Yared Ketema and his families all over Hararghe who put their faith in me and urged me to do better.

LIST OF FIGUREs

Figure 1: Chemical structures of Some important mycotoxins……..…..12

Figure 2: Chemical structures of major aflatoxins…….….14

Figure 3: Liver Biotransformation of aflatoxin …….….16

Figure 4: Aflatoxin disease pathways in humans ……..20

Figure 5: Risk assessment and management ……..….32

Figure 6: Extrapolation of dose dependence response…….……...33

Figure 7: Bench mark dose……..34

Figure 8: Map of Ethiopia indicating Hararghe region……...……...….37

Figure 9: Sorghum injera Sample collection procedure in Hararghe region…….38

Figure 10: Sample clean up procedure with IAC……....41

Figure 11: Aflatoxins standard chromatogram……..….47

Figure 12: Calibration curves for aflatoxins……....48

Figure 13: Chart shows the concentrations of total aflatoxins in sorghum injera sample…...52

Figure 14: Distributions of total aflatoxins in the total sampled analysed……..53

Figure 15: Distributions of AFB1 in the whole sample analysed……...53

Figure 16: **D**istribution of Aflatoxin B2…...……54

Figure 17: Distribution of Aflatoxin G1…...……...….54

Figure 18: Distribution of Aflatoxin G1…...……...…55

LIST OF TABLES

Table 1: Some Physical and chemical properties of Aflatoxins……..18

Table 2: U.S. Food and Drug Administration action levels for total aflatoxins in food and feed (µg kg-1) ……..35

Table 3: European Union for aflatoxins in human food (µg kg-1) ……...…36

Table 4: The average peak retention time……...…46

Table 5: Linearity and calibration curve ……48

Table 6: The concentration of individual aflatoxins in mix concentration……..…48

Table 7: Precision method validation…….49

Table 8: Limit of detection and quantification……49

Table 9:Level of Aflatoxin (µg/kg) determination in sorghum Injera sample from Eastern Ethiopia……50

Table 10: Percent of contaminated sorghum injera sample above the safe limit as per the EU and FAO food safety guidelines for direct human consumption……..…51

Table 11: Summary of total aflatoxin in Sorghum injera samples by HPLC analysis……52

LIST OF ABBREVIATIONS AND ACRONYMS

ADI…… Acceptable Daily Intake

AFs ……Aflatoxins

AOAC ……Association of Official Analytical chemists

EFSA…….. European Food Safety Authority

ELISA……..Enzyme-linked Immunosorbent Assay

EU……European Union

FAO …… Food and Agriculture Organization

FB1……Fumonisin B1

FDA ……..Food Drug Administration

FMHACA…….....Food, Medicine, Health Care Administration and Control Authority

GC-MS……..Gas Chromatography coupled to Mass Spectrometry

GAP…….. Good Agricultural Practice

GRAS ……..... Generally Regarded as Safe

HACCP…..…..…..…..…..…..…..…..…..…..…. Hazard Analysis and Critical Control Point

HCC …… …….Hepatocellular Carcinoma

HPLC DAD ……..Higher Performance Liquid Chromatography with diode array Detector

HPLC-FLD…….High-Performance Liquid Chromatography with fluorescence Detector

HPLC-MS……. High Performance Liquid Chromatography coupled to Mass Spectrometry

LAB…….Lactic acid Bacteria

IAC ……Immunoaffinity Column

IARC ……International Agency for Research on Cancer

JECFA ……..Joint Expert Committee on Food Additives

KAP ……. ……Knowledge, attitude and practice

LB ……. ……..Lower Bound

LC ……. ……Liquid chromatographic

LOD……..Limit of Detection

LOQ …… ……Limit of Quantification

MPC ……..... .Maximum permissible concentration

OTA ……..…Ochratoxin A

PMTDI…….. Provisional maximum tolerable daily intake

PPM ……..… Parts per million

PPB ……Parts per billion

RSD …….. ……Relative standard deviation

S/N …….. Signal to Noise Ratio

SPE …….....Solid phase extraction

STDEV …….. ……Standard Deviation

TDI ……Tolerable Daily Intake

TFA……Trifloroacetic acid

TLC ……..Thin-layer chromatography

UV ……..Ultraviolet

UB …….Upper Bound

ABSTRACTS

Sorghum Injera is a predominant human feed in Eastern Ethiopia. However, most of farmers in Hararghe, Eastern Ethiopia, store their grain in underground pit which is very conducive to produce Aflatoxin by Aspergillus Species. Thus, consumption of Injera made from sorghum grain contaminated with aflatoxin is a potential risk for human health. Aflatoxin is highly genotoxic, mutagenic, and hepatocarcinogenic substances. Therefore, this study was conducted with an objective to determine total aflatoxins in Sorghum Injera sample from Eastern Ethiopia. The analysis of the study was conducted on thirteen (30) duplicate samples collected from five districts of Harrghe, Eastern Ethiopia. The main analytical technique implemented for aflatoxin analysis was Immunoaffinity sample clean-up and Shimadzu High performance liquid chromatography using fluorescent as a detector. Questionnaire also implemented to assess the knowledge, attitude and practice (KAP) aspect of the participants.

The study revealed that 66.67% of the samples were contaminated with aflatoxins; B1, B2, G1 and G2 above lower limit of quantification. The maximum concentration of aflatoxin found in sorghum Injera sample was 53.33µg/kg with an overall mean of 11.2 µg/kg. On the other hand, the average AFB1, AFB2, AFG1, AFG2 concentrations were 4.63 µg kg-1, 0.406 µg kg-1, 5.75 µg kg-1 and 0.856 µg kg-1 respectively. The current result showed that there was significant contamination of Sorghum Injera samples with Aflatoxins. 33.33% of sample analyzed were unsafe for direct human consumption as per the FDA maximum tolerable intake limit (exceed 20 µg/kg). In addition, 53.33% of sample analysed were unsafe for human feed as per EU maximum tolerable intake level (exceed 4 µg/kg). The major underlined factor for the heavy contamination of sorghum injera with aflatoxin in the region might be because of poor Pre-and postharvest management of sorghum grain, mainly, the storage of sorghum grain in the un-sanitized underground pit was very favourable for the production aflatoxins by Aspergillus mould: *A. flavus, A. parasiticus* and *A. nomius.* Thus, Adequate pre- and postharvest management, adequate grain storage and suitable food processing steps shall be followed to get rid of aflatoxins from ingesting it along with food so that we can prevent the occurrence of disease with aflatoxins. The result of knowledge, attitude and practice assessment in this study revealed that, awareness of mold growth and formation of mycotoxin is very low among house holders, retailers and Farmers

Key words: Aflatoxin, Sorghum Injera, Ethiopia, HPLC, Immunoaffinity column.

Table Content

Acknowledgment……I

List of Figures……II

List of Tables……III

List of Abbreviations……IV

Abstract……...V

1 Introduction 3

1.1 Background 3

1.2 Statement of the problem 5

1.3 Significance of the study 6

1.4 Research question/Hypothesis 6

1.5 Objective 7

1.5.1 General Objective 7

1.5.2 Specific objectives 7

2 Literature Review 8

2.1 Overview of Sorghum, Sorghum Injera and Hararghe 8

2.2 Mycotoxins 10

2.2.1 Aflatoxins 12

2.2.2 Aflatoxin producing *Aspergillus* species 21

2.2.3 Management strategies against aflatoxins 21

2.2.4 Stability Aflatoxin in food processing 23

2.2.5 Aflatoxin Detection and quantification by HPLC 27

2.2.6 Immunoaffinity column (IAC) 29

2.2.7 Determination of total Aflatoxins 30

2.2.8 Aflatoxin exposure assessment 31

2.3 Legislation and detoxification 34

3 Materials and Method 37

3.1 Study area selection 37

3.2 Sample collection 37

3.3 Chemicals and reagent 38

3.4 Immunoaffinity columns 39

3.5 Mobile phase 39

3.6 Standards 39

3.7 Apparatus 39
3.8 Chromatographic condition 40
3.9 Procedures for Aflatoxin Analysis 40
3.9.1 Sample preparation, Extraction and clean Up 40
3.9.2 Aflatoxin Analysis by HPLC-FLD 42
3.9.3 Moisture Content 42
3.10 Chromatographic method validation 43
3.10.1 Identification 43
3.10.2 Linearity 43
3.10.3 Limit of detection (LOD) and Limit of quantification (LOQ). 43
3.10.4 Precision 43
3.10.5 Recovery 44
3.11 Standard preparation 44
3.12 Experimental Design and Questionnaire 44
3.13 Statistical analysis 45
3.14 Safety Measures 45
4 Results and discussion 46
4.1 HPLC method validation 46
4.1.1 Identification 46
4.1.2 Linearity and Range 47
4.1.3 Working range 48
4.1.4 Precision 49
4.1.5 Limit of detection and quantification 49
4.1.6 Recovery 50
4.2 Total Aflatoxins in sorghum Injera sample 50
4.3 Summary of Aflatoxins KAP Among Householders, retailers and Farmers 56
4.4 Discussion 58
4.5 Study Limitation 62
5 Conclusion and recommendations 63
5.1 Conclusion 63
5.2 Recommendations 63
6 References 65
Annex 1: Consent form and Questionaire, Amharic version 79
Annex 2: Statistical data report 82

1 Introduction

1.1 Background

Sorghum (*Sorghum bicolor* L.,) is a worldwide grass originated from the Africa continent: Ethiopia and Sudan border, which has spread to other temperate and tropical regions. Sorghum has been ranked as the seventh most cultivated grain in the world and the fourth in Africa (Hell. *etal*., 2008).

Sorghum grains are used as raw material for poultry, swine and bovine feeds, but are also destined for human use, constituting the staple food in India, China, and some African and Asian countries (Kange ., *et.al.,* 2015). Africa is a continent under pressure from climate stresses and is highly vulnerable to the impact of climate change. Many areas in Africa are recognized as having climate that is among the most variable in the world on seasonal and decadal time scales. Arid and semi-arid grazing systems in East Africa are highly vulnerable to combination of climate change and socio-economic factors (Dejene , 2004)).

Sorghum is a major economical food and nutritional security crop to more than 100 million people in Eastern horn of Africa, owing to its resilience to drought and other production constrains. In Ethiopia rain-fed agriculture is the backbone of the country's economy and rural livelihood and Ethiopia is highly vulnerable to the harmful effect of climate change due to global warming. In Ethiopia, Sorghum provides one third of the cereal diet and is grown almost entirely by the subsistence farmers and sorghum can grow under adverse soil, climatic, and poor management conditions in wide range of elevation from low lands to high land in the country and can supply food in drought prone areas (Dejene., 2004).

Hararghe in Oromia, Eastern Ethiopia where annual rainfall varies between 450 mm and 850mm. Approximately half of this region is in sorghum cultivation and as the result unlike other regions where sorghum is grown exclusively for food (Tesfaye, *et al.,*2008). The sole sorghum flour or mixed with other cereal flour is used for making injera (local circular pan-cake) and other consumable food items.

In the region sorghum grain is stored in their flask shaped traditionally underground storage pits until it is consumed or sold. The storage pits lead to moisture ingress in to the inter-

granular space elevating both the grain moisture content and the relative humidity inside the pit. Such a pit environment leads grain deterioration (Dejene., 2004).

The presence of deteriorative fungi with ability to produce mycotoxin in grains and food represents a great hazard for human and animal health, and it has been reported for sorghum in many countries with a high frequency of *Aspergillus* and *Fusarium* genera (Josefa, *et.al*., 2003).

Mycotoxins are fungal toxic metabolites which naturally contaminate food and feed. Aflatoxins (AFs), a kind of mycotoxins, are the main toxic secondary metabolites of some *Aspergillus* moulds such as *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *Aspergillus Nomius (*Alejandro Espinosa-Calderón, et.al., 2011). Such toxins can be separated into aflatoxins B1, B2, G1, B2a and G2a. Its order of toxicity is B1 > G1 > B2 > G2. Letters 'B' and 'G' refer to its blue and green fluorescence colours produced by these compounds under UV light. Numbers 1 and 2 indicate major and minor compounds, respectively (Hussein & Brasel, 2001; Weidenbörner, 2001).

A. flavus only produces B aflatoxins, while *A. parasiticus* and *nomius* also produce G aflatoxins (Alcaide-Molina, et al., 2009). Aflatoxins, when ingested, inhaled or adsorbed through the skin, have carcinogenic, hepatotoxic, teratogenic and mutagenic effects in human and animals even at very small concentrations. When aflatoxins B1 is ingested by cows, it is transformed into its hydroxylated product, AFs M1 and M2. Such aflatoxins are secreted in the milk and are relatively stable during milk pasteurization, storage, and preparation of various dairy products (Stroka & Anklam, 2002).

There are efforts in different countries to identify aflatoxin contaminations of sorghum grain. However, in Ethiopia such studies rare and adequate research based recommendations are not available with respect to safe storage, consumption sorghum grain and sorghum based humanfeeds.

Earlier report authored by (Geremew, *etal.,* 2016) reported on aflatoxin levels in Sorghum grain stored for different period and storage system in kewet districts, Northern shewa, Ethiopia, total aflatoxin contamination ranged from11.44µg/Kg to 344.26µg/Kg and the

mean total aflatoxin level was 123.85µg/Kg. This current study will generate further information on aflatoxin contamination in sorghum made Injera and such study will augment the future researcher to gather more information on aflatoxin contamination of sorghum injera and its associated health risks in the region. The aim of the study was to determine total aflatoxin concentration in sorghum Injera samples in Eastern Ethiopia.

1.2 Statement of the problem

In 2015 a nation-wide mycotoxin analysis of sorghum grain from major Sorghum growing Districts of Ethiopia were taken place in collaboration with FAO/WHO mycotoxins in Sorghum project. Among the major surveyed sampling area in FAO/WHO Mycotoxins in Sorghum project, districts from Eastern Ethiopia (Chiro, Haromaya, Harar, Babile) were most affected by Mycotoxins (Emana & Nigussie, 2015). The Survey was done in three rounds and the surveyed sites were categorized in to five agro-ecologies based on elevation and amount of annual rainfall. During the survey significant amount of individual aflatoxin were analysed in each round Survey.

Peasants in Hararghe store their sorghum in their flask shaped traditionally underground storage pits until it is consumed or sold. Such a pit environment leads moulding and grain deterioration. Major concern associated with grain mould is the production of mycotoxins which are harmful to both humans and animals. The species associated with mycotoxin production on contaminated sorghum grains are *Aspergillus* species and produces aflatoxins B1, B2, G1 and G2 which are very harmful to both humans and animals. Consumption aflatoxin contaminated food considered an important public health issue and have carcinogenic, hepatotoxic, teratogenic, mutagenic effects, immune disorder in human and had childhood stunting (Gong et al., 2004; Williams *et al*., 2004; Stronsnider, *et al.*, 2010).

Sorghum is the most preferred cereal for injera preparation in Eastern Ethiopia, most rural and some urban people of the region daily consumed Sorghum Injera. Such grain is not properly harvested, stored and processed and with high likely consumption of food contaminated with Aflatoxins.

In Ethiopia, cancer accounts for about 5.8% of total national mortality, exposure to environmental risk factors including aflatoxins one of the underlining preventable cause of cancer. Aflatoxin B1 is the most toxic and potent of the aflatoxins, naturally occurring chemical induce hepatocellular carcinoma (HCC), a form of liver cancer is classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC, 1993).

1.3 Significance of the study

The finding of this study will be helpful for the public health of the nation and the region by increasing knowledge and awareness of the public on food handling, storage, processing, hygienic and food safety system and hence, reduce the healthcare costs as well as increase the willingness and healthy life style of the consumers. It can also be an input for policy and decision makers to create and implement training programs, decision on the producers, processers, service deliverer, markets and trades associations. Researchers and academicians of the field can use the findings of the study as a reference material. Generally, the result will be used to strengthen the development of food safety and health strategies in Ethiopia.
Thus, regulations do little to help reduce aflatoxin and its related health effects in less developed countries. Rather, the focus should be on promoting the adoption of strategies that can control aflatoxin and its associated health risks, in the field, in postharvest conditions, or in the diet.

This study also helps the innovative Health Extension Workers programme for the successful implementation of cancer preventive activities in the country and to design training on food handling and prevention grain moulding and what preventative action to be taken.

1.4 Research question/Hypothesis

1. What is the level of Individual and total Aflatoxin in sorghum Injera sample from Eastern part of Ethiopia?
2. Does the regulatory authority of Ethiopia, will allow the level of aflatoxin to be present in human feed and its comparison relative to EU, FDA, FAO/WHO Tolerable limit?

1.5 Objective

1.5.1 General Objective

- To determine the level of total Aflatoxin in sorghum Injera sample in Eastern Ethiopia

1.5.2 Specific objectives

- To determine the level of individual Aflatoxins (B1, B2, G1, and G2) in sorghum Injera sample collected from Eastern part of Ethiopia.
- To determine the most predominant aflatoxin contaminated sorghum injera which people usually consumed.
- To evaluate the average concentrations of the aflatoxin found in sorghum injera from Eastern Ethiopia in accordance with minimum tolerance level of Ethiopian, FDA, European, FAO/WHO standards.
- To assess knowledge, attitude and practice (KAP) among Householders, Retailers and Farmers on Aflatoxin contamination.

2 Literature Review

2.1 Overview of Sorghum, Sorghum Injera and Hararghe

Sorghum, *Sorghum bicolor* (L) Moench, is the fifth most important cereal after rice, wheat, maize, and barley. It constitutes the main food grain for over 750 million people who live in the semi-arid tropics of Africa, Asia, and Latin America (FAO,1999).

Grain sorghums are generally grown in regions which are too dry or too hot for successful maize production. They are adapted to the drier climates due to several factors (Bennett et al. 1990): the ability to remain dormant during drought and then resume growth; Leaves roll up as they wilt reducing the area of leaf exposed for transpiration; Leaves and stalks contain an abundance of waxy coating which protects them from drying; Sorghum exhibits a low transpiration ratio (kg water required to produce a kg of plant material) e.g. 141 kg for sorghum, 170 kg for maize and 241 kg for wheat; Sorghums have a large number of fibrous roots that efficiently extract moisture from the soil (the absorption area is about twice that of maize); roots may be up to 2.5m in length; A large root absorption area and relatively large leaf area; Sorghums can withstand temperatures above 38 ° C, but dry winds coupled with hot weather during pollination reduce yields. Best yields are realised when temperatures during the season are 24-27 °C. The water requirements for sorghum vary within the range 350-700 mm depending on the length of the growing cycle; short growing cycle is 90 days; long growing cycle, more than 130 days, within many semi-arid areas of developing countries, typical temperatures range from 20°c-38°c with annual rainfall ranging from 300-750 mm. Since sorghum is predominantly a rain-fed crop grown by subsistence farmers, yields largely depend on the capacity for drought resistance of the variety used, Sorghums can tolerate a wide range of soil pH and textures.

In Africa, sorghum is still largely a subsistence food crop. In terms of tonnage, sorghum, quantitatively the second most important cereal grain in Africa after maize. Sorghum is a major economical food and nutritional security crop to more than 100 million people in Eastern horn of Africa, owing to its resilience to drought and other production constrains (T).

In Ethiopia Sorghum is a traditional food crop widely grown in the country, in 13 of the 18 major agro-ecological zones, covering over 1.3 million hectares. It is predominantly used for food, 80 percent of it for injera (flat pancake like traditional bread) making. The crop is

second only to tef for injera making. Ethiopia has a diverse wealth of sorghum germplasm adapted to a range of altitudes and rainfall conditions. The crop is mainly grown in the lowland arid and semiarid areas. Of the five morphological races of sorghum (bicolor, guinea, caudatum, durra, and kafir) all except kafir are grown in Ethiopia. Important traits reported from Ethiopian sorghum include cold tolerance, drought resistance, resistance to sorghum shoot fly, disease and pest resistance, grain quality and resistance to grain mould, high sugar content in the stalks, and high lysine and protein content (IBC,2007).

The Hararghe region in Oromia is located in Eastern Ethiopia; where annual rainfall ranges between 450mm to 850mm. Approximately half of this region is in sorghum cultivation and as the result unlike other regions where sorghum is grown exclusively for food item (Tesfaye *et al.,*2008). Sorghum flour or mixed with other cereal flour are used for production of naturally fermented traditional flat or semi leavened breads, injera (local circular pan-cake) of Ethiopia. Injera is a leavened, flat round Ethiopian traditional bread made from cereals such as tef and sorghum (Gebrekidan and Gebrehiwot, 1982).

Its surface has essentially evenly spaced gas holes, that make up a honeycomb-like structure formed due to the production of gas during fermentation and baking. The bottom surface of injera is smooth and shiny. A good injera is soft, fluffy and able to be rolled without cracking. It should retain these textural properties after two to three days of storage, which is traditionally done in a straw basket. A slight sourness is a characteristic taste of injera. Because injera is leavened bread made from non-gluten containing flour, it has great potential for commercial production internationally (Gebrekidan and GebreHiwot, 1982).

Sorghum is the second most preferred cereal for injera preparation in Ethiopia (Gebrekidan and Gebrehiwot 1982) with an annual grain production of 1.34 million metric tons (FAO, 2001). Preparing Injera from sorghum has considerable economic benefits over tef, as sorghum commands a much lower price. However, the problem is that sorghum Injera rapidly becomes firm and friable upon storage. In Eastern Ethiopia Sorghum Injera is the prior staple food consumed because the climatic condition was unfavourable for the growth of tef.

Fermentation to produce foods such as Injera involves the controlled souring by naturally occurring lactic acid bacteria (Chavan and Kadam, 1989). Like many other traditional

fermented foods, the fermentation in injera making is originally spontaneous and dependent upon the load and flora of microorganisms naturally present in the flour, mixing water and air borne contaminants. However, households are generally able to carry out consistently successful fermentations through practising a system of back slopping, whereby a 'portion of liquid from a successful fermentation is used to inoculate freshly prepared dough of sorghum flour. However, in general injera preparation involves two fermentation stages. The first takes 24-48 hr (depending on the sourness desired) from mixing the flour with water and adding the back -slopped culture. Then a portion of the fermented dough is cooked and added back to the fermented dough to initiate the second fermentation. The mixture is brought to a batter consistency and allowed to ferment for about 2-3 hr. After gas bubbles have formed and subsided the batter is poured on a hot clay griddle and baked covered. By cooking part of the dough to gelatinize the starch, the carbon dioxide produced by the fermentation is trapped and leavens the Injera on baking.

The presence of deteriorative fungi with ability to produce mycotoxin in grains and food represents a great hazard for human and animal health. A number of fungal species associated with sorghum belong to the genera *Fusarium*, *Aspergillus* and *Penicillium*, which have been known to produce mycotoxins that cause mycotoxicosis in animals and humans. Food contaminated with mycotoxins, particularly with aflatoxins, a subcategory, can cause sometimes fatal acute illness and are associated with increased cancer risk. To protect consumers from these health risks, many countries have adopted regulations to limit exposure to mycotoxins (Alexandera C. *et.al.,* 2013).

