ADDIS ABABA UNIVERSITY
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES
CENTER FOR FOOD SCIENCE AND NUTRITION

AFLATOXIN STATUS IN COMMERCIALLY PRODUCED TEFF IN ETHIOPIA

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A THESIS SUBMITTED TO THE CENTER OF FOOD SCIENCE AND NUTRITION, COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES, ADDIS ABABA UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER'S IN FOOD SCIENCE AND NUTRITION

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER'S IN FOOD SCIENCE AND NUTRITION

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Declaration

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

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AF	*Aflatoxin*
ATA	*Agricultural transformation Agency of Ethiopia*
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*
FMHAC	*Food, medicine, health care administration and control authority*
HACCP	*Hazard Analysis and critical control point*
HPLC-FLD	*High-performance liquid chromatography with fluorescence Detector*
IARC	*International Agency for Research on Cancer*
JECFA	*Joint Expert Committee on Food Additives*
KAP	*Knowledge, Attitude and Practice*
LC	*Liquid chromatographic*
LOD	*Limit of Detection*
LOQ	*Limit of Quantification*
MPC	*Maximum permissible concentration*
OTA	*Ochratoxin A*
PMTDI	*Provisional maximum tolerable*
RSD	*Relative standard deviation*
SPE	*Solid phase extraction*
WHO	*World health organization*

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Contamination of cereal commodities by moulds and mycotoxins results in dry matter, quality, and nutritional losses and represents a significant hazard to the food chain. Aflatoxins are toxic, hepatocarcinogenic, secondary metabolites of Aspergillus species produced in most agricultural commodities, stored at inappropriate temperatures and water activities. Aflatoxin contamination of teff in Ethiopia was reported in 1987, indicating that higher probability of contamination of teff, sampled from different part of Ethiopia.

The objective of this study was to investigate occurrence of total aflatoxin in teff produced by commercial farms of Ethiopia and the effect of stored temperature and relative humidity was also investigated. In addition, KAP study was also conducted on owners and employees of commercial farmers. A total of Eleven commercial teff producing farms were randomly selected and, Aflatoxin B1, B2, G1 and G2 were determined at microgram per kilogram (µg/kg) levels by immuno-affinity column clean up and reversed-phase liquid chromatography with fluorescence detection. The result showed all of them were below the maximum (4 µg/kg) tolerable level of Ethiopian teff flour standard. The highest AF concentration was 3.99 µg/kg and the lowest concentrations was 0.5 µg/kg. But 91% contaminated by AFB1 and 45% were unsafe for direct human consumption as per the Ethiopian and EU maximum (2 µg/kg) tolerable intake level and, 27% of the samples very close (above 3 µg/kg) to the 4 µg/kg. The highest aflatoxin B1 level was found in west Gojam 3.25 µg/kg and aflatoxin B1 is note detected in sample form Adam Ethiopia. From the samples analysed, 13.88%, 0%, 8.38% and 77.73% were contaminated with quantifiable concentrations of aflatoxin G2, G1, B2 and B1 respectively. Aflatoxin G2 (13.88%) and B1 (77.73%) concentrations were relatively higher than the others. In addition, KAP study was conducted on commercial farms and it shows that, the farmers had knowledge on mycotoxin related question but their knowledge to AFs practice towards reducing the contamination level of AFs is not enough.

Key words: Aflatoxin, Teff, KAP assessment, Immuno-affinity column

CHAPTER ONE
INTRODUCTION

1.1 Background

Teff (*Eragrostis tef(Zuccagni*) Trotter) is a tropical cereal that belongs to the family of *Poaceae*, subfamily *Eragrostoidae*, tribe *Eragrosteae*, and genus *Eragrostis*. About 350 species are known in the genus *Eragrostis (*Demissie *et al.,* 2001), of which teff is the only cultivated species. *Chloridoideae* is used synonymously for *Eragrostoidae* of teff (Stallknecht *et al.*, 1993).

Teff cultivars have been recognized and described based on colour of the grains and inflorescences, ramification of the inflorescences and the size of plants. Based on seed colour teff classified as, Netch (white), keye (red/brown) and Sergegna (mixed) (Demissie, 2001).

It is oval-shaped with size 0.9–1.7 *mm* (length) and 0.7–1.0 *mm* (diameter). The individual grain mass is in the range of 0.2–0.4 *mg*, perhaps the smallest among carbohydrate-rich kernels (Gebremariam *et al.*, 2014).

It was domesticated between 3,000 and 6,000 years ago, well before wheat, barley, and maize were introduced to Ethiopia. Today, 6 million smallholder-farming households grow teff and it is the most widely planted crop by area, 3 million hectares, in the country. Ethiopia is centre of both origin and diversity of teff and have a wealth of diversity in crop varieties. Fourteen species of "Lovegrass" are endemic to Ethiopia and it is still common to find teff growing wild in many parts of the country (Ethiopian Agricultural transformation Agency, 2016).