2.2 Mycotoxins

The term “mycotoxin” originates from the Greek word "Mykes”, meaning fungus, and from the Latin word “Toxicum”, meaning poison or toxin. Mycotoxins are classified as the most important chronic and noninfectious foodborne risk factor, more important than synthetic contaminants, plant toxins, food additives, and pesticide residues. Both humans and animals may show acute or chronic intoxication caused by mycotoxin ingestion, and the pathological condition that results from this ingestion is called mycotoxicosis (Mehdi Razzaghi-Abyaneh, 2013).

Mycotoxins are fungal secondary metabolites produced by the toxigenic strains of the fungi, which can produce acute or chronic toxic effects (e. g carcinogenic, mutagenic, and

teratogenic) on animals and probably on human at the levels of exposure (Darwish W., *et al,* 2014). The predisposing conditions for mycotoxin production relate mainly to poor hygienic practices during transportation and storage, high temperature and moisture content and heavy rains. These conditions are typically observed in different African countries.

The demand for the storage of food substances has been increased due to the increasing in the population in African continent. However, improper storage, transportation and processing facilities may facilitate fungal growth and subsequently lead to mycotoxin production and contamination of food and feedstuffs. The food-borne mycotoxins are of great importance in Africa and other parts of the world. The impact of such toxins on human health, animal production and economy has attracted worldwide attention.

Darwish, *et al,* 2010 reported that the occurrence of mycotoxins in Barley, sorghum, tef and wheat of Ethiopia. The authors also published that AFB1 and OTA were detected in samples of four crops. Most, peasants in Hararghe, Eastern Ethiopia, store sorghum grain in underground pits. These pits usually elevate grain moisture and storage temperature to levels that favour insect pests and fungi, causing grain spoilage. The storage pits are mostly neither lined nor plastered with any other material that would reduce moisture migration in to the stored grain and then the contact of the grain with the wet inner pit walls often leads to moisture ingress in to the inter-granular space elevating both the grain moisture content and the relative humidity inside the pit. Such a pit environment leads moulding and grain deterioration. At 15–19 % moisture content, spoilage fungi species grow resulting in a significant increase in respiratory activity. This results in temperature increase and sometimes spontaneous heating from colonization by succession of thermophile fungi (Dejene, M., 2004). *Aspergillus*, *Penicillium* and *Fusarium* are known to be the major mycotoxin-producing fungi.

The most important mycotoxins produced include aflatoxin (AF), ochratoxins (OT), deoxynivalenol (DON), zearalenone (ZEA), fumonisin (FUM) and trichothecenes (T). Furthermore, DON, ZEA, FUM and T are all produced by the *Fusarium* species and these compounds contaminate various food substances and agricultural crops including sorghum (Dereje, A. *et al,* 2010).

In Darwish, *et al*, 2010 study, the occurrence of mycotoxins in Ethiopia: barley, sorghum, tef and wheat AFB1 was detected in 8.8% of the samples analysed at concentrations ranging from trace amounts to 26 μg/kg. OTA occurred in 24.3% of the samples at a mean concentration of 54.1 μg/kg and a maximum of 2,106 μg/kg. DON occurred in barley, sorghum and wheat at 40–2,340 μg/kg with an overall incidence of 48.8% among the samples analysed. FUM and ZEA occurred only in sorghum samples with low frequencies at concentrations reaching 2,117 and 32 μg/kg respectively.

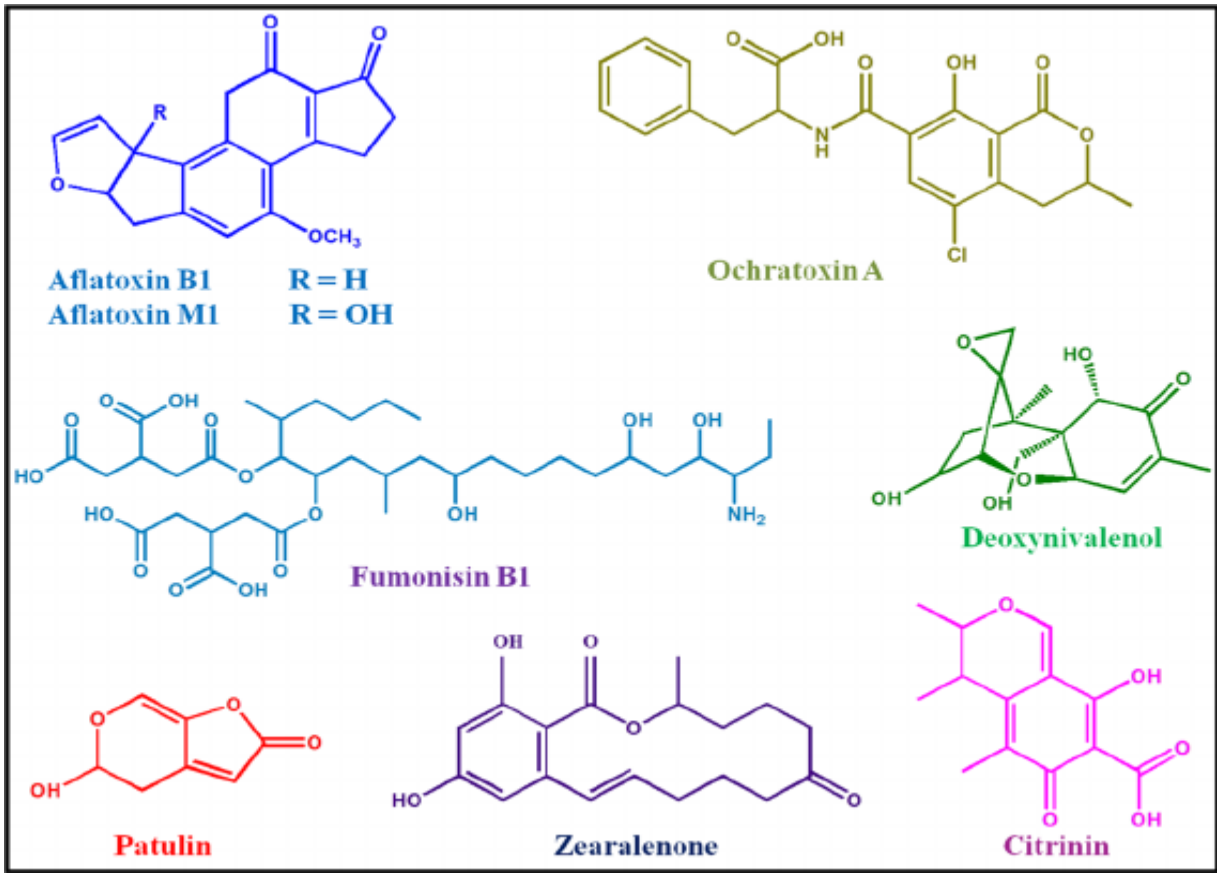


Figure 1: chemical structures of Some important mycotoxins

2.2.1 Aflatoxins

Aflatoxins are a group of polyketide toxic secondary metabolites produced by different species of toxigenic fungi, called mycotoxins. The discovery of aflatoxins date back to the year 1961 following the severe outbreak of turkey "X" disease, in the England, which resulted in the deaths of more than 100.000 turkeys and other farm animals. The cause of the disease was attributed to a feed using thin-layer chromatography (TLC) revealed that a series of fluorescent compounds, later termed aflatoxins, were responsible for the outbreak. The disease was linked to a peanut meal, incorporated in the diet, contaminated with a toxin

produced by the filamentous fungus *Aspergillus flavus*. Hence, the name aflatoxins, an acronym, has been formed from the following combination: the first letter, "A" for the genus *Aspergillus*, the next set of three letters, "FLA", for the species *flavus*, and the noun "TOXIN" meaning poison (Mehdi Razzaghi-Abyaneh, 2013).

Aflatoxins (AFs) are Aflatoxins are a group of approximately 20 related fungal difuranocoumarins metabolites that are classified into two broad groups according to their chemical structure and they include the difurocoumarocyclopentenone series (AFB1, AFB2, AFB2A, AFM1, AFM2, AFM2A and aflatoxicol) and the difurocoumarolactone series (AFG1, AFG2, AFG2A, AFGM1, AFGM2, AFGM2A and AFB3) and produced primarily by two species of *Aspergillus* fungus which are especially found in areas with hot, humid climates.

A. flavus is ubiquitous, favouring the aerial parts of plants (leaves, flowers) and produces B aflatoxins. *A. parasiticus* produces both B and G aflatoxins, is more adapted to a soil environment and has more limited distribution. *A. bombysis*, *A. ochraceoroseus*, *A. nomius*, and *A. pseudotamari* are also AFs producing species, but are encountered less frequently. From the mycological perspective, there are qualitative and quantitative differences in the toxigenic abilities displayed by different strains within each aflatoxigenic species. (Mansooreh, M., 2009).

Among the 18 different types of aflatoxins identified, the major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2). AFB1 is normally predominant in amount in cultures as well as in food products. *A. flavus* typically produces AFB1 and AFB2, whereas *A. parasiticus* produce AFG1 and AFG2 as well as AFB1 and AFB2. Four other aflatoxins M1, M2, B2A, G2A which may be produced in minor amounts were subsequently isolated from cultures of *A. flavus* and *A. parasiticus*. A number of closely related compounds namely aflatoxin GM1, parasiticol and aflatoxicol are also produced by *A. flavus.* The order of acute and chronic toxicity is AFB1 > AFG1 > AFB2 > AFG2, reflecting the role played by epoxidation of the 8,9-double bond and the greater potency associated with the cyclopentenone ring of the B series, when compared with the six-membered lactone ring of the G series. AFM1 and AFM2 are hydroxylated forms of AFB1 and AFB2. AFM1 and AFM2 are major metabolites of AFB1 and AFB2 in humans and

animals and may be present in milk from animals fed on AFB1 and AFB2 contaminated feed. The structure of six major compounds of aflatoxin B1, B2, G1, G2, M1 and M2 is shown (Figure 2).

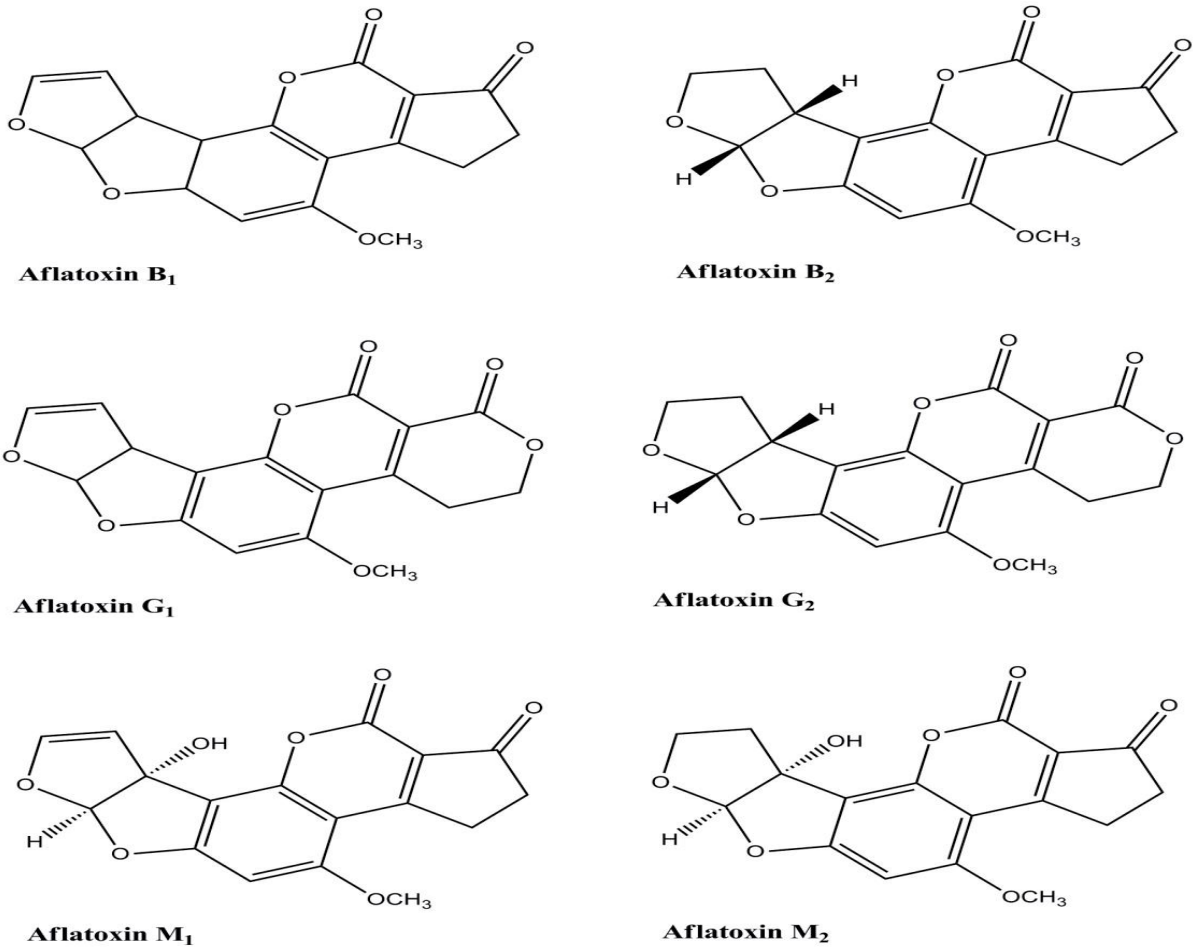


Figure 2: Chemical structures of major aflatoxins

2.2.1.1 Occurrence of Aflatoxin in Sorghum grain and Injera

Sorghum is the main foods for human consumption throughout the world and is found susceptible to AFs accumulation by aflatoxigenic fungus. Toxins are produced on cereals, both in the field and in storage; they involve both the grain and the whole plant. It is also the most important staple food crop in many countries and is grown in harsh environments where other crops do not grow well. Improvements in production, availability, storage, utilization, and consumption of this food crop will significantly contribute to the household food security and nutrition of the inhabitants of these areas. Sorghum is typically harvested as early as possible so that fields can quickly be planted with another crop. Sometimes the sorghum harvest coincides with heavy rainfall, hurricanes, and floods, all of which promote infection

by mycotoxin-producing fungi. As it is known, the consumption of large amounts of AFs contaminated food by starving people can cause toxic hepatitis and death. It is characterized by jaundice, rapidly developing ascites, and portal hypertension.

Sorghum flour or mixed with other cereal flour are used for production of naturally fermented traditional flat or semi leavened breads, *injera (local circular pan-cake)* of Ethiopia. *Injera* is a leavened, flat round Ethiopian traditional bread made from cereals such as tef and sorghum may also be served along with *injera* and *wot.* The normally sour *injera* and the spicy *wot* create a food taste combination that is considered ideal by Ethiopians depending on this staple (Gebrekidan and GebreHiwot, 1982).

To make injera the grains are dehulled manually or mechanically and milled into flour. After sufficient pounding, the bran is winnowed off to leave clean dehulled grain. This flour is mixed with water to form a dough, the starter (ersho) is added, and the dough is fermented for 2 or 3 days. The starter is a fluid saved from previously fermented dough. After fermentation the dough is thinned down to a thick batter and poured onto a lightly oiled pan, which is then covered with a tightly fitting lid to retain the steam (Parker, Melaku, & Faulks, 1989). Within about 2–3 min it is ready to be removed from the pan and then is placed on a basket. The storage period does not usually exceed 3 days at room temperature.

The microorganisms involved in fermentation of injera are mainly yeasts, some fungi including Pullaria sp., Aspergillus sp., Penicillium sp., Rhodotorula sp., Hormodendrum sp., Candida sp. and number of unidentified bacteria. Blandino and his worker reported that lactic acid fermentation is mainly carried out by lactic acid bacteria in cereals (Blandino A. et al.,2002).

Injera is seldom consumed alone or often taken with stew. The general name of the stew that is served with *injera* is *wot* (Amharic). Consumed aflatoxins in contaminated injera are highly lipid soluble compounds and are readily absorbed from the site of exposure usually through the gastrointestinal tract and respiratory tract into blood stream (Agag B, 2004). Human and animals get exposed to aflatoxins by two major routes (a) direct ingestion of aflatoxin contaminated foods or ingestion of aflatoxins carried over from feed into milk and milk products like cheese and powdered milk as well as other animal tissues mainly as AFM1

(Agag B, 2004) (b) by inhalation of dust particles of aflatoxins especially AFB1 in contaminated foods in industries and factories (Groopman, 2011). After entering the body, the aflatoxins are absorbed across the cell membranes where they reach the blood circulation. They are distributed in blood to different tissues and to the liver, the main organ of metabolism of xenobiotics. Aflatoxins are mainly metabolized by the liver to a reactive epoxide intermediate or hydroxylated to become the less harmful aflatoxin M1. In humans and susceptible animal species, aflatoxins especially AFB1 are metabolized by cytochrome P450 (CYP450) microsomal enzymes to aflatoxin-8,9-epoxide, a reactive form that binds to DNA and to albumin in the blood serum, forming adducts and hence causing DNA damage Various CYP450 enzymes isoforms occur in the liver and they metabolize aflatoxin into a reactive oxygen species (aflatoxin-8,9-epoxide), which may then bind to proteins and cause acute toxicity or to DNA and induce liver cancer (Wang, H., et al. , 1998)..

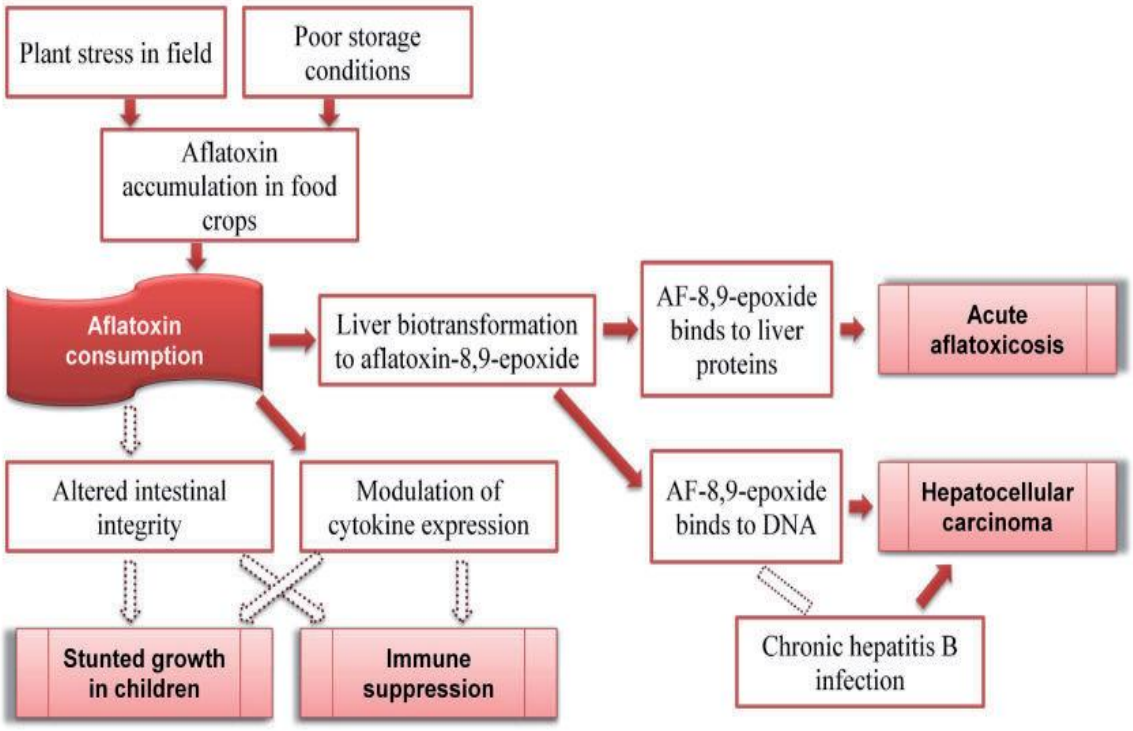


Figure 3: Liver Biotransformation of aflatoxin

2.2.1.2 Aflatoxin properties

All mycotoxins are low-molecular-weight natural products (i.e., small molecules) *(Bennett,1987 cited by Mehdi Razzaghi-Abyaneh,2013).* The hyphal structure of filamentous

fungi has evolved to utilize solid substrates efficiently by growing over surfaces and penetrating solid matrices. Moulds can secret enzymes to break down complex macromolecular compounds and utilize them for growth and metabolism. They can absorb low molecular weight nutrients, produce and secrete secondary metabolites, which are also relatively low molecular weight compounds but not associated with the process of growth and primary metabolism. Some aflatoxin derivatives are products of animal metabolism following ingestion of the mould metabolites, which are divided into the B and G groups based on their blue or green fluorescence under UV light when absorbed to solid substrates. *A. parasiticus* is the most toxigenic species, most strains producing both B and G toxins (Van Egmond, 1989 cited by Mehdi Razzaghi-Abyaneh, 2013). Aflatoxins are crystalline substances, freely soluble in moderately polar solvents such as chloroform, methanol, dimethyl sulfoxide; they dissolve in water to the extent of 10-20 mg L^{-1}.

The chemical and physical properties of aflatoxins are described as follows:

I. *Description*: Colorless to pale-yellow crystals. Intensely fluorescent in ultraviolet light, emitting blue (aflatoxins B1 and B2) or green (aflatoxin G1) and green–blue (aflatoxin G2) fluorescence, from which the designations B and G were derived, or blue–violet fluorescence (aflatoxin M1)
II. *Melting-points*: see Table 1.
III. *Absorption spectroscopy*: see Table 1.
IV. *Solubility*: Very slightly soluble in water (10–30 μg mL-1); insoluble in non-polar solvents; freely soluble in moderately polar organic solvents (e.g., chloroform and methanol) and especially in dimethyl sulfoxide
V. *Stability*: Unstable to ultraviolet light in the presence of oxygen, to pH extremes (< 3, > 10) and to oxidizing agents
VI. *Reactivity*: The lactone ring is susceptible to alkaline hydrolysis. Aflatoxins are also degraded by reaction with ammonia or sodium hypochlorite.

				UV absorption max (ε (L mol-1 cm-1)), methanol	
Aflatoxin	***Molecular formula***	***Molecular weight***	**Melting Point**	***265 nm***	***360-362 nm***
B1	C17H12O6	312	268-269	12,400	21,800
B2	C17H14O6	314	286-289	12,100	24,000
G1	C17H12O7	328	244-246	9,600	17,700
G2	C17H14O7	330	237-240	8,200	17,100

Table 1: Some Physical and chemical properties of Aflatoxins

Aflatoxins are quite stable in many foods and are fairly resistant to degradation. The effectiveness of some processes in reducing concentrations of aflatoxins in food can be affected by many factors, such as the presence of protein, pH, temperature and length of treatment. Commercial processing of raw commodities using cleaning regimes including the removal of broken particles, milling and sorting can reduce aflatoxin concentration considerably.

2.2.1.3 Health effects associated with consumption of Food contaminated with aflatoxins

Aflatoxicosis is a condition caused by aflatoxins in both humans and animals. It occurs in two general forms

1. the acute primary aflatoxicosisis produced when moderate to high levels of aflatoxins are consumed. Specific acute episodes of disease may include haemorrhage, acute liver damage, edema, alteration in digestion, absorption and/or metabolism of nutrients, and possibly death (Lizárraga-Paulín et al. 2011 & USAID., 2012).
2. The chronic primary aflatoxicosis results from ingestion of low to moderate levels of aflatoxins (USAID, 2012). The chronic forms of aflatoxicosis include (1) teratogenic effects associated with congenital malformations (2) mutagenic effects where aflatoxins cause changes (mutations) in the genetic code, altering DNA and these changes can be chromosomal breaks, rearrangement of chromosome pieces, gain or loss of entire chromosomes, or changes within a gene (3) the carcinogenic

effect in which the carcinogenic mechanisms have been identified such as the genotoxic effect where the electrophilic carcinogens alter genes through interaction with DNA and thus becoming a potential for DNA damage and the genotoxic carcinogens that are sometimes effective after a single exposure, can act in a cumulative manner, or act with other genotoxic carcinogens which affect the same organs. Chronic effects of aflatoxin have been reported to impair the normal body immune function (Hussein & Jeffrey, 2001, Turner *et al*., 2003, da Silva *et al*., 2004).

The aflatoxin problem is most serious in tropical and subtropical countries due to favourable climatic conditions for *Aspergillus flavus and Aspergillus parasiticus*. It is estimated that about 25,200 – 155,000 people worldwide have aflatoxin induced liver cancer. Of this population 40% occur in Africa. There are economic losses that result from contamination of crops and animal feeds with aflatoxins and public health problems that result from ingestion of products contaminated with aflatoxins (Mehdi Razzaghi-Abyaneh, 2013).

Naturally occurring aflatoxins (as a group) and other 107 agents were evaluated as carcinogenic to humans (Group 1) (Mohamed D. & Roquia A., 2011). These compounds can enter the food chain, mainly, by ingestion through the diet of humans and animals. There is sufficient evidence in humans for the carcinogenicity of aflatoxins, being liver cancer (hepatocellular carcinoma) the main effect. There is strong evidence that the carcinogenicity of aflatoxins operates by a genotoxic mechanism of action that involves metabolic activation to a genotoxic epoxide metabolite, formation of DNA adducts, and modification of the TP53 gene. Epoxide derivative of aflatoxin B1 binds with DNA and disrupts transcription and translation activities, thus initiating carcinogenesis. Oxidative nature of the toxic derivative releases free radicals and cause cell damage. In humans, hepatocellular carcinomas from areas of high exposure to aflatoxins, up to 50% of tumours have been shown to harbour a specific point mutation in the TP53 tumour suppressor gene (IARC, 2012). Carcinogenicity of naturally occurring mixtures of aflatoxins B1, G1 and M1 is also demonstrated in experimental animals. The intake of these toxins over a long period of time in very low concentrations may be highly dangerous.

AFB1 is the most potent carcinogenic substance naturally produced by *Aspergillus* species. Indeed, AFB1 is classified by IARC as Group 1 carcinogen (IARC,.2012). This compound is certainly acutely toxic to humans, is probably responsible for liver necrosis following chronic exposure, and may be involved in the epidemiology of human liver cancer in some parts of the world perhaps synergistically with hepatitis B virus. After ingestion, aflatoxin B is metabolized by enzymes to generate a reactive 8,9-epoxide metabolite that can be bound to DNA as well as to serum albumin forming Aflatoxin-N-7 guanine and lysine adducts, respectively. Covalent binding to DNA is a critical step in aflatoxin hepatocarcinogenesis (IARC, 1997).

Aflatoxin exposure has also been associated with childhood stunting (Gong et al., 2004) and it is a chronic form of malnutrition, is potentially associated with many health problems, including an increased rate of infectious illnesses, impaired learning capabilities, and reduced work productivity (Black et al., 2013).

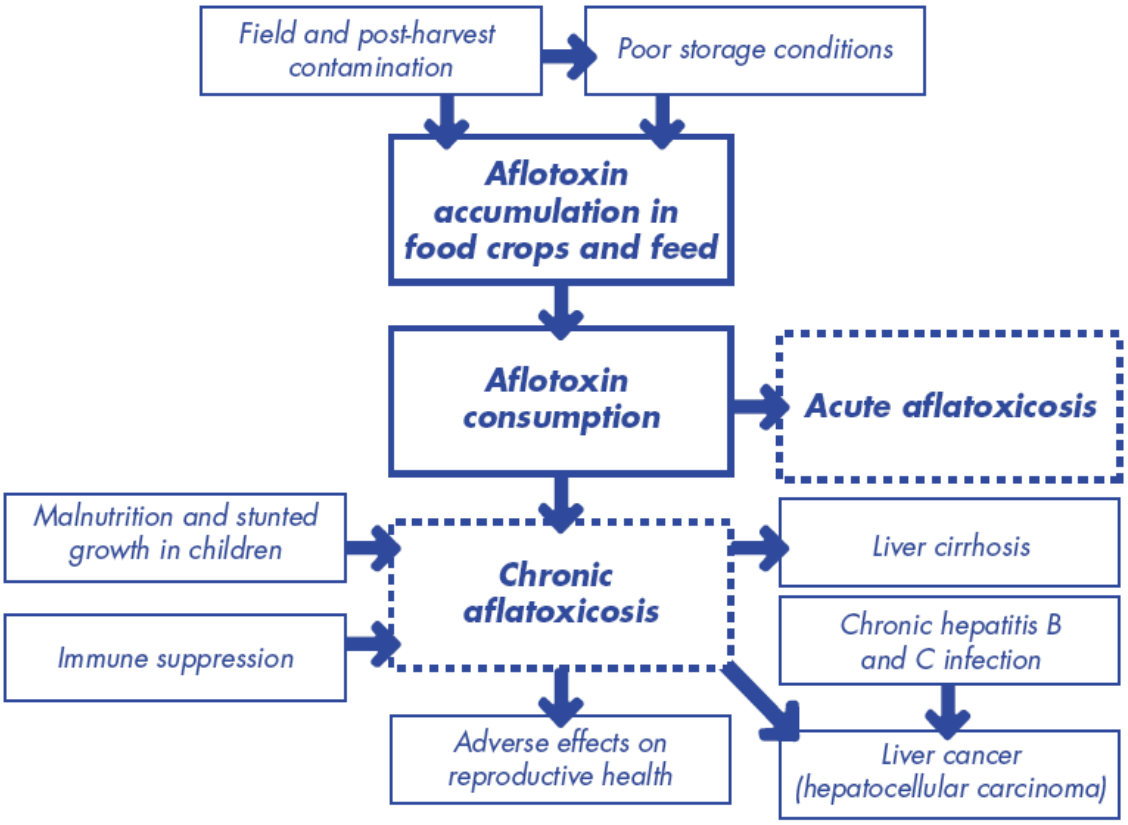


Figure 4: Aflatoxin disease pathways in humans (Adopted from Wu, 2010; USAID, 2012; WHO, 2011; Wu and Tritscher,2011)

2.2.2 Aflatoxin producing *Aspergillus* species

*Aspergillus s*ub-genus *Circumdati* Section *Flavi*, also referred to as the *Aspergillus flavus* group, has attracted worldwide attention for its industrial use and toxigenic potential. Section *Flavi* is divided into two groups of species. One includes the aflatoxigenic spp. *A. flavus, A parasiticus* and *A. nomius*, and the other includes non-aflatoxigenic spp. *A. oryzae*, *A. sojae* and *A. tamarii* (Rodrigues *et al*., 2007). Shephard (2005) reported that *A. flavus* and A. *parasiticus* only occur sporadically in both commercial and home-grown maize in South Africa and are not ear rot pathogens under local conditions.

A. flavus which has smooth spores may be distinguished from *A. parasiticus* with its rough-walled conidia (Klich & Pitt, 1988). *A. flavus* is the predominant spp. on maize and sorghum. *A. flavus* can be found worldwide but is predominantly a tropical to sub-tropical fungus which is more common in cultivated than uncultivated soil (Klich & Pitt, 1988).

A. flavus is a thermotolerant fungus, it is therefore more likely than many other fungi and bacteria to survive at temperatures of up to 48ºC (Brown *et al*., 1999) and under dry conditions (-35 MPa). Aflatoxins are produced between temperatures of 12 and 42°C and the optimum temperature is 25-35°C (Diener & Davis, 1966). The optimum water activity for growth of *A. flavus* is high (approximately 0.99 aw). The maximum is at least 0.998 aw whereas the minimum water activity for growth was reported by Pitt & Miscamble (1995) to be approximately 0.82 aw.

Aflatoxin production is particularly favoured by very moist conditions. Maximum moisture content for aflatoxin production in maize kernels is 25% at 30°C and the minimum relative humidity for aflatoxin production varies between 83% and 88% although Widstrom *et al*. (1990) found high maximum and high minimum daily temperatures, especially during periods with high nett evaporation, to be more important to the development of aflatoxin than humidity or average precipitation.

2.2.3 Management strategies against aflatoxins

To ensure global safety on food and feed supplies, extensive researches have been carried out to effectively control and manage AF contamination of crops. Aspergillus infection increase with high temperature, high humidity, insect damage and nitrogen deficiency. Temperature and humidity are therefore important in aflatoxin management. A. flavus and A. parasiticus

are unable to grow or produce aflatoxin at water activity of less than 0.7 (relative humidity below 70% or temperature below 100°C, however under stress condition such as drought, aflatoxin contamination can be higher. Various strategies have been suggested in management of aflatoxins. The strategies for preventing AF contamination are generally divided into two categories including pre- and post-harvest controls (Kabak *et al.*, 2006).

2.2.3.1 Pre- harvest strategies

These include;

a. Good agricultural practices (GAP) that involve adequate fertilizer application and crop rotation with non-host.
b. Management of insect pests that predispose crops to fungal infection through availability of infection channels such as wounds and other entry points.
c. Optimal harvest time so that crops are not left in the field exposed to environmental factors that predispose crops to pathogen infection. Harvesting immediately after physiological maturity is recommended since aflatoxin level can increase with delayed harvest interval.
d. Suitable management of crop residues as they harbour pathogens that are able to survive saprophytically.
e. Management with environmentally friendly fungicides.
f. Biological control is use of one microorganism to control another microorganism such as Pseudomonas strains. It has been noted that Aspergillus flavus strains differ in aflatoxin production and this influences crop contamination. There are strains that produce a lot of aflatoxins and produce numerous small sclerotia (<400μm). These are the 'S' strains (toxigenic strain). Another strain the 'L' strain produces low aflatoxin levels and a few large sclerotia that are about >400μm and are atoxigenic.
g. There is competitive exclusion when one strain competes to exclude another in the environment. Atoxigenic strains of A. flavus from Nigeria have been combined as a bio-control product and registered as AflaSafe.

2.2.3.2 Post-harvest management

Aflatoxin is preferably controlled in the standing crop, since contamination of harvested crops increases with storage time. Aflatoxin contamination in Africa is compounded by

Good Agricultural practice (GAPs) for aflatoxin management

Preharvest GAPs	**At-harvest and postharvest GAPs**
1. Use of *A. flavus* resistant/tolerant varieties	1. Harvesting the crop at the correct maturity
2. Selection of healthy seeds	2. Use of water-harvesting to preserve available
3. Early planting	3. moisture.
4. Avoidance of mono-cropping	4. Use of *A. flavus* resistant/tolerant varieties
5. Ploughing before sowing	5. Avoidance of damage to pods during harvest
6. Appropriate weeding	6. Drying seed to 8 percent moisture level
7. Application of farmyard manure	7. Stripping the pod immediately after drying
8. Treatment of foliar diseases	8. Removing immature pods attached to the haulms
9. Application of lime or gypsum	9. Removing damaged, shrivelled, and immature pods
10. Mulching with crop residues at 40 days after planting	10. Not mixing clean harvested pods with gleaned pods
11. Maintenance of optimal density of plants in the field	11. Avoidance of re-humidification of pods during
12. Avoidance of end-of-season drought through irrigation	12. shelling or in storage.
(if possible)	13. Fumigation of pods with insecticide to avoid insect
13. Removal of dead plants from the field before harvest	14. damage during storage.

excessive heat, high humidity, lack of aeration in the storage area, and insect and rodent damage. Postharvest strategies include:

1. Minimize times between harvesting and drying
2. Effective cleaning of maize prior to storage

14. Efficient drying to <14% m.c.

14.. Effective hygiene and management of silos

15. Absence of pests in store which can provide metabolic water and initiate heating
16. Clear specifications and traceability from field to store

SOURCE: International food policy research institute

2.2.4 Stability Aflatoxin in food processing

Aflatoxin content in food stuff. However, regard to the fact that AFs prevention is not always possible, recently, decontamination methods have gained attention as alternative way of reducing Aflatoxin uptake through food chain (Ahmadzadeh F, *et al.,* 2015). In general, process to degrade the toxin to safe levels should meet the following requirements: 1) inactivate, destroy, or remove the toxin, 2) not produce or leave toxic residues in the food/feed, 3) retain the nutritive value of the food/feed, 4) not alter the acceptability or the technological properties of the product, and, if possible, 5) destroy fungal spores. So far, detoxification of AFs is achieved by removal or elimination of contaminated commodities or

by inactivation of the toxins present in these commodities by physical, chemical, or biological methods. Therefore, food processes that may have effects on mycotoxins include sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization (tortilla process) and extrusion (Bullerman B. & Bianchini A., 2007). However, for the interest of this paper cleaning, milling, heating (thermal processing) and fermentation will be reviewed in short.

2.2.4.1 Sorting, trimming and Cleaning

Sorting and trimming may lower mycotoxin concentrations by removal of contaminated material. However, these operations do not destroy mycotoxins (Bullerman B. & Bianchini A., 2007). Cleaning is a multistep process such as removing dust, husks and products colonized by moulds, mechanical sorting and washing. Hulling of some products can reduce mycotoxins. some cereals are subjected to a dehulling step, which must be done as efficiently as possible since it has been demonstrated that the husks are very susceptible to mycotoxin contamination.

Due to the low solubility of AFs in water, it is generally hard to remove AFs by washing. However, in a study conducted by Hwang about 40% of AFB1 was removed from contaminated wheat, by washing (Hwang & Lee KG, 2006). Fandohan reported that since AFs are usually attached on surface of wheat, it's possible to remove them by washing. But, it is very difficult to remove aflatoxin bonded or attached strongly to the inner texture of food (Fandohan et al, 2005). While sorting, trimming and cleaning may reduce mycotoxin concentrations in commodities, this operations may not completely remove all the contamination. The initial condition of the grain, or commodity, and extent of the contamination will influence cleaning efficiency (Bullerman B. & Bianchini A., 2007).

2.2.4.2 Milling

In the milling process mycotoxin contamination, may be redistributed and concentrated in certain mill fractions, but there is no step or operation that destroys mycotoxins. Mycotoxins tend to be concentrated in germ and bran fractions in the dry milling process (Scudamore et al., 2003; Brera et al., 2004). Katta *et al*. 1997 showed that during the dry milling of corn, fumonisin B1 was found in highest amounts in the bran fraction that is used as animal feed, followed by the germ fraction, which may be used as animal feed or for oil extraction. Brera *et al.* 2004 also observed this while studying the effect of industrial processing on the

distribution of fumonisin B1 in dry milled corn fractions. Also with the dry milling of wheat, barley, and other cereals, DON, zearalenone, aflatoxins and ochratoxin A were found in highest amounts in fractions of the commodity that are less likely to be used for food production (germ and bran fractions) (Park, 2002; Scudamore *et al*., 2003).

In the wet milling of corn, mycotoxins may be dissolved into the steep water or distributed among the by-products of the process, but not destroyed. By the end of the wet milling process mycotoxins, including aflatoxin, zearalenone and fumonisins, can be found in the steep water, gluten fiber and germ, while the starch tends to be relatively free of these mycotoxins (Park, 2002).

2.2.4.3 Heating

AFs have high decomposition temperatures ranging from 237 °C to 306 °C. Solid AFBl is quite stable to dry heating at temperatures below its thermal decomposition temperature of 267 °C. However, it has been reported all heat treatment (boiling, roasting, baking and steaming) still provides a feasible mechanism for reducing the AFs concentration in foodstuffs. All processing methods (boiling, roasting, baking and steaming) destroyed AFs to a considerable extent. The percentage destruction ranged from 50-70% (Reddy UM. &Rani PC., 2004). The efficacy and extent of reduction method is depending on several factors, including AFs concentration, the extent of binding between AFs and food constituents, heat penetration, moisture content, pH, ionic strength, processing conditions (Hwang &Lee KG., 2006) and source of contamination (naturally or artificially) (Hussien *A. et al.*, 2011).

The relationship between moisture content of foods and reduction of AFs has been demonstrated several times (Torres P, Mendez-Albores A. *et al.*, 2004). According to these reports, by increased moisture content the destruction of AFs is increased during cooking or baking. Kabak and his co-workers also reported that the moisture content is a critical factor in AFs reduction and in presence of water decontamination of food by heating is easier and more effective. They suggested that the presence of water helps in opening the lactone ring in AFBl to form a terminal carboxylic acid. The terminal acid group thereafter undergoes heat-induced decarboxylation (Kabak B. *et al*., 2006).

However, in contrast with this idea, Mendez Albores (Mendez-Albores A. *et al.*, 2004) reported that higher reductions in AFs levels were achieved during the toasting process and

only a moderate extra-reduction occurred during the boiling. Moreover, Hussain and co-workers (Hussien A. et al., 2011) reported that roasting resulted in a significant decrease in the AFs content of nuts, corn and oilseed meals. Degradation of aflatoxins by roasting was both time and temperature dependent. Roasting at 150° C for 120 min degraded more than 95% of AFB1 in peanuts. It seems that different samples showed different behaviour under heat treatment and more research must be done to evaluate the effect of heat treatment on AFs.

2.2.4.4 Fermentation

Traditional fermentation is one of the oldest methods of food preparation, processing and preservation. Fermented foods have improved taste and texture, extended shelf life, improved nutritional value, and in some cases improved levels of safety. Fermentation of locally consumed foods is considered a cost-effective and nutritionally beneficial technology for communities with food safety and malnutrition problems, especially in developing countries. Changes that occur during the fermentation process are predominantly the result of enzymatic activity brought about by bacteria, yeasts and moulds. Some microorganisms produce desirable characteristics while others are responsible for spoilage or toxicity. Several microorganisms may be involved in a particular single fermentation; and each may be responsible for a specific change or series of changes in the entire process. However, there are only four main fermentation processes: alcoholic, lactic acid, acetic acid and alkali fermentation (Soni & Sandhu, 1990 sited by Blandino A.*et al.*, 2002). Alcohol fermentation results in the production of ethanol, and yeasts are the predominant organisms (e.g. wines and beers). Lactic acid fermentation (e.g. fermented milks and cereals) is mainly carried out by lactic acid bacteria (Blandino A. *et al.*, 2002). By tradition, lactic acid bacteria (LAB) are the most commonly used microorganisms for preservation of foods. Their importance is associated mainly with their safe metabolic activity while growing in foods utilising available sugar to produce organic acids and other metabolites.

Lactic acid bacteria (LAB) are Gram-positive, catalase-negative, non-spore forming rods and cocci. They are generally non-motile and utilize carbohydrates fermentative to form lactic acid as the major endproduct. A few LAB are aero-tolerant, most LAB are used for preservation of food products by inhibiting the growth of spoilage and pathogenic bacteria through production of lactic acid, acetic acid, diacetyl, and acetaldehyde. Accumulation of

these products causes inhibition of spoilage bacteria in some foods and beverages. The use of LAB in food preservation and to enhance food safety has recently gained momentum, as consumers are increasingly becoming more concerned with products containing artificial preservatives. All LAB used in food fermentation have GRAS (Generally Regarded As Safe) status (Frida A, 2013).

LAB is reported to be able to metabolize or transform mycotoxins into compounds that are less toxic. The ability of some of the LAB strains to repress mycotoxins by producing low-molecular-weight metabolites and/or binding of the toxic to bacteria cell wall has been reported (Me´ndez-Albores A. *et al.,* 2007). Microorganisms can either use sorption and/or enzymatic degradation to detoxify mycotoxins. Lactic acid bacteria can absorb mycotoxins either by attaching them to their cell wall components or by active internalization and accumulation (Gobbetti M. *et al,* 2005). During cell rupture, it is postulated that LAB can release molecules that potentially inhibit mould growth and therefore lead to a lower accumulation of their mycotoxins (Zindine A. *et al,* 2005).

2.2.5 Aflatoxin Detection and quantification by HPLC

High-performance liquid chromatography (HPLC) is the most popular chromatographic technique for separation and determination of organic compounds. About 80% of organic compounds in the world are determined using HPLC (Li, P., *et al.,* 2011). The HPLC technique makes use of a stationary phase confined to either a glass or a plastic tube and a mobile phase comprising aqueous/organic solvents, which flow through the solid adsorbent. When the sample to be analysed is layered on top of the column, it flows through and distributes between both the mobile and the stationary phases. This is achieved because the components in the samples to be separated have different affinities for the two phases and thus move through the column at different rates. The liquid (mobile) phase emerging from the column yields separate fractions containing individual components in the sample.

In practice, the HPLC technique employs a stationary phase such as C-18 chromatography column, a pump that moves the mobile phase(s) through the column, a detector that displays the retention times of each molecule, and mobile phase. The sample to be analysed is usually injected into the stationary phase and the analytes are carried along through the stationary phase by the mobile phase using high pressure delivered by a pump. The analytes are

distributed differently within the stationary phase (Malviya, R., 2010) through chemical as well as physical interactions with the stationary and mobile phases (Rahmani A. *et al* ,2009). The time at which a specific analyte elutes is recorded by a detector as its retention time. The retention time depends on the nature of the analyte and composition of both stationary and mobile phases (Xiang P., *et al*., 2006). Programmable detectors such as either the fluorescent detector (FLD) or the ultraviolet (UV) detector or the diode array detector (DAD) may be used in the detection and identification of aflatoxins.

High pressure liquid chromatography methods used for the determination of aflatoxins in foods include the normal-phase and reversed phase high pressure liquid chromatography techniques (Rahmani A. *et al* ,2009). The reversed phase HPLC method is the most widely used for separation and determination of aflatoxins. Occasionally, chemical derivatization of aflatoxins B1 and G1 may be required to enhance sensitivity of HPLC during analysis since the natural fluorescence of aflatoxins B1 and G1 may not be high enough to reach the required detection limit (Kok, W., *etal.,* 1986).

High-performance liquid chromatography provides fast and accurate aflatoxins detection results within a short time. However, the disadvantage of using HPLC for aflatoxins analysis is the requirement of rigorous sample purification using immunoaffinity columns. In addition, HPLC requires tedious pre- and post-column derivatization processes to improve the detection limits of aflatoxins B1 and G1 HPLC (Li, p., *et al.*, 2011). Therefore, to overcome the challenges associated with derivatization processes in aflatoxins HPLC is coupled to mass spectroscopy, has been made and is currently employed in the determination of aflatoxin (Takino M. and Tanaka T., 2008). Since the mass spectrometer requires neither use of UV fluorescence nor the absorbance of an analyte, the need for chemical derivatization of compounds is eliminated. The HPLC-MS/MS uses small amounts of sample to generate structural information and exhibits low detection limits (Rahmani A. *et al* ,2009). However, HPLC-MS/MS is bulky and very expensive equipment which can only be operated by trained and skilled personnel. Besides, this also limits its use to only laboratory environment and not field conditions.