The use of teff as a cereal for human's consumption is surpassing the boundaries of Ethiopia, United States and South Africa starts Commercial production of teff, and international markets are opening (Yewondwosen, 2017).

In Ethiopia teff and other cereals are affected by fungi infection, due to predisposing reasons such as frequent end season drought, soil water stress, lack of resistant varieties, Storage facility and conditions and harvesting method (Chala *et al.,* 2013).

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 is reported in many countries with a high occurrence of Aspergillus and Fusarium (Silva *et al.*, 2004).

Aflatoxins, a mycotoxin, are the main toxic secondary metabolites of some Aspergillus moulds such as *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *Aspergillus nomius* (Goldblatt, 2012).

Aflatoxins are produced on various cereals, grains and nuts, for example, corn, sorghum, cottonseed, peanuts, pistachio nuts, copra, fruits, oilseeds, dried fruits, cocoa, and spices in the field and during storage. AFs occur mainly in hot and humid regions where high temperature and humidity are ideal for moulds growth and toxins production (Espinosa-Calderón, 2011). Humans are exposed to aflatoxins by direct consuming contaminated foods, such exposure is difficult to avoid because fungal growth in foods is not easy controllable.

Evidence of acute *aflatoxicosis* in humans has been reported from several parts of the world, especially the developing Countries, like Kenya, Tanzania, Taiwan, Uganda, India, and others. In 2004 outbreak of acute aflatoxicosis in Kenya was one of the most severe episodes of human aflatoxin poisoning in history. A total of 317 cases were reported by 20 July 2004, with a case fatality rate of 39%. This epidemic resulted from ingestion of contaminated maize (Azziz-Baumgartner *et al.*, 2005).

In June 2016, an outbreak of an aflatoxicosis was occur in clusters of families in two regions of the central part of Tanzania. A total of 68 cases occurred between 14 May and 14 November 2016, of which 20 died (Kamala *et al.*, 2018)

The syndrome is described by vomiting, abdominal pain, pulmonary edema, convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidneys, and heart. Conditions which increasing the likelihood of acute *aflatoxicosis* in humans include insufficient availability of food, environmental conditions that support fungal development in crops and commodities, and lack of regulation for aflatoxin monitoring and control (Chala *et al.*, 2014).

This study has focused on the investigation of the level of aflatoxin in exported teff from commercial farms of Ethiopia. In addition, knowledge, attitude and practice of the commercial farms were studied.

1.2. Problem Statement

Several surveys on the occurrence of aflatoxins have been reviewed and clearly shows, occurrence of aflatoxins in food and feed is still a relevant issue in food safety. It was shown that in Ethiopia aflatoxins could be found in significant fractions of different food staff. For instant the research done by Samuel shows that 66.67% of Sorghum Injera analysed were quantified with total aflatoxin, above lower limit of quantification. From 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. The results of samuel 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 (Samuel, 2009).

Another study on Sorghum and Finger millet shows, all the tested sorghum and finger millet samples were found to be contaminated by Fusarium and Aspergillus Species Aflatoxins B1, B2, G1 and G2 were detected in at least one sorghum sample while only aflatoxins B1 and G1 were present in finger millet samples. The average aflatoxins B1 and G1 concentrations in sorghum have been higher than European standards (Chala *et al.*, 2014). In 1987 the research done by Aberra and Admassu shows the aflatoxin content of 486 samples of six cereal varieties was determined and 71 (14.6%) of these were found to be positive, ranging from 9 *μg/Kg* to 39 *μg/Kg*. Of these, 23 (32.4%) were maize grains followed by the white sorghum, 12 (16.9%) and mixed sorghum, 11 (15.5%). The positive numbers of white, mixed and red teff samples were significantly as low as 7 (9.8%),8(11.3%) and 10 (14.1%), respectively (Aberra *et al.*, 1987).

Aflatoxin in Ethiopia is known, due to influencing reasons such as frequent end season drought soil water stress, lack of resistant varieties, Storage facility and conditions and harvesting method among others (Chala *et al.*,2013).

Now a days Ethiopia is suffering by aflatoxin specially on exporting cereals and spices. Recently hot pepper powder worth ten million USD has returned to Ethiopia from European markets when

it was found to have unsafe levels of Aflatoxins and Ochratoxins during testing at entrance (Yewondwosen, 2017).

The next big concern related to aflatoxin is teff, since Ethiopia starts to export teff for Europe and North America. Ethiopia is to begin the export of around 180,000ql off teff flour, by the next five years following the ban set by the government on the export of teff in 2006. To produce teff for export purposes by the fiscal year 2017/2018, 6,000ha of land is cultivated. From these farms, a yield of 20ql to 30ql off teff per hectare is expected, all of which will be export. In the past year, export licenses have been granted to 40 commercial farmers. (Ethiopian agrcultural transformation agency, 2016).

Recently teff become the new "super grain" of choice in Europe and North America, overtaking the quinoa. Ethiopia is ready to export teff for Europe and North America markets, and the global market for teff is growing by the day. Gluten-free products are a £3.7bn industry globally with demand estimated to grow 10% each year between 2015 and 2020 (Ethiopian agricultural transformation agency, 2016).