2.2.6 Immunoaffinity column (IAC)

Immunoassays were first developed more than 50 years ago and the exploitation of antibodies in various formats has continued making a significant impact in the testing and diagnostic fields. Antibodies in user-friendly formats have even been exploited in the home-testing arena, e.g. pregnancy tests. In the clinical field, urine or blood plasma, being relatively uncomplicated liquid samples, readily lend themselves to direct analysis. Although immunoassays have changed the way some food testing is conducted, arguably the biggest impact of antibody technology has probably been in the development and application of immunoaffinity columns (IAC) to trace analysis of foods. A number of other terms are also used to describe immunoaffinity column to explain the same process of sample extraction and clean-up. Thus, the process of using an IAC is sometimes described as immunoextraction, immunoaffinity based solid-phase extraction or sol–gel immunoaffinity chromatography. Immunofiltration or immuno-ultrafiltration (IUF) are like IAC except with IUF the antibodies are not bound to a solid support material but are used in free form. The principle of the IAC is relatively simple in that an antibody (polyclonal or monoclonal) raised against the analyte is immobilised on a gel, and generally about 0.2–0.5 ml of gel is packed into a small plastic column. The column is initially conditioned with phosphate buffered saline (PBS) and then the crude sample extract is applied slowly to the column at around 1–2 ml/min. The sample can be applied under gravity flow or under positive pressure from a syringe or can be sucked through the column under vacuum, but maintaining a uniform flow rate in line with manufacturers recommendation is important.

In general, the sample extract must be in aqueous solution because organic solvents can damage the antibody and can interfere with the antibody–antigen interaction and this may be a limitation when wishing to use this approach to analyse non-polar target analytes. During application of the sample extract the analyte becomes bound to the antibody and thereby bound to the IAC gel. The specificity of the antibody is important, in terms of the extent of recognition of the analyte (antigen) compared to structurally similar co-extractives which should not become bound to the antibody. The strength of the binding to the antigen (avidity) is also important as binding strength will influence recovery during this extraction stage. The capacity of the column in terms of the total number of antibody sites (quantity of antibody) available for binding will also be important as overloading the column will lead to poor recovery. After loading the extract onto the IAC, the gel is washed with PBS to completely

remove any co-extractives. Finally, the analyte is eluted from the IAC by breaking the antibody–antigen bond. For small molecules, this can be achieved with a small volume of methanol or acetonitrile, which is generally the procedure used with commercial columns (Hamide, Z, *etal.*, 2010).

2.2.7 Determination of total Aflatoxins

Calculate concentration of aflatoxin in test sample as follows: Plot data the peak area (units; y-axis)] against [concentration of aflatoxin (ng/mL; x-axis)] from the calibrant solution experiments into a table and calculate the calibration curve using linear regression.

Use the resulting function (y = ax + b) to calculate the concentration of aflatoxin in the measured solution. For a linear calibration, the formula describes the correlation between the detector signal (y) and the corresponding concentration of the analyte (x).

This means that (y) is a function of (x) [$y = (f)\ x$]. The constant (a) is the corresponding value of the slope of the linear function, while (b) is the value where the calibration function intercepts the y-axis of the coordinate system.
Calculation of the calibration curve (function) obtained by linear regression:

$$\boldsymbol{signal\ sample(units) = a\ x\ concentration\ sample(ng/ml) + b}$$

$$\boldsymbol{Contam\left(\frac{ng}{g}\right) = \frac{Csamp\ x\ solvent\ x\ Elution}{wt\ x\ Aliquot}\left[\frac{ngxmlxml}{ml\ x\ g\ x\ ml}\right]}$$

Where:

Wt (g) = sample material taken for analysis
Solvent (mL) = solvent taken for extraction;
Aliquot (mL) = aliquot taken for immunoaffinity clean-up;
Elution (mL) = final volume collected after elution from IAC;
Csmp (ng/mL) = concentration of aflatoxin calculated from linear regression;
Contam (ng/g) = contamination of sample material with aflatoxin;
Signal smp (units) = area of aflatoxin peak obtained from the measured solution

2.2.8 Aflatoxin exposure assessment

Exposure assessment – an estimation of the potential human exposure to the hazard and includes the use of data such as the occurrence in the food and/or potential consumption rates of the food. One of the most important aspects in risk analysis of chemical substances is to determine the degree of human exposure, particularly difficult task for contaminants present in foodstuffs. However, it is possible to indirectly estimate the degree of exposure based on data on consumption of contaminated foodstuffs, and on the average occurrence of the toxin. In this estimation, the degree of exposure is measured in terms of probable daily intake (PDI) per unit of body weight, and is generally expressed in ng/kg of body weight (BW) / day.

In risk analysis, PDI is compared with tolerable daily intake (TDI) determined in toxicological studies.

For dietary exposure to Aflatoxin, exposure can be defined as:

$$Ei = \frac{\Sigma\, Qi,k \; x \; Ci,k}{Bwi}$$

Where:

Ei is the exposure of individual i to some chemical at some specified point in time, Qi,k is the amount of food k consumed by individual i, Ci,k is the concentration of aflatoxin in food k consumed by individual i and Bwi is the body weight of individual I (Peter C., and John R., 2016).

Exposure assessment can be based on the average intake of aflatoxins from the general population of individuals with a value at the 90th percentile of the general population, or to evaluate only those individuals who consume food contaminated with aflatoxins. Exposure can be made for different age groups or target, depending on the scenario of the study (i.e. sharp intake compared with chronic intake. Assessment of exposure to aflatoxins and their metabolites may be based on the measurement of specific biomarkers, taking calculated based on pharmacokinetic interactions.

Risk characterization is qualitative and/or quantitative evaluation, including accompanying uncertainty and the burden and the likely occurrence or absence of known or potential adverse health effects on the population at risk of exposure to aflatoxins. Risk characterization is based on three main components: hazard identification, hazard

characterization and exposure assessment. Risk characterization can also be establishing levels of daily exposure in which the risk is negligible lifetime (i.e. exposure should be below tolerable daily intake - TDI or another measure of safe dose). The latter determination can be relevant, considering the uncertainties.

Along with the average population, the risk characterization for aflatoxins should also consider those groups that are most vulnerable to exposure as children (due to their lower body weight), and other groups that may be a difference in bioavailability, metabolism or genetic predisposition, and the elderly. In this respect, the adequacy of the safety factor of ten times to eliminate the differences in sensitivity between individuals arising from variability should be examined and assessed separately. Detailed risk assessments in relation to aflatoxins are periodically reviewed to receipt of new information in terms of exposure and the mechanism of action (Valentina *et al*, 2013).

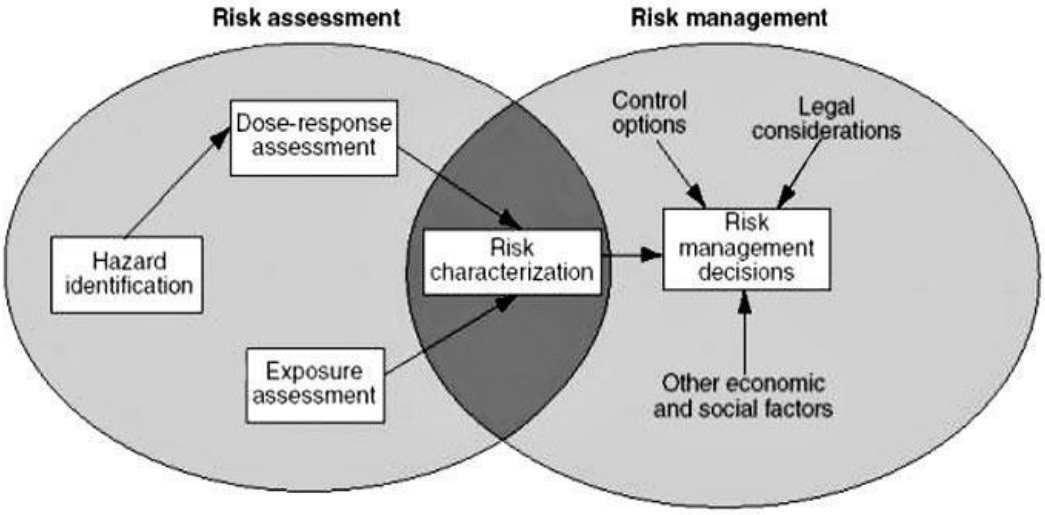


Figure 5: Risk assessment and risk management

In the final assessments of risk assessment of exposure to aflatoxins can be based on actual residue levels (not MRL), a worst-case scenario. The concern of regulators is that if allowed higher synoptic for Maximum residue limit (MRL), it will be an acceptable level for the industry and there would be a change in the upward direction to reach that level that this will lead to increased exposure.

A mathematical model is used in most of which it is assumed that the effects of low doses are linear, to extrapolate the possibility of side effects at lower doses. Dose corresponding risk level 10^{-5} or $10^{-6,}$ in some jurisdictions, was assessed as presenting negligible risk. Per other jurisdictions, the most appropriate method for regulating the genotoxic carcinogens or even genotoxic agents (eg, Aflatoxins) that the carcinogenic potential has not been proven to determine the levels that are "the lowest reasonably possible point "is (ALAR-"as low as is reasonably achievable") or as low as technologically achievable.

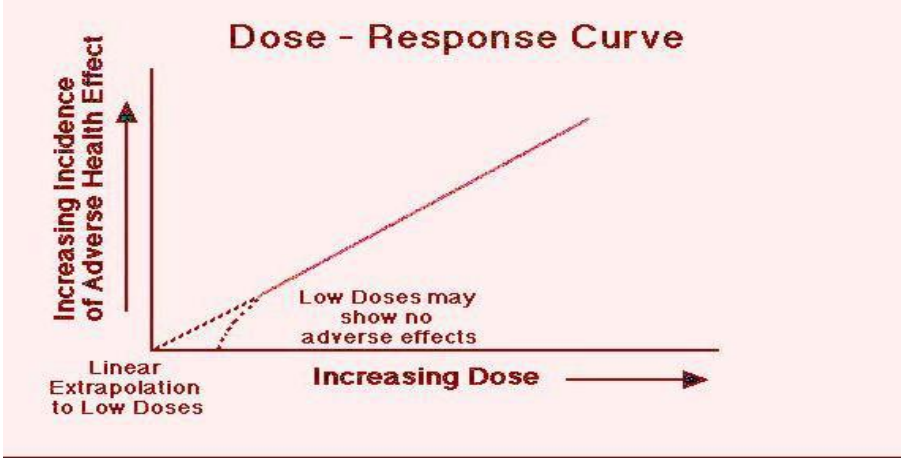
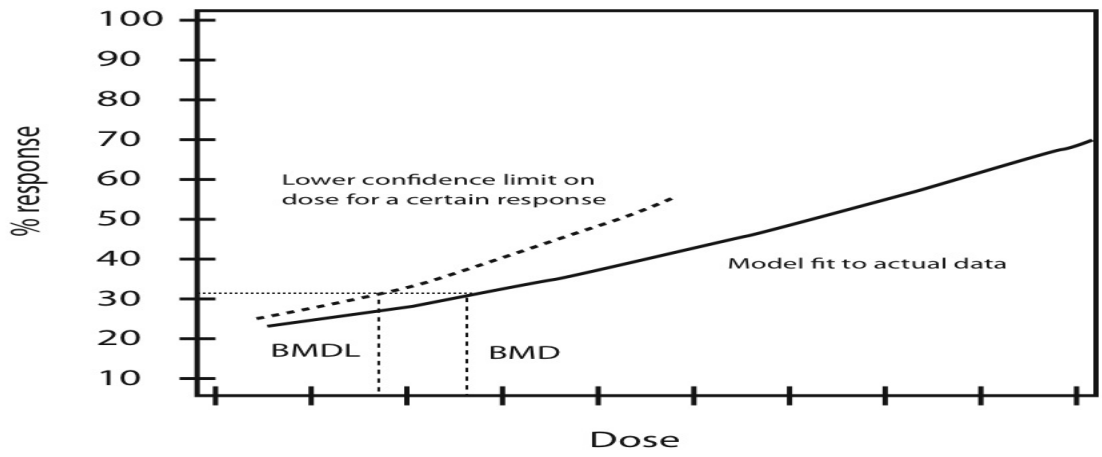


Figure 6: Extrapolation of dependence "dose-response" at low doses (Valentina *et al*, 2013).

Alternative regulation can be based on biological factors such as mode of action and the burden of proof, which is certified by the severity of the resulting injury. This information can be combined with assessment of tumor potential, as is the magnitude in the TD05 (Salomon, S. *et al*., 2011).

A new concept is to use a Benchmark dose (BMD). This approach finds application in both threshold and non-threshold at toxicologically compounds. Indicator BMD (Fig 8) is defined as a dose that corresponds to a given change in adverse reaction in comparison to unexposed individuals in response and less than 95% confidence limit, called indicator of dose level. BMDL = Benchmark dose (BMD) is modelled to 10% above the critical risk. The approach is an alternative to the no-observed adverse effect levels (NOAEL) - an approach that has been used for many years in a dose-response relationship. BMDL10 = 95% lower confidence limit of BMD for an additional 10% risk of a critical effect (Valentina *et al*, 2013)**.**



.

Figure 7: Benchmark dose (Valentina *et al*, 2013).

2.3 Legislation and detoxification

Most countries, established various regulations for aflatoxin levels (either total aflatoxins or for AFB1) in food and/or feed in order to limit exposure to this group of mycotoxins (Van-Egmond et al., 2007). Worldwide, aflatoxins because of their prevalence and toxicity are important in public health. Public health concerns center on both primary poisoning from aflatoxins in commodities, food and feed stuffs, and relay poisoning from aflatoxins in milk. The allowable levels of aflatoxins in animal feedstuff and human foods vary with governmental jurisdictions (Coppock & Christian, 2007).

Aflatoxins are of great concern because of their detrimental effects on the health of humans and animals, including carcinogenic, mutagenic, teratogenic and immunosuppressive effects. AFB1 is the most potent hepatocarcinogen known in mammals and is classified by the International Agency of Research on Cancer as Group 1 carcinogen (Eaton & Gallagher, 1994 as cited in Zinedine, 2009).

The first legislative act was undertaken in 1965 by the Food and Drug Administration (FDA) of the USA, which proposed a tolerance level of 30 pg kg-1 of total aflatoxins (Bl + Gl + B2 + G2). With increasing awareness of aflatoxins as potent toxic substances, the proposed level was lowered to 20 pg kg-1 in 1969. The FDA has action levels for aflatoxins regulating the levels and species to which contaminated feeds may be fed (Giniani Carla Dors et al., 2011) (Table 2).

In 1973, the European Economic Community (EEC) established legislation on maximum permitted levels of AFBl in different types of feedstuffs. The European Community levels are more restrictive (Tables), 4 μg kg-1 total aflatoxin in food for human consumption are the maximum acceptable limits in the EU, the strictest in standard worldwide. Human foods are allowed 4–30 ppb aflatoxin, depending on the country involved (Table 3) (John, 2007). Several African countries still have to put in place regulatory mechanisms for aflatoxins. However, Kenya's limit for aflatoxin in products for human consumption is 20ppb. In the case of Ethiopia, Ethiopian standards may be either mandatory or voluntary. mandatory standards have the force of law as do other technical regulations in Ethiopia. They are enforced by laws and administrative regulations and concern the protection of human health, personal property and safety (Ethiopian mandatory standards catalogue, 2016). Regarding to mycotoxin regulation, the country doesn't limit for aflatoxins and other mycotoxin in products for human consumption in Ethiopian mandatory standard catalogue.

Commodity	***Concentration*** (μg kg-1)
Cottonseed meal as a feed ingredient	***300***
Corn and peanut products for finishing beef cattle	***300***
Corn and peanut products for finishing swine	***200***
Corn and peanut products for breeding beef cattle, swine and mature poultry	***100***
Corn for immature animals and dairy cattle	***20***
All products, except milk, designated for humans	***20***
All other feedstuffs	***20***
Milk	***0.5***

Table 2: U.S. Food and Drug Administration action levels for total aflatoxins in food and feed (μg kg-1).

Human food	*AFB1*(µg kg-1)	*AFB1, B2, G1 and G2* (µg kg-1)	*AFM1*(µg kg-1)
Groundnuts, dried fruit and processed products thereof	***2***	***4***	**-**
Groundnuts subjected to sorting or physic treating	***8***	***15***	**-**
As above but for nuts and dried fruits	***5***	***10***	**-**
Cereals (including maize) and processed products thereof	***2***	***4***	**-**
Milk	**-**	**-**	***0.05***

Table 3: European Union for aflatoxins in human food (µg kg-1).

Aflatoxins can be detoxified or removed from contaminated food and nutrients by physical, chemical or biological methods. The inactivation of these compounds by physical and chemical methods have not proved to be effective and economically viable (Mishra & Das, 2003). However, biological degradation offers an attractive alternative to eliminate these toxins retaining food nutritional value. In the last decade, it became clear that fungi are among the microorganisms that play a major role in mycotoxin degradation particularly AFB1 (Zucchi et. al., 2008 cited in Giniani Carla Dors et al., 2011).

Aflatoxins are thermostable, so the physical treatment by heat results in only small changes in their levels (Tripathi & Mishra, 2010). Chemical treatments using solvents are able to extract these compounds causing minimal effect on nutritional quality, however, this technology is still impractical and expensive, besides inducing odors and flavors. Ammonization is also used as an effective and practical application for decontamination of agricultural products containing aflatoxins (Allameh *et al.*, 2005). Ozonation is the chemical method that has been most studied for the decontamination of aflatoxins in foods, once ozone has been recognized as safe by the Food and Drug Administration in 2001 (Zorlugenç *et al.*, 2008 sited in Giniani Carla Dors *et al.*, 2010)

3 Materials and Method

3.1 Study area selection

The study was conducted in five districts of Hararghe: Assebe Teferi (Chiro), Chelenko, Haromaya, Harar and babile, Eastern Ethiopia (Fig. 8) was selected for the following reasons: First, Sorghum is the first food crop in the region, due to its drought resistance it is sometimes called 'the camel crops of cereals'. Second, in hararghe majority sorghum grain are stored in Underground pits, which are not underlined with protective materials until the grains are consumed or sold, such storage environment is very conducive for moulding and mycotoxin production such as aflatoxins (Dejene M., 2004). Third, Sorghum is the second most preferred cereal for injera preparation in Ethiopia next to tef *[Eragrostis tef*]. However, Sorghum Injera mainly consumed staple food in East Ethiopia: Hararghe where it is difficult to grow other food grains.

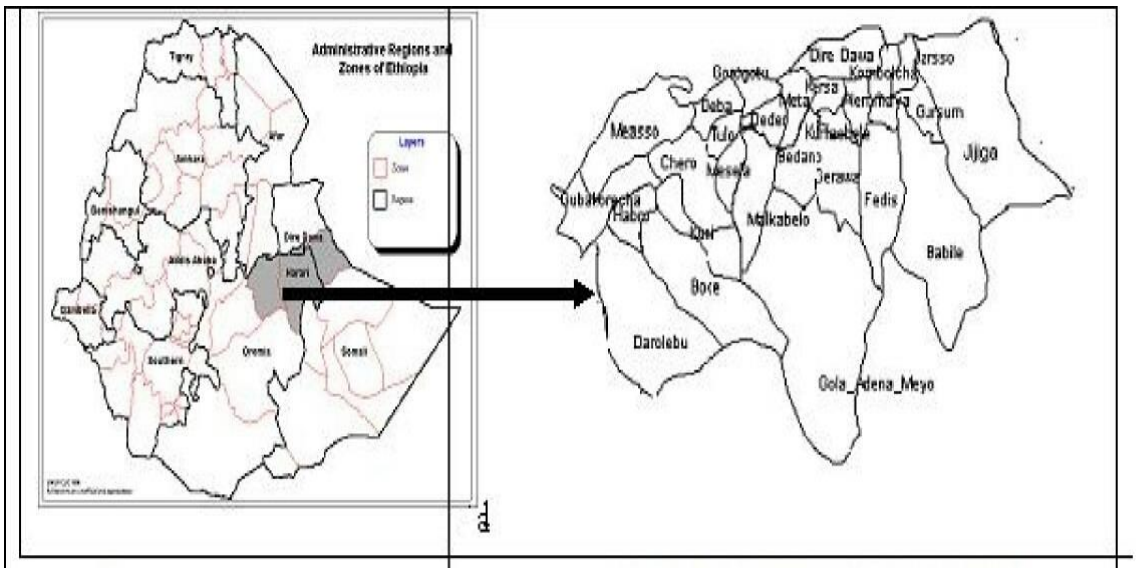


Figure 8: Map of Ethiopia indicating Hararghe region

3.2 Sample collection

30 duplicate sorghum Injera sample were randomly collected from five districts of Eastern Ethiopia (Eastern and western Hararghe): Assebe Teferi (Chiro), Chelenko, Haromaya, Harar and Babile. From each district of Hararghe six sorghum Injera were sampled. Sampling were covered both at the retailers and householders level (Figure 1). Samples were collected from the site in duplicate for the result to be expressed as a mean of duplicate. Samples were properly labeled with the name of the location, code and sample collection date and properly

wrapped with zipper freezer bags (Falcon®) inorder to preserve samples from physical and microbial damage. During the survey, data were collected to assess knowledge, practice and attitude of the population on food processing, handling and storage of grain at sample collection area.

Figure 9: Sorghum injera Sample collection procedure in Hararghe region

3.3 Chemicals and reagent

Acetonitrile, methanol, sodium chloride, ultrapure water and phosphate buffered saline (PBS: NaCl 8g l^{-1}, KCl 0.2 g l^{-1}, Na_2HPO_4 1.15 g l^{-1}, KH_2PO_4 0.2 g l^{-1}; and adjust pH to 7.4 using 0.1M HCl or 0.1M NaOH) were purchased. Reagents for HPLC separation were suitable for

UPLC filtered through a 0.2µm membrane (Merck Chemicals, Darmstadt, Germany). All other chemicals and reagents used were of analytical grade.

3.4 Immunoaffinity columns

AFLAPREP® (product code P07, P07/500, Batch number: CL 577, Glasgow UK) immune affinity column which contain antibodies against B1, B2, G1 and G2 with a capacity greater than 100 ng for aflatoxin B1 and recovery (70%-110%) was used for sample clean up. IAC was purchased from R-Biopharma Rhone Ltd, Scotland, UK.

3.5 Mobile phase

The mobile phase used for aflatoxin analysis was a mixture of a de-ionized water-methanol-acetonitrile (65:25:15, v/v/v) at flow rate 1.2 ml/min. The mobile phase was vacuum filtered and sonicated for 30 minutes to degassed and Isocratic method was applied for analysis for better resolution. Distilled and ultrapure water were used throughout mobile phase preparation.

3.6 Standards

Aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 and mixed Aflatoxin standards purchased from Sigma Aldrich (St. Louis, MO, USA).

3.7 Apparatus

Immunoaffinity column (Aflaprep®,1ml), laboratory stand with clump, Graduated pipettes (1ml, 5ml, 10ml, 25ml and 50ml), volumetric flask (10ml, 25ml, 50ml, 100ml, 500ml and 1000ml), Measuring cylinder (50ml and 100ml), Beaker (50ml, 100ml and 500ml), conical flask(250, 500 and 1000ml),Hot air oven for moisture analysis, Mixer, Stirrer, Ultra bath sonicator, ultrapure water machine (Vivagen Korea) Wash bottle, Micropipettes, Micropipette tips, Syringe Filter (0.45µm pore size), Electronic balance type AX120 (capacity, readability 0.1mg, shimadzu corporation), syringes (5ml and 10ml), Paraffin, Sample label, Vials with screw cap. HPLC system setup contains auto sampler, injector, oven, column (C18 column 250 L×4.6 mm, 5µm), Link, Degasser, fluorescence detector and desktop computer with shimadzu LC software.

3.8 Chromatographic condition

Chromatographic separation and detection was carried out using Shimadzu USA, HPLC instrument with LC software and fluorescence detector were used for analysis. separation was achieving with A Shim-pack FC-ODS reversed phase column (5µm, 250L x 4.6mm diameter). The operating condition were as follows: column temperature at 25°c temperature; flow rate 1.2ml min^{-1}; 25 minutes running time, 20µl injection volume; detection wavelength: excitation wavelength 365 nm/emission wavelength 440 nm; diluent methanol and Needle wash (Water: Methanol 90:10 v/v)

3.9 Procedures for Aflatoxin Analysis

3.9.1 Sample preparation, Extraction and clean Up

The collected Injera sample were initially analysed for moisture content according to weight loss after drying method using an oven at 115°c and its average moisture content was determined. The sample was completely dried and crushed for 15 minutes with mill to medium sized powder and passed through an appropriate mesh size sieve i.e. 20µm mesh at the Center for Food Science and Nutrition laboratory using metal and powder free sterile latex gloves. Fifty grams of ground sample and 5 grams of sodium chloride were weighed and place into a 1 liter capacity, solvent resistant blender jar and then 100ml of well mixed extraction solvent (80% v/v HPLC grade methanol and 20% v/v Ultrapure water) was added to blender jar.

The mixture was blended for 2 minutes at high speed and then the extract was filtered through a filter paper (Whatman® No.4) and 2 ml of clear filtrate (equivalent to 1 g of product) was transferred to a beaker and 14 ml of PBS (PH=7.2: NaCl 8g l^{-1}, KCl 0.2 g l^{-1}, Na_2HPO_4 1.15 g l^{-1}, KH_2PO_4 0.2 g l^{-1}) solution was added. Immunoaffinity column (AFLAPREP®Product code: P07, batch number CL577 R-BIOPHARM RHONE Ltd Scotland park, Glasgow United Kingdom) was used for sample clean up prior to aflatoxin analysis.

The columns were placed at ambient temperature before conditioning and 0.45µm Millipore was attached at the tip of 10 ml syringe for sample filtration. The conditioned IAC would be filled with 1 ml of the diluted extracts.

The samples passed slowly and continuously through the columns (approximately 2ml/min) and the syringes were filled with residual samples solutions then gradually to the column. The passed solutions were discarded and the columns were rinsed by 20 ml of PBS and some air was pressed through the column to make sure that all the residual fluids were removed from the columns. Then aflatoxins were eluted with 1 ml of 100% methanol (at flow rate of approximately 1 drop/sec) and second 1ml of ultrapure water was added to the column to make the total elute volume 2ml in amber color glass vial. All eluted residues were collected by pressing air thoroughly through the column.

Figure 10: Sample clean-up procedure with immunoaffinity column

3.9.2 Aflatoxin Analysis by HPLC-FLD

Aflatoxin was quantified using HPLC-fluorescence detector (Shimadzu LC RF-20A prominence L20495273405, US), equipped with a reverse phase C18 column (Hichrom C18, 250 mm x 4.6 mm, 5µm, UK). The operating condition were as follows: column temperature at 25°c temperature; flow rate 1.2ml min^{-1} isocratically; 25 minutes running time per analysis, 20µl injection volume; detection wavelength: excitation wavelength 365 nm/emission wavelength 440 nm.

Concentrations of aflatoxins were quantified with reference to AFs standards. The calibration curve for the standards was established with a concentration range from 2ppb-100 ppb.

The toxin level was calculated with the formula total AF, $\mathbf{ng/g = \frac{Csamp\ x\ solvent\ x\ Elution}{wt\ x\ Aliquot}\left[\frac{ngxmlxml}{ml\ x\ g\ x\ ml}\right]}$,

where Wt (g) = sample material taken for analysis; Solvent (mL) = solvent taken for extraction; Aliquot (mL) = aliquot taken for immunoaffinity clean-up; Elution (mL) = final volume collected after elution from IAC; Csmp (ng/mL) = concentration of aflatoxin calculated from linear regression; Contam (ng/g) = contamination of sample material with aflatoxin; Signal smp (units) = area of aflatoxin peak obtained from the measured solution.

Calibrant solutions were prepared by diluting the total aflatoxin standard stock with 100% methanol at concentrations of 2, 5, 10, 20, 30, 50 and 100 ng/ml. They were injected into the HPLC at a volume of 20 µl.

3.9.3 Moisture Content

Fungal spoilage of stored commodities and aflatoxin production highly depends on several important factors including moisture content, relative humidity in the air and temperature of the environment. The activities of molds are also governed by the relative humidity of surrounding air and moisture content of stored products and Consumables. There is defined relationship between water content in the grain and relative humidity of the surrounding atmosphere. The Injera sample in the present study were analysed for Moisture by AOAC (2000) official method based on weight loss during drying in hot air oven at 115°C.

3.10 Chromatographic method validation

In order to Obtain consistent, reliable and accurate data, method validation for the suitability of the analytical method for the determination of aflatoxins in the sample was performed on the following validation attributes:

- Toxin identification linearity and calibration curve, LODs and limits of quantification (LOQ), precision, recovery and working range

3.10.1 Identification

Toxins Identification were determined by injecting each four aflatoxin standards and mixed aflatoxin standard at the same HPLC condition to see their retention time and their precision were determined by calculating their percentage RSD (%RSD).

3.10.2 Linearity

The linearity was studied by plotting analytical curves built up from 7 different concentrations of the aflatoxin standards (2µg/kg, 5µg/kg,10µg/kg ,20µg/kg, 30µg/kg, 50µg/kg, 100µg/kg) in methanol. Each curve, for the aflatoxins B1, B2, G1 and G2, was prepared by plotting regression line peak area (Y-axis) against [concentration (x-axis)]. The coefficient of correlation (R^2) was considered appropriate when > 0.99.

3.10.3 Limit of detection (LOD) and Limit of quantification (LOQ).

LOD the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, whereas LOQ as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were found by injecting blank sample (solvent: methanol) and decreasing concentrations (0.01, 0.2,0.01 and 0.2) of individual standard (AFB1, AFB2, AFG1 and AFG2) solution respectively. The limit of detection (LOD) was determined as the analyte concentration that gives a signal equal to: **3xS/N ratio**. Similarly, the limit of quantitation (LOQ) was defined as: **10 x S/N ratio**.

3.10.4 Precision

The closeness of agreement between a series of measurement of was evaluated in terms of repeatability (Intraday precision). Repeatability was assessed by injecting by ten replicates of 30 PPB mixed aflatoxin standards the same mixed aflatoxin standard under the same

operating conditions over a short interval of time. The result expressed as% RSD of peak areas.

3.10.5 Recovery

Recovery experiments were carried out by spiking two mixed aflatoxin standard concentration (30 PPB and 50 PPB) in to sample. Spiked and un-spiked sample were treated with the same extraction and clean up procedure for sample analysis. Recovery specifications with AFLAPREP® with immunoaffinity clean-up are greater or equal to 85% and less than or equal to 110% for aflatoxins B1, B2 and G1, and greater or equal to 80% and less than or equal to 110% of aflatoxin G2 (Certificate of analysis, R-Biopharm Rhone Ltd).

Recoveries which were calculated as:

$$\%\ \boldsymbol{recovery} = \frac{\boldsymbol{x' - x}}{\boldsymbol{x\ spiked}}\boldsymbol{x100}\%$$

Where x'= The concentration of the spiked sample

X= The concentration of non-spiked sample

X spiked= Is the concentration added

3.11 Standard preparation

Aflatoxin standards (AFB1, AFB2, AFG1, AFG2 >98% purity) were purchased from Sigma-Aldrich (Saint Louis, MO USA) and from them individual aflatoxin standard stock solution was prepared. From Individual stock solution, serious of aflatoxin mixed standard which had a concentration of (2, 5, 10,20,30,50 and 100) PPB were prepared by using HPLC grade methanol as a diluent in 10 ml volumetric flask. The prepared standards transferred to vials and stored at 4 °C and protected from light to avoid deterioration of the aflatoxins in the solution.

3.12 Experimental Design and Questionnaire

Completely randomized experimental designs were applied to see the level of aflatoxin in sorghum injera sample collected in East Ethiopia. Purposive sampling technique were followed and questionnaire evaluation were done on sorghum grain moulding, handling and safety knowledge and practice.

3.13 Statistical analysis

All statistical analyses were performed using SPSS software, version 20 and the data were expressed as mean ± standard deviation (SD). A repeated measures analysis of variance (ANOVA) were used to compare the mean of aflatoxins concentration in Sorghum samples in the differences were considered significant at values of $P < 0.05$.

3.14 Safety Measures

AFs are carcinogens and care should be exercised to avoid personal exposure and potential risk of contamination. All handling of pure compounds was done in the fume hood with protective wear such as safety glasses, gloves, laboratory coat and a disposable face mask. The glass wares were washed with hypochlorite and dilute acid before re-using and the waste materials treated with hypochlorite before disposal.

4 Results and discussion

4.1 HPLC method validation

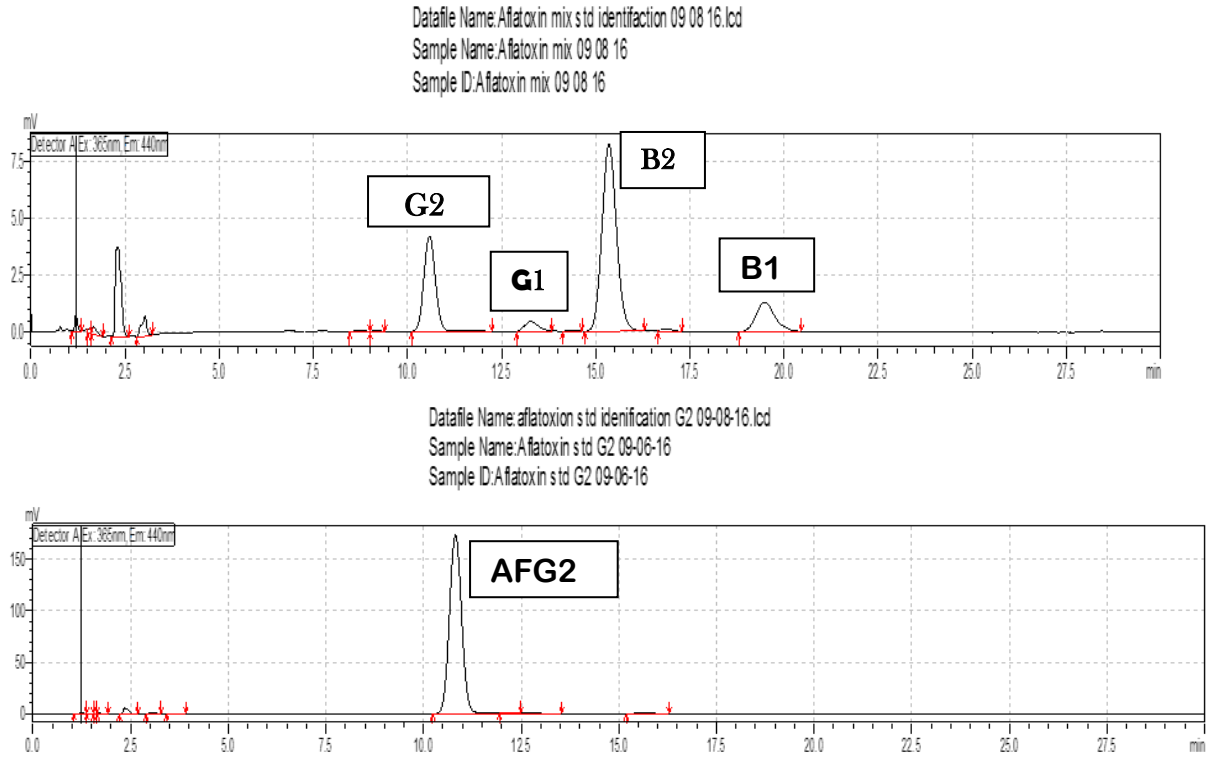
4.1.1 Identification

Aflatoxins peak were Identified by running 100 PPB of mixed aflatoxin and each 100 PPB of AFB1, AFB2, AFG1 and AFG2. The peak was identified in the order of mean retention time AFG2, AFG1, AFB2, AFB1 with 10.702, 13.237,15.352 and 19.284 respectively.

Table 4: The average peak retention time at the time of chromatographic run with current condition was tabulated in the following Table:

	Retention times				
Aflatoxins	**Mixed run**	**Single run**	**mean**	**Std dev.**	**% RSD**
AFG2	10.586	*10.818*	10.702	0.1160	1.08%
AFG1	13.277	*13.197*	13.237	0.056568	0.427%
AFB2	15.354	*15.109*	15.354	0.04202328	0.273%
AFB1	19.487	*19.082*	19.2845	0.0391918	0.203%

Mixed Aflatoxins



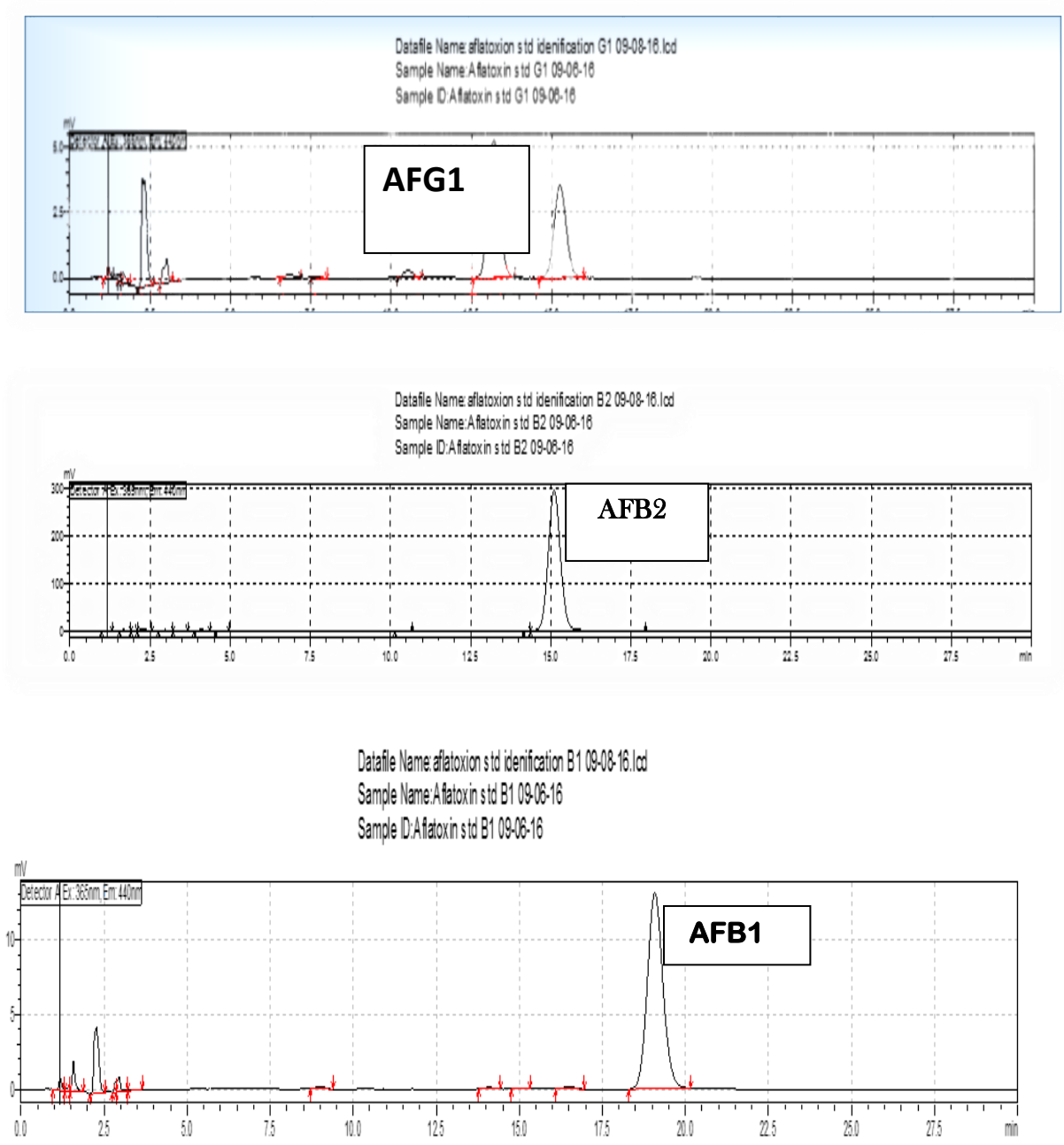


Figure 11: Aflatoxins standard chromatogram

4.1.2 Linearity and Range

Linearity was determined with series of seven injection of standard Aflatoxin concentration ranging from 2 PPB to 100 PPB. A linear regression equation was plotted against peak area on the Y- axis as a function of analyte concentration on x- axis. The equation showed that individual aflatoxin had excellent relationship the respective coefficient of variation R^2 0.998, 0.999,0.9978 and 0.9985 for AFG2, AFG1, AFB2 and AFB1.According to FDA R2> 0.998 was found to be acceptable and had excellent relationship.

Table 5: Linearity and calibration curve

Aflatoxins	N	Regression equation	Correlation coefficient (R2)
Aflatoxin G2	6	Y=42517X+2308	R^2=0.998
Aflatoxin G1	6	Y=1388.3X-85.317	R^2=0.999
Aflatoxin B2	6	Y=105943X+5031.8	R^2=0.9978
Aflatoxin B1	6	Y=5235X+1316.2	R^2=0.9985

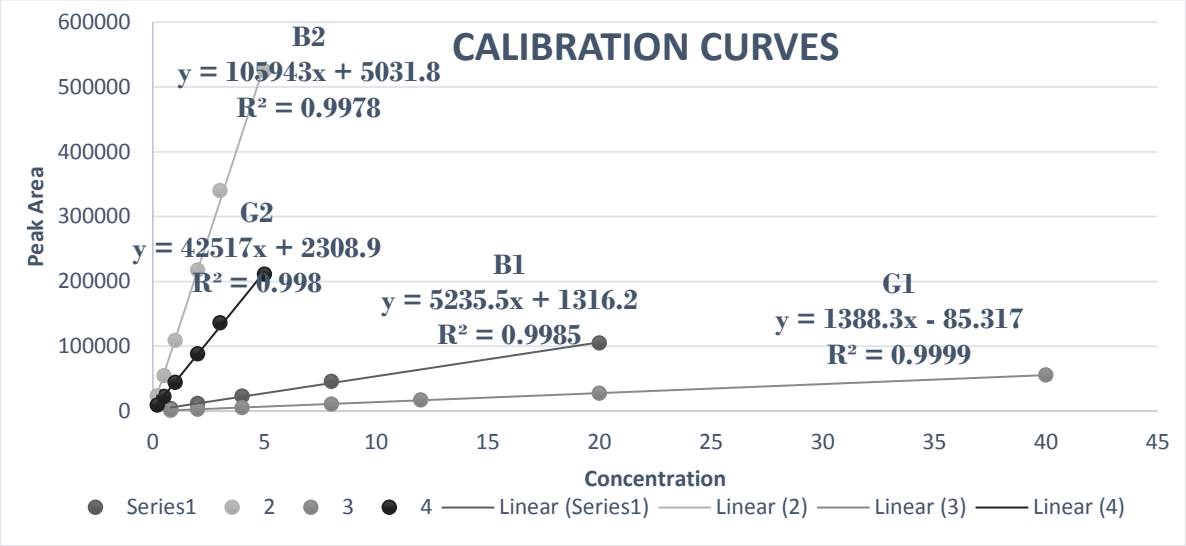


Figure 12: Calibration curves of four aflatoxins

4.1.3 Working range

The range of an analytical method is the interval from the upper to the lower levels that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (parts per billion, μgkg^{-1}) obtained by the analytical method.

Table 6: The concentration of individual aflatoxins in mix concentration

	Aflatoxin (μgkg^{-1})			
Aflatoxin sum (μgkg^{-1})	**AFG2**	**AFG1**	**AFB2**	**AFB1**
2	0.20	0.80	0.20	0.80
5	0.50	2.00	0.50	2.00
10	1.00	4.00	1.00	4.00
20	2.00	8.00	2.00	8.00
30	3.00	12.0	3.00	12.0
50	5.00	20.0	5.00	20.0
100	10.00	40.0	10.0	40.0

4.1.4 Precision

The precision chromatographic method was expressed by the Relative Standard deviation (RSD%) and calculated according to the repeatability of the subsequent Injection of 30 µg/kg mixed aflatoxin standard concentration repeated Injection experiment with the same chromatographic condition to be used for sample analysis. According to the result obtained described on the table Peak area RSD calculation for individual aflatoxin 3.0423,3.80121, 4.25 52 and 2.8272 and Retention time RSD calculation 1.0378, 1.8499, 1.0702 and 1.0856 for AFG2, AFG1, AFB2 and AFB1 respectively. The acceptable % RSD for Peak area <5% and <% RSD for analyte retention time <2% and hence chromatographic method precision studies was acceptable for sample determination precisely.

Aflatoxins	N	% RSDr (Peak area)	% RSDr (Retention time)
AFG2	10	3.04234	1.03783
AFG1	10	3.80121	1.84992
AFB2	10	4.25523	1.07021
AFB1	10	2.82723	1.08562

Table 7: Precision method validation

4.1.5 Limit of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were found by injecting decreasing concentrations of standard solution containing the four aflatoxins. The limits of detection and quantification found were 0.01 µg kg-1, 0.2 µg kg-1, 0.01 µg kg-1, 0.2 µg kg-1 and 0.05 µg kg-1, 0.80 µg kg-1, 0.05 µg kg-1 and 0.80 µg kg-1 for aflatoxin AFG2, AFG1, AFGB2 and AFB1 respectively. LOD and LOQ were determined by the amount of analyte that can be detected above baseline noise of blank run; typically, three times the noise level S/N > 3 and ten times the noise level of blank, methanol run S/N>10.

Aflatoxin	LOD		LOQ	
	µg kg-1	S/N	µg kg-1	S/N
AFG2	0.01	4.67	0.05	11.92
AFG1	0.20	3.34	0.80	10.41
AFB2	0.01	5.41	0.05	12.86
AFB1	0.20	4.08	0.80	11.16

Table 8: Limit of detection and quantification

4.1.6 Recovery

The recovery studies were conducted by spiking two known concentrations of standard solution containing four individual aflatoxins and the results revealed from the recovery testing were the following: Aflatoxin B1(110%), aflatoxin B2 (110%), Aflatoxin G1(108%) and Aflatoxin G2 (107%).

4.2 Total Aflatoxins in sorghum Injera sample

After determination of moisture content of sorghum Injera sample, the level of individual and total aflatoxins in Injera samples Collected from five districts of Eastern Ethiopia were determined with shimadzu HPLC by using Fluorescence as detector.

The Injera samples Collected in the present study contained moisture in the range 58%-64%.

Table 9: level of Aflatoxin (µg/kg) in Sorghum Injera sample from East Ethiopia

Site	MC %	Aflatoxin type and concentration (µg/kg)				
		AFG2	AFG1	AFB2	AFB1	Total
Chiro-1	63%	<LOQ	<LOD	<LOD	<LOQ	**<LOQ**
Chiro -2	65%	<LOQ	ND	<LOQ	<LOQ	**<LOQ**
Chiro-3	58 %	4.06816	24.90050	<LOQ	<LOQ	**28.9687**
Chiro-4	64.0%	1.48053	16.02201	0.197034	4.05034	**21.75**
Chiro-5	62.0%	1.42281	30.31431	1.63791	4.5386	**37.914**
Chiro-6	59%	0.53686	<LOQ	0.10160	4.2637	**4.9021**
Chelenko-1	59.6%	0.92819	31.45239	1.17258	19.783	**53.3361**
Chelenko-2	59.2%	ND	ND	0.08458	2.2748	**2.35935**
Chelenko-3	57.8%	0.16713	14.58428	0.3241	6.831	**21.9065**
Chelenko-4	58.8%	ND	ND	0.10203	3.7211	**3.8231**
Chelenko-5	62%	ND	ND	0.11611	4.5386	**4.6547**
Chelenko-6	60%	ND	ND	0.05743	2.161	**2.2184**
Haromaya-1	61%	ND	ND	0.02132	<LOQ	**0.0214**
Haromaya-2	59%	ND	ND	<LOQ	<LOQ	**<LOQ**
Haromaya-3	62%	ND	<LOQ	<LOQ	<LOQ	**<LOQ**
Haromaya-4	61.5%	ND	ND	ND	<LOQ	**<LOQ**
Haromaya-5	62%	ND	ND	ND	<LOQ	**<LOQ**
Haromaya-6	61%	ND	<LOQ	<LOQ	<LOQ	**<LOQ**

Harar-1	63%	<LOQ	ND	ND	ND	**<LOQ**
Harar-2	59%	ND	ND	<LOQ	<LOQ	**<LOQ**
Harar-3	58%	ND	ND	0.1014	<LOQ	**0.1014**
Harar-4	60%	ND	<LOQ	<LOQ	<LOQ	**<LOQ**
Harar-5	61%	ND	ND	0.00396	<LOQ	**0.00397**
Harar-6	63%	ND	ND	0.07685	<LOQ	**0.0769**
Babile-1	60.7%	1.00127	11.37460	0.97793	27.769	**40.1214**
Babile-2	59%	4.77743	17.32721	1.57953	12.798	**31.706**
Babile-3	58%	1.14991	<LOD	0.79242	7.222	**9.16450**
Babile-4	62%	6.48333	7.82061	1.35548	10.95	**20.1261**
Babile-5	58%	1.92217	11.90042	2.22015	16.24	**30.3605**
Babile-6	61%	1.75224	6.96633	1.26833	11.884	**21.871**
Total		**25.69007**	**172.6627**	**12.1908**	**139.025**	**335.384**
Average		**0.856335**	**5.75542**	**0.407**	**4.6342**	**11.17945**
STD		**1.929**	**8.8024**	**0.7030**	**7.373**	**11.7284**

Where, MC- moisture content, ND- None detected, < LOD less than limit of detection, < LOQ less than limit of detection

Table 10: Percent of contaminated sorghum injera sample above the safe limit as per the EU and FAO food safety guidelines for direct human consumption

Location in sites	**Sample size**	**% of samples above Aflatoxin-safe levels***	
		Above 2 ppb	**Above 30 ppb**
Location1	6	66.66%	16.66%
Location 2	6	100%	16.66%
Location 3	6	Nil	Nil
Location 4	6	Nil	Nil
Location 5	6	100%	50%
Total	30		
mean		53.33%	16.66%

Table 11: Summary of total aflatoxin in Sorghum injera samples by HPLC analysis

Location	Number of samples	Aflatoxin total	Aflatoxin(PPB) (Mean±SD)	Contamination range
Chiro	**6**	**4 (66.67%)**	**15.58±16.21^{a}**	**4.90-28.96**
Chelenko	**6**	**6 (100%)**	**14.71±20.35^{ab}**	**2.21-53.33**
Haromaya	**6**	**1 (16.67%)**	**0.03±0.01^{ab}**	**0.021**
Harar	**6**	**2 (33.33%)**	**0.03±0.04^{ab}**	**0.07-0.1**
Babile	**6**	**6 (100%)**	**25.56±10.81^{b}**	**9.16-40.12**
Total	**30**	**19 (63.33%)**		**0.07-53.33**

*EU Maximum Tolerated Intake Level for direct human consumption (< 2ppb); FAO unfit limit of unprocessed samples for human consumption (> 30ppb)

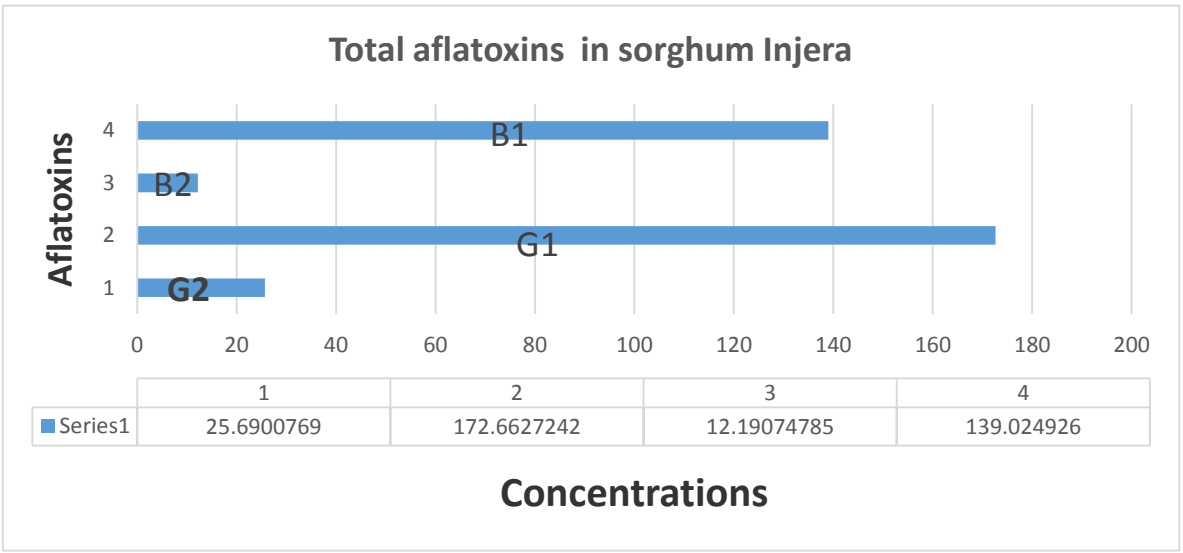
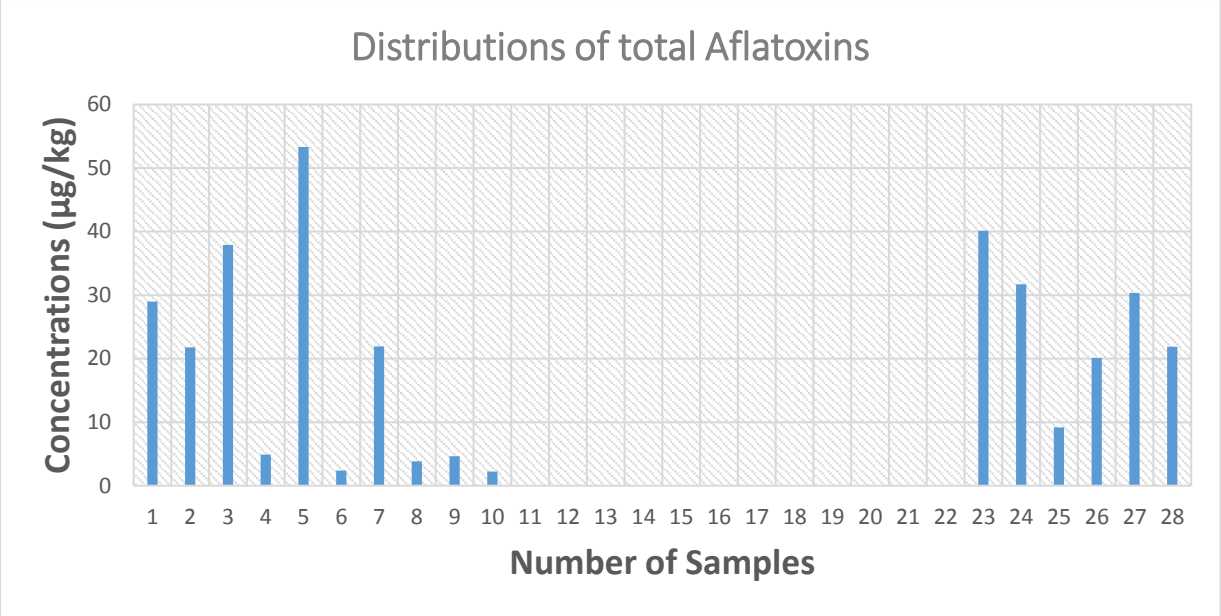


Figure 13: Chart shows the concentrations of total aflatoxins in sorghum injera sample



Where: 1-6 chiro, 7-12 chelenko, 13-18 Haromaya, 19-24 Harar, 25-30 Babile

Figure 14: Distributions of total aflatoxins in the total sampled analyzed

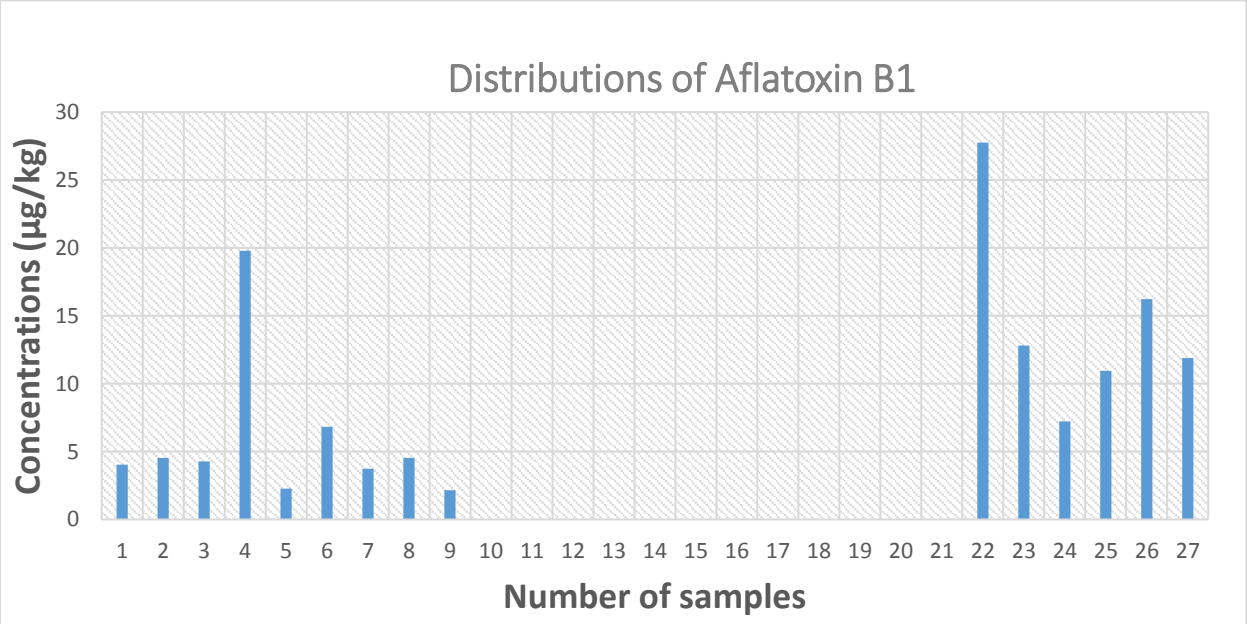


Figure 15: Distributions of AFB1 in the whole sample analysed

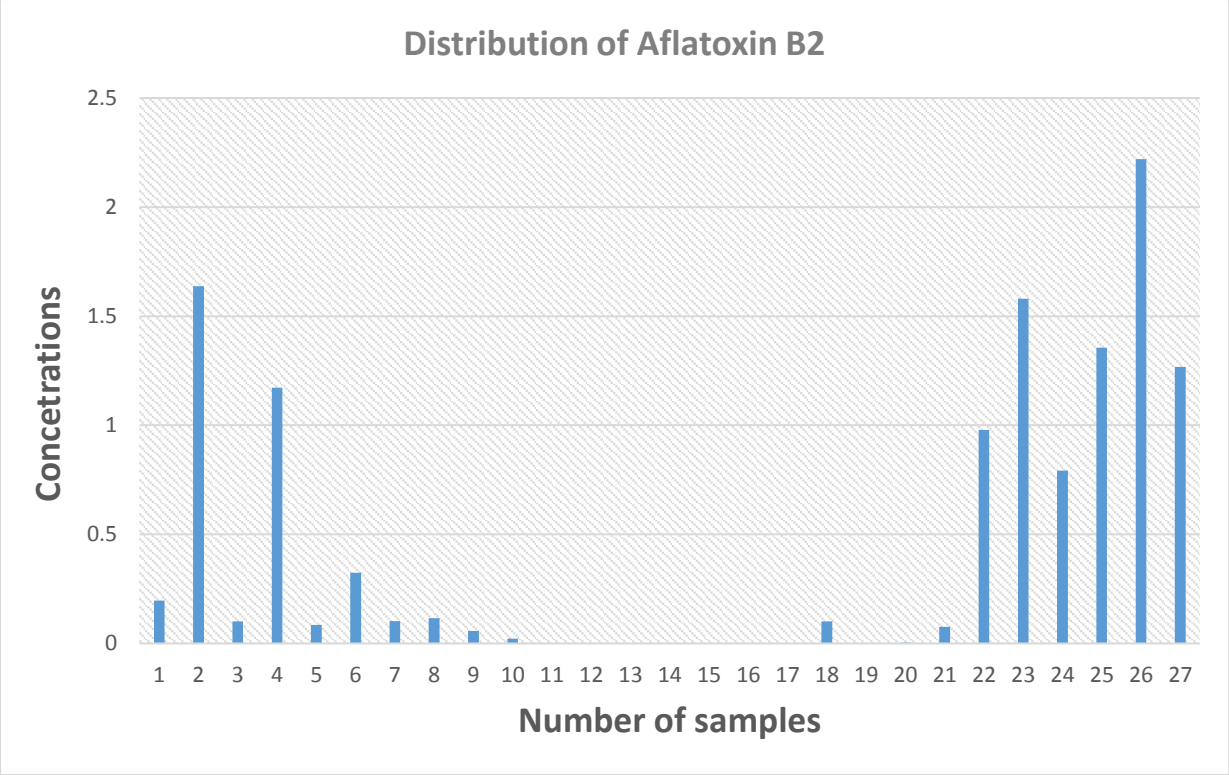


Figure 16: Distribution of Aflatoxin B2

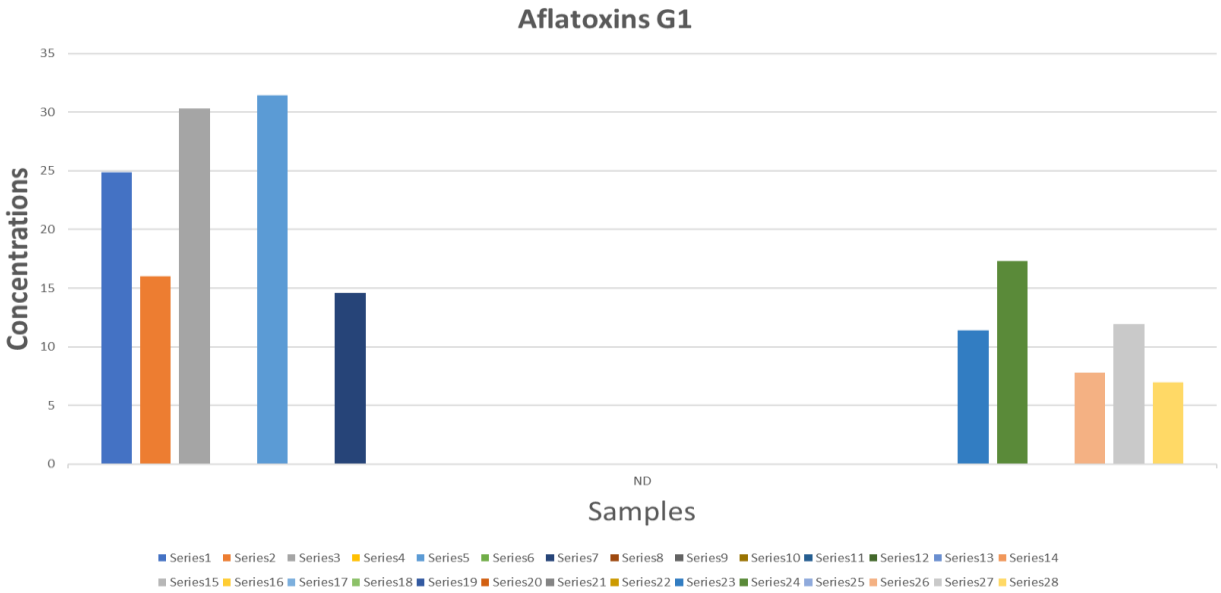


Figure 17: Distribution of Aflatoxin G1

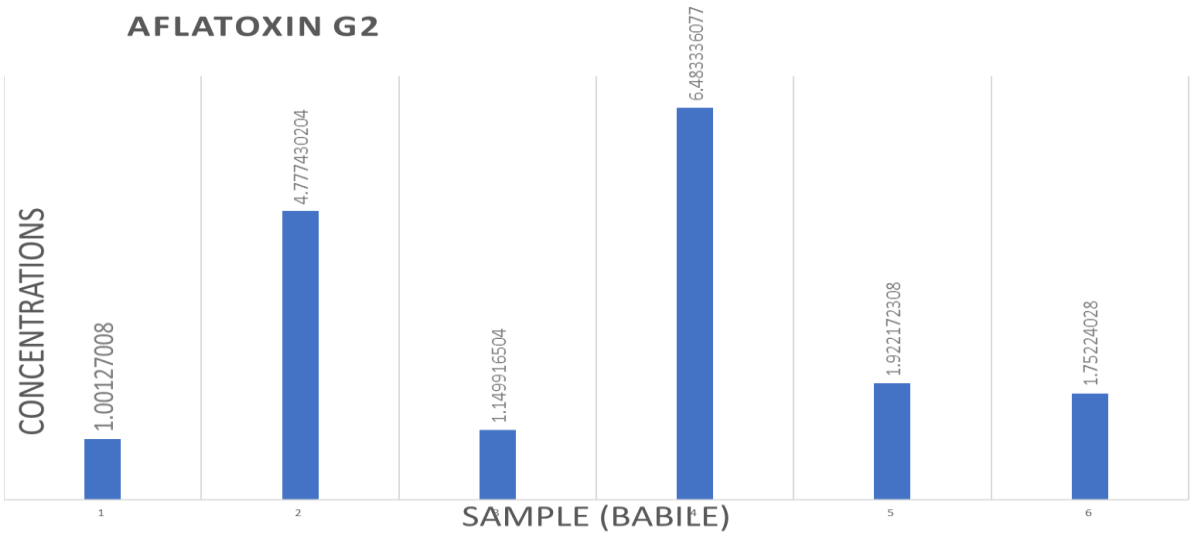


Figure 18: Distribution of Aflatoxin G2

4.3 Summary of Aflatoxins KAP Among Householders, retailers and Farmers

Statement		Response		
Knowledge		**Response**	**Frequency**	**Percentage**
1.	Knowledge on mold growth and formation of mycotoxin.	Yes	7	23
		No	23	67
2.	Knowledge on Conditions that favourable for mold growth on stored Sorghum	Yes	6	20
		No	24	80
3.	Do you know the effect of mold contamination with on Sorghum grain	Yes	2	7
		No	28	93
4.	Knowledge how to store Sorghum grain to prevent molding	Yes	9	30
		No	21	70
5.	Ability to differentiate contaminated Sorghum grain from non-contaminated	yes	23	77
		No	7	23
6.	Knowledge on the use of Underground pit for molding of stored grain	Pit	30	100
		Gotera	0	0
7.	Knowledge on the risks associated with consumption of contaminated food with mycotoxins	Yes	0	0
		No	30	100
Practice				
1.	Cleaning and drying of the storage area, prior to Store Sorghum grain.	yes	9	30
		No	0	0
2.	Post storage activities like visual inspection, Moisture, pest control and ventilation	yes	22	73.3
		No	8	26.7
3.	For how long did you stored Sorghum grain	<1 year	21	70
		>1year	9	30
4.	Did you mix your Sorghum grain with other grains?	yes	0	0
		No	30	100
5.	For How long did you ferment your sorghum flour dough??	1 day	12	40
		2 days	18	60
		3 days	0	0
6.	Cleaning, drying of Sorghum grain before milling	Yes	30	100
		No	0	0
7.	Where did you got Sorghum grain to make Injera??	Farm	9	30
		Market	21	70
Attitude				
1.	Attitude on consumption food contaminated with mold on health Impacts	yes	5	17
		No	25	83
2.	Inadequate Sorghum storage on mold growth	yes	28	93.33
		No	2	6.66
3.	Cleaning of dough preparation utensils before making Injera	yes	30	100
		No	0	0
4.	Pre-and post-harvest precaution for mold deterioration of Sorghum grain	Yes	9	30
		No	21	70
5.	What is the color of contaminated	colored	30	100

	Sorghum grain and what it looks like?	Noncoloured	0	0
6.	How do know deteriorated sorghum grain because of storage	By color	8	27
		By Odor	6	20
		Odor and color	16	53

4.4 Discussion

All sorghum Injera samples contained detectable concentration of aflatoxins. Most of the samples contaminated by aflatoxins were found to fall in the range 0-20 µg/kg, some in 21-40 µg/kg, and 2 samples of each were contaminated by total aflatoxins found >40 µg/kg. The maximum concentration of aflatoxin in sorghum injera was 53.33µg/kg with an overall mean of 11.2 µg/kg (Table 9).

Results presented in Table 9 indicate 66.667% of Sorghum Injera analysed were quantified with total aflatoxin B1, B2, G1 and G2 above lower limit of quantification and there were no quantified aflatoxins in 33.33% sample analysed was below lower limit of quantification moreover; 10 samples were not safe for human consumption according to U.S. Food and Drug Administration action levels for total aflatoxins in food (20 µg/kg).

Of the 30 samples of injera analysed, 12.40%, 33.3%, 63.3% and 50% were contaminated with quantifiable concentrations of aflatoxin G2, G1, B2 and B1 respectively. Aflatoxin B2 (63.3%) and B1(50%) concentrations were higher than Aflatoxin G1 and G2. According to cancer research center aflatoxin B1 is the top ranked carcinogenic chemicals, its average concentration was 4.63 µg kg-1, and 50% of sorghum injera analysed was not safe for human consumption in accordance with European Union for aflatoxins in human food (2 µg kg-1 for AFB1).

As there are no standards set in Ethiopia, the aflatoxin concentration levels of sorghum Injera samples collected from the East Ethiopia were evaluated and compared against all the three limits, FDA, EU and FAO safety guidelines. Accordingly, the comparison reveals that from the total samples analyzed, 33.33% and 53.33% were unsafe for direct human consumption as per the FDA and the EU maximum tolerable intake level respectively and on the basis of the FAO maximum tolerable intake level, 16.66% of the samples exceeded the 30 PPB limit.

The level of total Aflatoxins in Sorghum Injera sample from five locations mentioned above, collected from Chiro and Babile were Significantly different ($P<0.005$). Level of aflatoxins from sample Chiro, chelenko, Haromaya, and Harar not significantly different and Babile, Harar, Haramaya,and chelenko were not significantly different.

By all standards, the qualities of Sorghum Injera samples were in general low. The average concentration for the total aflatoxins samples had over 3-fold of the EU maximum tolerable intake level (4 µg kg-1). This indicates that the likelihood of aflatoxin exposure to humans in the region is high, where the frequency of Sorghum injera consumption is relatively high.

According to Mycotoxin survey done in FAO/WHO mycotoxins in sorghum Project, among the Samples collected from Major Sorghum growing Districts: Chiro, Haramoya, Babile, Harar were more affected by mycotoxins for which storage structure was underground pit. In each round of Survey Significant number of Individual aflatoxins were analysed in addition to other existed mycotoxins. According to Isolated Aflatoxins in Sorghum grain in Round one, two and three Surveys AFG1, AFG2, AFB1, AFB2 on average contaminant level in PPB were (18.38, 8.3, 63.00, 3.95), (32.53, 16.00, 16.41, 4.05), (14.8, 7.2,30.33,42.8) respectively (Emana and Nigussie, 2015). The Current study will support researcher findings in terms occurrence of Aflatoxins in Sorghum based Food in the country's major sorghum growing districts.

(Geremew *etal.,* 2016) reported on aflatoxin levels in Sorghum grain stored for different period and storage system in kewet districts, Northern shewa, Ethiopia, from the sample analysed 96.66%, 93.33%, 96.7% and 90% were contaminated by aflatoxin B1, B2, G2 and G1 respectively. Total aflatoxin contamination was ranged from11.44µg/Kg to 344.26µg/Kg and the mean total aflatoxin level was 123.85µg/Kg. In this study, significant aflatoxins were contaminated sorghum grain because of poor underground storage practice by farmers. These results revealed the widespread occurrence of aflatoxigenic strains of A. flavus, A. parasaticus and A. niger in the region, and highlight the importance of the post-harvest care of grains.

In this existing study from all sample analysed 50%,63.3%,33.3% and 12.40% were contaminated with AFB1, AFB2, AFG1 and AFG2 respectively and 11.17µgKg^{-1} average total aflatoxin was found in sorghum injera and such result was in line with the Geremew's study in terms of detection of aflatoxins in sorghum based food; though several food processing steps have been done to come up to injera however; the contamination level was much smaller than the Geremew's result. Although, the sample were collected at different site, such results discovered that relatively high contamination of sorghum grain comparing

to sorghum product, Injera. Therefore, some food processing methods have been shown to result in reduction aflatoxins (Mutungi et al., 2007). Sorting, cleaning, trimming, dehulling, milling, fermentation and thermal processing (baking) are the steps to process sorghum grain to sorghum injera and such stepwise process has a positive impact on the reduction of aflatoxin levels (Bullerman B. & Bianchini A., 2007).

Milani J. *et al*, 2014 found that fermentation and baking had a great effect in the reduction of aflatoxin in the study conducted to observe the effect of bread making process on aflatoxins levels changes. The researcher also emphasized that reduction of aflatoxin during heat processing depends on the moisture content of the product and he found 85% reduction in aflatoxin was recorded following heat treatment at 100°c for 2 hours when the meal moisture content was 30% and another temperature-time combination was studied with the 50% reduction in aflatoxin with 6.6% moisture content. Shapira and paster sited by Milani J., etal, 2014 reported that in the light of data accumulated from several studies on the effect of moisture content on aflatoxin degradation, it can be concluded that degradation is enhanced at high moisture content. Different research revealed that, there are three different methods to made injera: traditional (wood), electric and renewable methods. With all methods to be able to make injera in the right way, the baking plate be 220°c.

According to the report made by Milani J.*etal*, 2014 heat treatment at 100°c for 2 hours to a meal with 30% moisture content found 85% reduction in aflatoxin. This result agreed with this study in terms of injera baking temperature (≈220°c) and injera moisture content (58%-64%) which is enough to aflatoxin degradation.

Other worker (Oluwafemi, 2014) fermentation of maize for a minimum of three days is a relatively effective form of detoxification method that can reduce aflatoxin by 50% that lactic acid bacteria are known to detoxify aflatoxin in fermented foods. The antiaflatoxigenic effect of lactic acid bacteria might also be because of the secretion of bacteriocins (Yurdugul and Bozoglu, 2002) and some other metabolites such as hydrogen peroxide.

The responses collected from questionnaires regarding to attitude, practice and knowledge on aflatoxin contamination, storage and handling sorghum grain by respondents in this study clearly showed that most participants do not identify fungal contamination of grains until

there are obvious signs of spoilage such as discoloration, insect infestation or rotting. Most, respondents did not know of aflatoxin let alone its harmful health effects. Given the level of poverty and the lack of awareness of the health effects of consuming aflatoxin contaminated foods, the amounts of contaminated injera made from the grain sorghum consumed are likely to be very high.

Based on the information collected in the sample collection site through questionnaires for retailers who are not farmer their sorghum injera was made from sorghum grain either directly purchased from local market or grain storage warehouses owned by local merchant and those who are farmer made injera from the grain they harvested and stored in the pit. From the total 30 sample injera, for twenty-one of the sample in the districts the grain was directly purchased from the market and nine of householders was farmers in which the sample was collected. Though in the region to increase sensory attributes, Injera is made of sorghum flour mixed with tef flour or maize flour however; information gathered from questionnaires implementation revelled that, the sampled injera were made only sorghum flour. Most of the householders and retailers fermented their dough for 2 days and a day. Farmers in the districts stored their sorghums in the traditional underground pits that were cone-shaped. The underground pit used in Hararghe was found to be unsuitable for safe grain storage.

The respiration by organisms associated with the grain and the migration of moisture from the surrounding soil walls into the grain was the main cause for elevations of the carbon dioxide, relative humidity, and temperature levels inside the storage environment, even to the extent of the process called grain heating. In turn, the high temperature and the concomitant high relative humidity in the store reduce seed viability due to an increased degree of invasion by storage fungi. Severe invasion also leads to other tremendous quantitative and qualitative grain deterioration including moulding, mycotoxin production, offensive odour, rancidity, seed-cake and grain weight loss. Farmers in Hararghe are often tempted to reject a significant proportion of their grain due to moulding. The situation is intolerable since it has a direct link with shortage of grain and human starvation in the region. The sorghum grain loss due to moulding in Haromaya was estimated to range from 2 to 25% for full pits and 7 to 35% for half full storage pits (Gilman, 1968) sited by Dejen, 2004). Grain losses vary from one geographic location to another and from pit to pit, depending on original grain

conditions, season, and associated organisms. Losses could be even higher if aggravated by high initial grain moisture content, direct rainwater leakage into the store, pest infestation, and poor storage sanitation.

4.5 Study Limitation

The study had the following limitations because of financial challenge and resource inaccessibility.

1. The study didn't cover sufficient sample size and the samples were not collected from all districts of Eastern Ethiopia.
2. The sorghum Injera sample was not collected at different seasons
3. This study only looked at the level aflatoxin in Injera made from sorghum grain but not from the sorghum grain.
4. Such study didn't show the effect of baking, fermentation and the effect of heating temperature, fermentation time on the level of aflatoxins Experimentally.
5. Such Experiment not compared the level aflatoxin from sorghum flour and its sorghum injera after processing.

5 Conclusion and recommendations

5.1 Conclusion

Sorghum Provides one third of the cereal diet in Ethiopia and its flour is mainly used to make injera (pancake) for human consumption in Eastern Ethiopia. Poor pre-harvest and Post-harvest management, storage temperature and relative humidity of underground pit used for grain storage are conducive environment for moulding with toxigenic fungi. Aspergillus flavus and A. parasticus are the most common fungi species associated with sorghum contamination to produces aflatoxin B1, B2, G1 and G2 and these toxins are eventually consumed through sorghum injera which is made from improper harvested, stored and processed grain.

The results of current study demonstrated that 11.17µgKg^{-1} average total aflatoxin and 4.63 µgKg^{-1} for aflatoxin B1 were found in the ready to be consumed sorghum injera sample collected from householder and retailer, Eastern Ethiopia at a level much higher than EU standard. This indicates that the likelihood of aflatoxin exposure to humans in the region is high, where the frequency of Sorghum injera consumption is relatively high. As aflatoxins are associated with health risks, reducing their level in food stuff to a level accepted by international standard is paramount importance through good agricultural practice, appropriate pre- and post-harvest management, proper food processing practices to ensure the safety of these food stuff to consumers thereby preventing aflatoxin induced health risks.

5.2 Recommendations

- Using of agricultural extension workers as a tool to incorporate knowledge of the key critical control points during pre-harvest, harvesting, drying and storage stages in the cereal production chain are essential in developing effective prevention strategies.
- It is essential to aggressively follow that Good Agricultural Practice and operation approved supplier chains are in place.
- Using a safe and highly cost-effective biocontrol product developed by international institute of Tropical agriculture (IITA) called **aflasafe®** together with other good management practices.

- Establish aflatoxin awareness for regulatory authorities and policy makers to set standards and creation of awareness through awareness raising campaigns to institution, individuals and targeted education to farmers and value chain actors.
- Ministry of health/agriculture should establish regional serum based laboratories and low-cost diagnostic kits for urine and serum screening that can be used on-site at community, hospitals and health care systems and incorporate the idea as one of the strategies to prevent cancer into ‘’National cancer control plan of Ethiopia, 2016-2020’’ since aflatoxins are graded as number one carcinogenic substances by IARC.
- provide sensitization training in aflatoxin and food-borne disease diagnostics, prevention, and management for selected medical health professionals (midwives, nursing staff, physicians, paediatricians, obstetrician/gynaecologist doctors and community health workers), agriculture, and trade professionals.

- Mainstreaming of nutrition and food safety within existing structures and inclusion of information about aflatoxin in Ethiopian health extension package and include information about aflatoxin in celebrations of World Cancer Day.

- Future study should be done on the correlation between average daily intake of Aflatoxins or Aflatoxin levels in food sample and the incidence of liver cancer in eastern Ethiopia.

6 References

Agag, B. I. (2004). Mycotoxins in foods and feeds: Aflatoxins. *Association of Universal Bullettin of Environmental Research*. 7(1) :173-191.

Ahmadzadeh, F., Mirlohi, M., Madani, G. (2015). Reduction of aflatoxin M1 by Some Lactic Acid Bacteria and the Effect of pH and Temperature in Phosphate Buffer Saline Solution. *J Chem. Bio. Phy. Sci*.3: 2748-2755.

Alemayehu, C., Abdi, M., Amare, A. and Helge, S. (2013). Natural occurrence of Aflatoxins in ground nut (Arachis hypogaea L.) from eastern Ethiopia. *Food control.* 30:602-605.

Alemayehu, C., Wondimen, T., Amare, A., Rudolf, K., Micheal, S and Antonio (2014). Multimycotoxim analysis of sorghum (sorghum bicolour L.Moench) and finger Millet (Eleusine Coracana L.Garten) from Ethiopia. *Food control* 45:29-35.

Alex P.W, Wendiro D, Vuzi PC, Hawumba J.F, (2014). Methods for Detection of Aflatoxins in Agricultural Food Crops. *J. appl. chem.,* 11-15.

Alexandra C. W, Todd See, M., Jeff A.H, Yong B. K, Anna L. P., Teena F.M. and Sung Woo Kim, (2013). The Use of Feed Additives to Reduce the Effects of Aflatoxin and Deoxynivalenol on Pig Growth, Organ Health and Immune Status during Chronic Exposure. *Toxins 5* :1261-1281.

Allameh, A., Safamehr, A., Mirhadi, S.A.M., Shivazad, M., Razzaghi-Abyaneh, M. & Afshar- Naderi, A. (2005). Evaluation of biochemical and production parameters of broiler chicks fed ammonia treated aflatoxin contaminated maize grains. *Animal Feed Science Technology* 122 (3): 289-301.

Amar, R., Noureddine, B., Salim, M., Flourence, M. and Ahmed, L., (2010). Aspergillus section flavi and aflatoxin in Algerian wheat and derived products. *Food and chemical Toxicology.* 48: 2772-2777.

Amare A, (2010). Mycotoxins and surface & internal fungi of maize from Ethiopia: *African Journal of food, agriculture Nutrition& development* 10(9): 4109-4123.

Amezqueta, S., Gonzalez-Penas, E., Murillo, M., Lopez de Cerain, A., (2005). Occurrence of

ochratoxin A in cocoa beans: Effect of shelling. *Food Addit Contam*; 22(6): 590–96.

Angele N. T, Paul, F.M &Fe´licite´, M. T., (2010). Aflatoxin contamination in food and body fluid in relation to malnutrition and cancer status in Cameroon. *Int.J.Environ.Res.Public health*, 7:178-188.

Anna Chiara Manetta, (2011). Aflatoxins: Their Measure and Analysis, Aflatoxins Detection, Measurement and Control 6:93-108

Atmac, E, GÜvenc, D., AKsoy, A., (2015). Determination of Aflatoxin levels in maize by High performance chromatography using an immunoaffinity column clean Up. *Kafkas Univ vet Fak Derg*. 21(4):619-622.

Awika, J. M., and Lloyd W. R., (2004). Sorghum phytochemicals and their potential impact on human health. *Phytochemistry*. 65(9): 1199-1221.

Bennett, J.W., Kale, S and Juijiang Y., (1994). Aflatoxins: Background, Toxicology and molecular biology. Infections disease: Food borne diseases. 13:355-373.

Bilal T, Aksakal DH, Sünnetci S, Keser O and Eseceli, H, (2014). Detection of aflatoxin, zearalenone and deoxynivalenol in some feed and feedstuffs in Turkey. Pak Vet J, 34(4): 459-463.

Blandino A., Al-Aseeri, M.E, Pandiella, J.S, Cantero, D., Webb, C. (2003). Cereal based fermented foods and beverages. *Food research International*. 36: 527-543.

Brera, C., Debegnach, F., Grossi, S., Miraglia, M., (2004). Effect of industrial processing on the distribution of fumonisin B1 in dry milling corn fractions. *Journal of Food Protection.* 67 (6):1261–1266.

Brhane Gebrekidan & Belaynesh Gebrehiwot. (1981). Sorghum injera preparations and quality parameters. In Proceedings of the International Symposium on Sorghum Grain Quality, 21-31 October, 1981. *ICRISAT*. pp. 55-65.

Bucheli, P.,Taniwaki, M.H., (2002). Review Research on the origin, and on the impact of post-harvest handling and manufacturing on the presence of ochratoxin A in coffee. *Food Addit Contam*.19(7): 655–65.

Bullerman, L.B, Bianchini, A., (2007). Stability of mycotoxins during food processing. *International journal of food microbiology* .119: 140-146.

Coppock, W.R. & Christian, R.G., (2007). Aflatoxins, In: *Veterinary Toxicology – Basic and Clinical Principles*. pp. 939-950.

Cotty, P. J., Bayman, D. S., Egel, D. S., & Elias, K. S., (1994). Agriculture, aflatoxins and Aspergillus. In K. A. Powell, A. Renwick, & J. F. Peberdy (Eds.), The genus Aspergillus: From taxonomy and genetics to industrial applications (pp. 1–27). New York: Plenum Press.

Darwish, W.S, Ikenaka Y, Nakayama, M.M, Ishizuka, M., (2014). An overview in mycotoxin contamination of foods in Africa. *J.vet.med.sci*.76(6):789:797.

Dejene M, (2004). Grain storage methods and their effects on sorghum grain quality in Hararghe, Ethiopia. *Department of Ecology and Crop Production Science, Uppsala (PHD thesis).*

Dereje, A., Muez, T., Helges, (2012). Natural Occurrence of toxigenic Fungi Species and Aflatoxin in freshly Harvested groundnut kernels in Tigray, Northern Ethiopia. *Journal of the Drylands* 5(1):377-382.

Diao, E., Dong, H., Hou, H., Zhang, Z., Ji, N. & Wenwen, M., (2015). Factors Influencing Aflatoxin Contamination in Before and After Peanuts: A Review. *Journal of Food Research.* 4 (1).

Diedhio, P.M, Ba, F., Kane, A., Mbaye, N., (2012). Effect of different cooking methods on aflatoxin fate in peanut products. *African Journal of Food Science and Technology* 3(2):53-58.

Emana G and Nigussie R, (2015). Mycotoxin Analysis of sorghum grain Samples collected

from Major Sorghum Growing districts of Ethiopia. FAO/WHO Mycotoxins in Sorghum Project. Unpublished

Enes, A., GÜVENÇ, D., AKSOY, A., (2015). Determination of Aflatoxin Levels in Maize Grain by High Performance Liquid Chromatography Using an Immunoaffinity Column Cleanup**.** *Kafkas Univ Vet Fak Derg* 21 (4): 619-622.

Eva G. Lizárraga-Paulín, Ernesto Moreno-Martínez and Susana P. Miranda-Castro, (2011). Aflatoxins and Their Impact on Human and Animal Health: An Emerging Problem, Aflatoxins. *Biochemistry and Molecular Biology.* 255-282.

Eva G. Lizárraga-Paulín, Susana Patricia Miranda-Castro, Ernesto Moreno-Martínez, Irineo Torres-Pacheco and Alma Virginia Lara-Sagahón. (2013). Novel Methods for Preventing and Controlling Aflatoxins in Food: A Worldwide Daily Challenge. *Biochemistry and molecular Biology*. PP 93-121

Eva G. Lizarraga-Paulin, Ernesto Moreno-Martinez and Susana P. Miranda-Castro, (2011). Aflatoxins and Their Impact on Human and Animal Health: An Emerging Problem, Aflatoxins - *Biochemistry and Molecular Biology*.

Fandohan, P., Zoumenou, D., Hounhouigan, D.J., Marasas, W.F., Wingfield, M.J, Hell, K. (2005). Fate of aflatoxins and fumonisins during the processing of maize into food products in Benin. *Int. J. Food Microbiol*. 98(4): 249–259.

Federal Ministry of health Ethiopia, (2015). National cancer Control plan, 2016-2020.

Food & Agricultural Organization of the United States (FAO), 1999. Sorghum: Post-harvest Operations.

Frida A, (2013). Potential of lactic Acid fermentation in Reducing Aflatoxin B and Fumonisim B1, Tanzanian maize-based complementary Gruel. *MSc thesis Michigan state university*

Gabriel, O.A &Puleng, L., (2013). Strategies for the prevention &Reduction of mycotoxins in developing countries. *INTECH mycotoxin and food safety in developing countries. pp 123-136.*

Geiser, D. M., Pitt, J. I., & Taylor, J. W., (1998). Cryptic speciation and recombination in the aflatoxin-producing fungus Aspergillus flavus. *PNAS*. 95: 388–393.

Geremew, TW., Tilahun BZ, Ashagrie ZW, Habtamu FG. (2016). Study on Aspergillus Species and Aflatoxin levels in Sorghum (Sorghum bicolor L) for different period and Storage system in Kewet Districts, North Shewa, Ethiopia. J Food Sci Nutr 3:010.

Gibbons, J. G., Salichos, L., Slot, J. C., Rinker, D. C., McGary, K. L., King, J. G., (2012). The evolutionary imprint of domestication on genome variation and function of the filamentous fungus Aspergillus oryzae. *Current Biology*. 22:1403–1409.

Gilman, G.A. (1968). Storage problem in Ethiopia with special reference to deterioration by fungi. *Reports of Tropical products institute*

Giniani Carla Dors, Sergiane Souza Caldas, Vivian Feddern, Renata Heidtmann Bemvenuti, Helen Cristina dos Santos Hackbart, Michele Moraes de Souza, Melissa dos Santos Oliveira, Jaqueline Garda-Buffon, Ednei Gilberto Primel and Eliana Badiale-Furlong. (2011). Aflatoxins-Contamination, Analysis and Control. Aflatoxins. Biochemistry and Molecular Biology. 20: 415-438.

Gobbetti, M., De Angelis, M., Corsetti, A., & Di Cagno, R., (2005). Biochemistry and physiology of sourdough lactic acid bacteria. *Trends in Food Science & Technology, 16*(1):57-69.

Gonc¸alves, S. S., Stchigel, A. M., Cano, J. F., Godoy-Martinez, P. C., Colombo, A. L., & Guarro, J., (2012). Aspergillus novoparasiticus: A new clinical species of the section Flavi. *Medical Mycology*. 50:152–160.

Gong, Y.Y, Hounsa, A., Egal, S., Turner, P.C, Sutcliffe, A.E, Hall, A.J, Cardwell KF, Wild, CP., (2004). Post-weaning exposure to aflatoxin results in impaired child growth: A Longitudinal study in Benin, West Africa. *Env. Health Persp*.112: 1334-1338.

Groopman, J. D., Kensler, T. W., & Wild, C. P. (2008). Protective interventions to prevent

aflatoxin induced carcinogenesis in developing countries. *Annu Rev Public Health*. 28:187-203.

Haiyan, Z., Shuai, W., Ying, X., &Mingtao,F., (2015). Reduction of aflatoxin B1 in peanut meal by extrusion cooking. *LWT_Food science and technology*. 64:515-519.

Hamide, Z., John,G., (2010). Immunoaffinity column clean Up techniques in food analysis: A review. Journal of chromatography B. 878:115:132.

Hamidou F, Rathore A, Waliyar F & Vadaz V (2014). Although drought intensity increases aflatoxin contamination, drought tolerance does not lead to less aflatoxin contamination. *Field crops research* 156: 103-593.

Hell, K. & Mutagi, C. (2011). Aflatoxin control and prevention strategies in key crops of sub-Saharan Africa. *African journal of microbiology Research*.5(5):459-466.

Hell, K., Fandohann, P., Bandyopadhay, R., Kiewnick, S., Sikora, R., and cottyP, (2008). Pre- and post-harvest management of Aflatoxin in maize. *An African prespective. Management of Aflatoxin in maize*. 219-249.

Hirut K, Abbas H, Fisher D, Bella Loui, (2012). Relationship between Aflatoxin contamination and physiological Response of corn plants under drought and heat stress. *Toxins* 4:1385-1403.

Houbroken, J., Deveries, R., Samson, R., (2014). Modern Taxonomy of Biotechnologically Important Aspergillus and Penicillium species. *Adv appl Microbiol* 86:199_249.

Hussain, A., Ali, J., Akther, S., Shafqatullah, (2011). Degradation of aflatoxins by roasting in contaminated peanuts. *Pak. J. Biochem. Mol. Biol*. 44(2): 56-59.

Hussaini, A., Timothy, A., Olufunmilayo, H., Ezekiel, A., Godwin, H., (2009). Fungi and some mycotoxins found in mouldy sorghum in Niger state, Nigeria. *World Journal of Agriculture sciences*.5(1):05-17.

Hwang, J.H, Lee, K.G. (2006). Reduction of aflatoxin B1 contamination in wheat by various cooking treatments. *Food Chem*. 98(1): 71–75.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. (2012). *Agents Classified by the IARC Monographs*. http://monographs. iarc.fr/ENG/ Classification/,1-105.

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. (2012). *A Review of Human Carcinogens: Chemical Agents and Related Occupations*, 100F, http://monographs.iarc.fr/ENG/Monographs/vol100F/mono100F-23.

Ian-Johnston., Rebeca, S., Cedic, R., Darrel, S, (2012). The fate of Aflatoxin in corn fermentation. *Natural resources.* 3:126-136.

IARC. International Agency of Research on Cancer. (1997). Evaluation of carcinogenic risks to humans: some naturally occurring substances: aromatic amines and mycotoxins. Lyon. *IARC Monographs*, 56. p. 245-39.

Idris Y.MA, Mariod, A.A, Elnour I.A, Mohammed A.A, (2010). Determination of Aflatoxin levels in Sudanese edible oils. *Food and chemical toxicology* 48:2539-2541.

Jafar, M.M, Seyed, S.N., Gisso,M., Elmira, B., Elmira, P.B. (2014).Effect of Bread making process on aflatoxins levels changes. *Journal of chemical health risks*.4(4):1-7.

Jin, H.D., Dong-kug, C., (2007). Aflatoxins: Detection, Toxicity &Biosynthesis. *Biotechnology & Bioprocess Engineering*. 12:585-593

JiujiangY & Kenneth, C (2004). Aflatoxins-Biosynthetic pathway genes. *USDA/ARS, Southern Regional Research center, New or leans, Louisiana, USA.*

John, L.R. (2007). Some major mycotoxins and their mycotoxicoses: an overview. *International Journal of Food Microbiology*, Vol.119, No.1-2, pp. 3-10.

Jonathan, H.W, Timothy D.P, Pauline E.J., Jonathan, K.S., Curtis, M.J., and Deepar, A (2004). Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences and interventions. *Am J clin Nutr*.80:1106-22.

Jong-Gyu K (2007). Anti-aflatoxigenic activity of some bacteria related with fermentation. *Communicating current research &Educational Topics and trends in applied microbiology*.322-328.

Kabak, B., Dobson, A.D. (2009). Biological strategies to counteract the effects of mycotoxins. *J. Food Prot.* 72: 2006-2016

Kabak B, Dobson ADW, Var I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: A review. *Crit. Rev. Food Sci. Nutr*. 46(8): 593-619.

Kange, M., Cheruiyot ,K., Ogend, O. and Arama F., (2015). Effect of sorghum (*Sorghum bicolor* L. Moench) grain conditions on occurrence of mycotoxin producing fungi. *Agriculture and food security*. 4:15

Kok, W.T, van Neer, T.C., Traag, A., and L. G. T. Tuinstra,L.G.(1986). Determination of aflatoxins in cattle feed by liquid chromatography and post-column derivatization with electrochemically generated bromine. *Journal of Chromatography A.* vol. 367: 231–236.

Lee, C. Z., Liou, G. Y., & Yuan, G. F. (2006). Comparison of the aflR gene sequences of strains in Aspergillus section Flavi. *Microbiology*.152:161–170.

Malviya, R., Bansal,V.,Pal O.P, and P. K. Sharma,P.K,(2010).High performance liquid chromatography: a short review. *Journal of Global Pharma Technology*. 2(5) :22–26.

Mansooreh, M. (2009). Determination of aflatoxins in imported rice to Iran. *Food and Chemical Toxicology.* 47: 2064–2066.

Mansour, K., Azita, M., Ferzaneh, M. (2013). Determination of aflatoxin in the imported Rice in zahedan, south East of Iran. Health scope 2(3): 125-9.

Maryam, J. (2015). A review on aflatoxins Reduction in food. Italian Journal of Health, safety and Environmental. 3(1):445-459.

Mcdaniel, A., Holmes, P., Williams, K., Armbrust, L., Sparks,D and Brown, A. (2011). Effect of Matrix Clean-Up for Aflatoxin Analysis in Corn and Dried Distillers Grains. *Natural Resources*. 2: 250-257.

Mendez-Albores, A., De Jesus-Flores, F., Castaneda-Roldan, E., Arambula-Villa, G., Moreno- Martinez E., (2004). The effect of toasting and boiling on the fate of B-aflatoxins during pinole preparation. *J. Food Engin*. 65(4): 585.

Mendez-Albores,A., Martinez-Sustos, F,, Gaytan-Martinez, M.& Moreno-Martinez,E. (2008). Effect of Lactic and citric acid on the stabilityof B-aflatoxin in extrusion-cooked sorghum. *Applied microbiology,letters in applied microbiology* 47:1-7.

MehdiRazzaghi A, (2013). Aflatoxins Recent Advances and future approaches. *Mehdi Razzaghi- Abayneh*

Mohammed-Hasseni, F., Mirlohi, M., Mosharraf, L., &Hasanzade, A., (2016). Occurance of aflatoxins in wheat flour specified for sangak bread and its reduction through fermentation and baking. *Quality assurance and safety of crops and Foods.* pp 1-8.

Mukthar,M., Farooq,.Z, and Manzoor,M.(2016). Determination of Aflatoxins in superkernel Rice Type consumed in different regions of Punjab, Pakistan. *The journal of Animal and plant science.*26 (2):542:548.

Mutangi, C., Lamuka, P., Arimi, S., Gathumbi, J., Onyango, C., (2008). The fate of Aflatoxins during processing of Maize in to Muthokoi-A Traditional Kenyan food. *Food control* 19:714-721

Mokoena, M.P, Chelule, P.K, Gqaleni, N (2016). The toxicity and decreased concentration of

aflatoxin B1 natural lactic acid fermented maize meal. *Journal of applied Microbiology* 10:773-777.

Narash M and David A (2007). Postharvest control strategies: *Minimizing mycotoxins in the food chain. International Journal of food microbiology*.119 (1&2):131-139.

Oluwafeni F, (2004). Fate of aflatoxin in cereals and cereals products during processing. *Journal of food, agriculture and Environment.* 2(3&4):57-60.

Olumafemi, F and Ikeowa, M.C, (2008). Length of fermentation and detection methods of Aflatoxin B1 During fermentation of maize in to 'Ogi'. *Nigerian Journal of microbiology* 22(1):1698-1703.

National Library of Medicine, (2002). Aflatoxins Hazardous Substance Data Base. *Toxnet (National Data Network)*.

Nidhine, N., Bhavya, M.L, Bhaskhar N, Muthukumar, S.P,&Murthy P.S, (2016). Aflatoxin production by Aspergillus in rumen liquor and its implications. *Food control.* 71: 26-31.

Park, D.L., (2002). Effect of processing on aflatoxin. Advances in Experimental *Medicine and Biology*. 504:173–179.

Pearson, S.M, Candish, A.A, Aidoo, K.E, Smith, J.E (1999). Determination of aflatoxin levels in Pistachio and cashew nuts using immunoaffinity column clean up with HPLC & fluorescence detection. *Biotechnology techniques* 13:97-99.

Park, J.W., Kim, Y.B., (2006). Effect of pressure cooking on aflatoxin B1 in rice. *Journal of Agricultural and Food Chemistry*.54: 2431–2435.

Li,P., Zhang, Q., D. Zhang ,D., (2011). Aflatoxin measurement and analysis. *Aflatoxin Detection, Measurement and Control*. pp. 183–208.

Rahmani, S. Jinap and Soleimany,F., (2009). Qualitative and quantitative analysis of

mycotoxins. *Comprehensive Reviews in Food Science and Food Safety*, vol. 8(3): 202–251.

Rajarajan P. N, Rajasekaran &Ashadev, N.K., (2013). Aflatoxin contamination in agricultural commidities. *Indian J.pharm.Biol.Res*.(1-2):131-139.

Reddy KRN, Salleh B, saad B, Abbas HK, Abel CA, & Shier, W.T, (2010). An overview of mycotoxin contamination in foods & its implications for human health. *Toxin Reviews.* 29(1): 3-26.

Reddy, U.M, Rani, P.C. (2004). Effect of processing on detoxification of aflatoxins in maize. *Indian J. Nutr. Diet*. 43(1): 54-59.

Salomon S, Christopher J, Daniel K, (2011). A signal-to-Noise Crossover Dose as the point of departure for health risk Assessment. Environmental health perspectives. 119 (12):1766-1774.

Samson, R. A., Yilmaz, N., Houbraken, J., Spierenburg, H., Seifert, K. A., Peterson, S. W., (2011). Phylogeny and nomenclature of the genus Talaromyces and taxa accommodated in Penicillium subgenus Biverticillium. *Studies in Mycology*.70:159–183.

Schuster, E., Dunn-Coleman, N., Frisvad, J. C., & van Dijck, P. W. (2002). On the safety of Aspergillus niger: A review. *Applied Microbiology and Biotechnology*.59: 426–435.

Scudamore, K.A., Banks, J.N., Guy, R.C.E., (2004). Fate of ochratoxin A in the processing of whole wheat grain during extrusion. *Food Additives and Contaminants* .21 (5):488–497.

Stroka, J., Von Holst, C., Anklam, E., Reutter, M., (2003). Immunoaffinity column clean- up with liquid chromatography using post-column for determination of aflatoxin B1 in cattle feed: Collaborative study. *Research Gate article in journal of AOAC international.*

Stroka, J., Anklame**,** E., Dr.Wierz , Dr. Jörissen, and John Gilbert (2000) . Immunoaffinity

Column Cleanup with Liquid Chromatography Using Post-Column Bromination for Determination of Aflatoxins in Peanut Butter, Pistachio Paste, Fig Paste, and Paprika Powder: Collaborative Study. *Food chemical contaminant*: journal of AOAC International vol. 83: 2.

Strosnider, H., Azziz-Baumgartner, E., Banziger,. Bhat, R.V, Breiman R,Brune MN, DeCock K, Dilley A, Groopman J, Hell K, Henry SH,Jeffers D, Jolly c, Jolly P, Kibata GN. Lewis L, Liu X, Luber G, McCoy L, Mensah P, Miraglia M, Misore A, Njapau H, Ong CN, Onsongo MTK, Page SW, Park D, Patel M, Phillips T, Pineiro M, Pronczuk J, Rogers HS, Rubin C, Sabino M, Schaafsma A, Shephard G, Stroka J, Wild C, Williams JT, Wilson D., (2006). Workgroup report: public health strategies for reducing aflatoxin exposure in developing countries. *Environ. Health Perspect*.114: 1898-1903.

Takino, M. and Tanaka, T. (2008) *Determination of Aflatoxins in Food by LC-MS/MS*, Agilent Technologies.

Thrasher, J. D. (2012). Aflatoxicosis in animals. *Aflatoxins and Health*, www.alphaboostjuice. com/AFLATOXICOSIS_IN_ANIMALS.pdf.

Tominaga, M., Lee, Y. H., Hayashi, R., Suzuki, Y., Yamada, O., Sakamoto, K. (2006). Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in Aspergillus oryzae RIB strains. *Applied and Environmental Microbiology*:72: 484–490.

Torres, P., Guzman-Ortiz, M., Ramirez-Wong B., (2001). Revising the role of pH and thermal treatments in aflatoxin content reduction during the tortilla and deep frying processes. J. Agric. Food Chem. 49(6): 2825-29.

Tripathi, S. & Mishra, H.N. (2010). Enzymatic coupled with UV degradation of aflatoxina B1 in red chili powder. *Journal of Food Quality*. 33 (s1): 186-203.

Unnevehr & Grace, (2013). Aflatoxins: Finding solutions for improved food safety. International food policy research, Institute, 2020 Focus.

USAID. (2012). Aflatoxin: A Synthesis of the Research in Health, Agriculture and Trade.

*Feed the Future: The Office of Regional Economic Integration USAID East Africa Regional Mission.*www.eastafrica.usaid.gov/esearch_in_Health_Agriculture_and_Trade 10-15.

Valentina, Yulina and Lyubomir, (2013). Aflatoxins analysis and risk management. Mintage Journal of pharmaceutical and Medical supplies. 2(4):1-6.

Van-Egmond, H.P.; Schothorst, R.C. & Jonker, M.A. (2007). Regulations Relating to Mycotoxins in Food: Perspectives in a Global and European Context. *Analitical and Bioanalytical Chemistry*. 389 (1):147-157.

Varga, J., Frisvad, J. C., & Samson, R. A. (2011). Two new aflatoxin producing species, and an overview of Aspergillus section Flavi. *Studies in Mycology*.69:57–80.

Wang, H. (1998). Structure-function relationships of human liver cytochromes 450A: aflatoxin B1 metabolism as a probe. *Biochemistry*, 37, 12536-12545.

Wild C.P and Turner P.C (20020. The toxicology of Aflatoxins as a basis for public health decisions. *Mutagenesis* 17(6): 471-481.

Williams, J.H, Phillips, T.D, Jolly, P.E, Stiles, J.K, Jolly, C.M, Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences and interventions. *Am. J. Clin. Nutr*.80: 1106-1122.

Xiang,Y., Liu, Y., and Lee, M.L. ,(2006). Ultrahigh pressure liquid chromatography using elevated temperature. *Journal of Chromatography A*. 1104(1-2). 198–202.

Yurdugul, S. and Bozoglu, F. (2002). Studies on an inhibitor produced by lactic acid bacteria of wines on control of malolactic fermentation. *Eur. Food Res. Technol*. 215(1): 38-41.

Zinedine, A. & Mañes, J. (2009). Occurrence and legislation of mycotoxins in food and feed from Morocco. *Food Control*. 20(4): 334-344.

Zinedine, A., Faid, M., & Benlemlih, M. O. (2005). In vitro reduction of aflatoxin B1 by

strains of lactic acid bacteria isolated from Moroccan sourdough bread. *International Journal of Agriculture and Biology*: *7*(1): 67-70.

Zorlugenç, B., Zorlugenç, F.K., Öztekin, S. & Evliya, I.B. (2008). The influence of gaseous ozone and ozonated water on microbial flora and degradation of aflatoxina B1 in dried figs. *Food and Chemical Toxicology*. 46(12):3593-3597.

Zucchi, T.D., Moraes, L.A. & Melo, I.S. (2008). *Streptomyces* sp. ASBV-1 reduces aflatoxin accumulation by *Aspergillus parasiticus* in peanut grains. *Journal of Applied Microbiology.*105(6): 2153–2160

Annex 1: Consent Form and Questionnaire, Amharic Version

በአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና ስነምግብ ትምህርት ክፍል በአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና ስነ-ምግብ ትምህርት ክፍል የአፍላቶክሲን ብከላ በማሽላ እንጀራ ላይ በተመለከተ ለሚደረግ ጥናት የተዘጋጀ መጠይቅ

1.ቃለ መጠይቅ የተካሄደበት ቀን _____/_____/_____

2.መለያ ቁጥር _____

3.የጠያቂው ስም_____ፊርማ_____

የሀላፊው ማረጋገጫ. ፊርማ _____ቀን_____/____/___

መግቢያ

ሰላምታ።

ስሜ _____ይባላል። እኔ ለአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና ስነ ምግብ ትምህርት ክፍል ለሚደረግ ጥናት መረጃ ሰብሳቢ ነኝ፡፡ እኛ እዚህ አካባቢ ለምግብ አገልግሎት በሚውለው የማሽላ እንጀራ ላይ የአፍላቶክሲን ብከላ በማሽላ እንጀራ ለሚደረግ ጥናት ቃለ መጠይቅና የማሽላ እንጀራ እየሰበሰብን እንገኛለን፡፡ አፍላቶክሲን ማለት ፈንግስ በተባለ በተለይም አስፓርጅን በተባለ የሚፈጠር መርዛማ ነገር ሲሆን በሰዎች ጤና ላይ ጉዳት የሚያመጣ ሲሆን በተጨማሪም በምርታማነት ላይ ጉዳት ያመጣል፡፡

የሚሰጡት መረጃ ሚስጥርነቱ ሙሉ ለሙሉ የተጠበቀ ነዉ። መጠይቁ ላይ የእርሰዎን ስም የሚገልፅ ማንኛዉም አይነት ነገር አይጠቀስም ወይም አይያያዝም፣ በመጠይቁ ወቅት የማይፈልጉትን ማንኛዉንም አይነት ጥያቄ መተዉ ወይም በማንኛዉም ሰዓት መጠይቁን ማቋረጥ ይችላሉ። ሆኖም ግን እርሰዎ የሚሰጡን እዉነተኛ መረጃ ወደ ፊት አፍላቶክሲን በማሽላ እንጀራ ላይ በተመለከተ ለሚያደርጉ ጥናቶች ጠቃሚ ይሆናል። ለዚህ ጥናት ለሚያደርጉልን ትብብር ምስጋናችን ከልብ የመነጨ ነዉ። ስለዚህ በመጠይቁ ለመሳተፍ ፈቃደኛ

ነዎት?_____

(የጥናቱን ተሳታፊ ሙሉ ፈቃደኝነት ያረጋግጡ የጥናቱ መረጃ ሰብሳቢ

ፊርማ……..)

ተቁ	ጥያቄ	መልስ	ይለፉ
1	ስለ ማሽላ ሻጋታ ስምተው ያውቃሉ?	1. አዎ…… 2. አላውቅም…….	
2	እባክዎ ቀለሙ ምን ዐይነት እንደሆነ ይግፁልን		
3	ማሽላ ከየት ያገኛሉ	1. ከገቢያ……. መልስዎ 1 ከሆነ ወደ	

		ተራ ቁጥር 11 ይለፉ 2. ከምርት……..	
4	አምራች ከሆኑ ምርትዎን ለርጅም ግዜ ስያስቀምጡ ችግር ገጥሞወት ያዉቃል	1. አዎ…… 2. አልነበረም……	
5	አዎ ካሉ ምን አይነት ?	1. ነፍሳት…… 2. አይጦች…….…… 3. ወፎች…….....……..…….. 4. ሻጋታ……..…… 5. ሌላ _____	
6	እነዚህን ለማጥፋት ምን ተጠቀሙ?	1. ምንም…….…… 2. ተባይ ማጥፍያ……..…… 3. ጭስ……..…… 4. ሌላ _____	
7	ምርተዎን ለማድረቅ ምን ይጠቀማሉ	1. ፀሀይ …….……. 2. የማድረቅያመሳርያ…….……..... 3. ሌላ _____	
8.	ምርተዎን የት ያስቀምጣሉ?	1. ጎተራ ዉስጥ…….. 2. መሬት ቆፍረዉ…….	
9	ምርተዎን ለርጅም ግዜ ከማስቀመጠዎ በፊት ቦታዉን ያፀዳሉ?	1. ሜዳ ላይ……..…… 2. ቤት ዉስጥ…….…….. 3. ከቤት አጠገብ…….…	
10	የማሽላ ምርተዎን ለምን ያህል ግዜ ያስቀምጣሉ?		
11	ተራ ቁጥር 3 መልስዎት አዎ ከ ገቢያ ከ ሆነ ምን ዓይነት ማሽላ ከገቢያ ይገዛሉ		
12	ማሽላዉን ከምን ጋር ቀላቅለዉታል?	1. ከጤፍ 2. ከበቆሎ 3. ከሩዝ 4. አልተቀላቀለም	
12	ከማስፈጨትዎት በፊት ማሽላዉን አበጥርዎታል (ታጥቧል)? መልስዎ አዎ ከሆነ በምን? እንዴት?		
13	በሚያስፈጩበት ወቅት ወፍጮዉ ምን ዕይነት እህል ተፈጭቶበታል ?		
14	የማሽላዉን ዱቄት ከማቡካቶት በፊት የማቡኪያዉ ዕቃዎች ተፀድተዋል?	1. አዎ 2. አይደለም	

15	ለምን ያህል ጊዜ ብኬቱን ያቆዩታል?		
16.	በአፍላቶክሲን የተበከለ ምግብ መመገብ በጤና ላይ ያለውን ጠንቅ ያዉቁታል;	1. አዎ 2. አይደለም	

Annex 2: Statistical analysis SPSS version 20.

Descriptive

The level of total aflatoxin was determined

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Chiro	6	15.5867	16.21886	6.62132	-1.4340	32.6073	.00	37.91
Chelenko	6	14.7100	20.35689	8.31067	-6.6533	36.0733	2.21	53.33
Haromaya	6	.0033	.00816	.00333	-.0052	.0119	.00	.02
Harar	6	.0307	.04642	.01895	-.0180	.0794	.00	.10
Babile	6	25.5550	10.81575	4.41551	14.2046	36.9054	9.16	40.12
Total	30	11.1771	15.42352	2.81594	5.4179	16.9364	.00	53.33

ANOVA

The level of total aflatoxin was determined

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2926.474	4	731.619	4.605	.006
Within Groups	3972.187	25	158.887		
Total	6898.661	29			

Multiple Comparisons

Dependent Variable: The level of total aflatoxin was determined

Tukey HSD

(I) Location where Enjera samples were collected for aflatoxin analysis	(J) Location where Enjera samples were collected for aflatoxin analysis	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Chiro	Chelenko	.87667	7.27753	1.000	-20.4965	22.2498
	Haromaya	15.58333	7.27753	.235	-5.7898	36.9565
	Harar	15.55600	7.27753	.236	-5.8172	36.9292
	Babile	-9.96833	7.27753	.652	-31.3415	11.4048
Chelenko	Chiro	-.87667	7.27753	1.000	-22.2498	20.4965
	Haromaya	14.70667	7.27753	.286	-6.6665	36.0798
	Harar	14.67933	7.27753	.287	-6.6938	36.0525
	Babile	-10.84500	7.27753	.578	-32.2182	10.5282
Haromaya	Chiro	-15.58333	7.27753	.235	-36.9565	5.7898
	Chelenko	-14.70667	7.27753	.286	-36.0798	6.6665
	Harar	-.02733	7.27753	1.000	-21.4005	21.3458
	Babile	-25.55167*	7.27753	.014	-46.9248	-4.1785
Harar	Chiro	-15.55600	7.27753	.236	-36.9292	5.8172
	Chelenko	-14.67933	7.27753	.287	-36.0525	6.6938
	Haromaya	.02733	7.27753	1.000	-21.3458	21.4005
	Babile	-25.52433*	7.27753	.014	-46.8975	-4.1512
Babile	Chiro	9.96833	7.27753	.652	-11.4048	31.3415

	Chelenko	10.84500	7.27753	.578	-10.5282	32.2182
	Haromaya	25.55167*	7.27753	.014	4.1785	46.9248
	Harar	25.52433*	7.27753	.014	4.1512	46.8975

*. The mean difference is significant at the 0.05 level.

The level of total aflatoxin was determined

Tukey HSD

Location where Enjera samples were collected for aflatoxin analysis	N	Subset for alpha = 0.05	
		1	2
Haromaya	6	.0033	
Harar	6	.0307	
Chelenko	6	14.7100	14.7100
Chiro	6	15.5867	15.5867
Babile	6		25.5550
Sig.		.235	.578

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.