Ethiopian agricultural transformation agency aims by 2020 to make annual revenues of 99 million USD, of this total, 677,000 USD will be earned through teff export to the US and Western Europe. From which 90% of the products being processed within the farmers and 10% through contractual agreements with processors in Addis Ababa (Ethiopian agricultural transformation agency, 2016).

Therefore, assessing aflatoxin content of teff is crucial before exporting to assure the exported teff is free from aflatoxin and to save the loss during exporting.

1.3. Significance of the Study

The main concern of this study is to determine the level of aflatoxins content of exported teff, produced by commercial farms of Ethiopia. Disseminates the level of significance to agricultural transformation agency of Ethiopia (ATA) and government regulatory bodies and aimed to determine the food safety and hygiene practices in minimizing aflatoxins among teff exporters. Also, the finding of this study will be helpful for the public health of the nation and the region to

increase knowledge and awareness of the farmers on food handling, storage, processing, and food safety system and hence, reduce the healthcare costs and loss on exports.

1.4. Research Question

1. Does the commercially produced teff contaminated with aflatoxin?
2. Does teff exporters have the knowledge, attitude and practice of aflatoxin contamination?
3. Does the level of aflatoxin detected in commercial teff exceed the Ethiopian, EU, FDA, FAO and WHO tolerable limits?

1.5 Objectives

1.5.1 General Objective

To assess the level of aflatoxin contamination in commercial produced teff in Ethiopia.

1.5.2 Specific Objectives

- To determine the level of aflatoxins (B1, B2, G1, and G2) in teff sample collected from commercial farm in Ethiopia.
- To assess the knowledge, attitude and practice of commercial farms regarding aflatoxin contamination and mitigation mechanism.
- To compare the aflatoxins level in commercial teff with Ethiopian and international standards.

CHAPTER TWO
LITERATURE REVIEW

2.1 Overview of teff

Teff (*Eragrostis* Teff) is an intriguing grain, ancient, minute in size, and packed with nutrition. It is believed to have originated in Ethiopia between 4000 and 1000 before Christ (BC) (Bekele *et al.*, 1981). Teff was grown primarily as a cereal crop in Ethiopia. It is the smallest grain in the world, ranging from 1–1.7mm long and 0.6–1mm diameter with l000 seed weight averaging 0.3–0.4 grams and taking 150 grains to weigh as much as one grain of wheat. The common English names for teff are, love grass, and annual bunch grass. It is intermediate between a tropical and temperate grass (Tadesse, 1969).

2.1.1 Agronomy and taxonomy aspects of teff

Eragrostis is a member of the tribe *Eragrosteae*, sub-family *Eragrostoidae*, of the Poaceae (Gramineae). Teff is a tetraploid plant 2n = 40. There are approximately 350 species in the genus *Eragrostis* containing of both annuals and perennials which are found over a wide geographic range. *Eragrostis* teff is one of those species. The closest relative of teff is *E.Pilosa* (Ingram, 2003).

Eragrostis species are classified based on characteristics of culms, spike lets, lateral veins, pedicels, panicle, flowering scales, and flower scale colours. Recently, the taxonomy of teff has been clarified by numerical taxonomy techniques, cytology and biochemistry, including leaf flavanoids and seed protein electrophoretic patterns (Peterson *et al.*, 2007).

Teff is a fine stemmed, tufted annual grass characterized by a large crown, many shoots, and a shallow fibrous diverse root system. The plants germinate quickly and are adapted to environments ranging from drought stress to water logged soil conditions. The inflorescence is an open panicle and produces small seeds (1.000 weigh 0.3 to 0.4 g). The florets consist of a lemma, three stamens, two stigmas and two lodicules. Floret colours vary from white to dark brown. Plant height of teff varies from 25–135 cm which dependents on cultivar type and

growing environments. Panicle length 11–63 cm, with spike lets numbers per panicle varying from 190–1410. Panicle types vary from loose, lax, compact, multiple branching multi-lateral and unilateral loose to compact forms. Maturity varies from 93–130 days (Stallknecht *et al.*, 1993).

Teff is an annual warm season grass crop. Teff is a self-pollinating chasmogamous plant. It is a reliable low risk crop and can be planted in late May like millets. Late plantings have the advantage to control emerged weeds by tillage before planting, which can be significant since, teff is a poor competitor with weeds during the early growth stages. Planting of teff requires a firm moist seedbed. To affect good soil moisture-seed contact because of the small seed size. Seeding rates varies from 2.3 to 9 kg/ha, with 5 to 8 kg/ha generally recommended (Bekele, 2006).

Teff should be seeded 12–15 mm deep either broadcast or in narrow rows. Teff is relatively free of plant diseases when compared to other cereal crops. In Ethiopia, where humidity's are high, rusts and head smuts are important diseases, and 22 fungi and 3 pathogenic nematodes have been identified on teff (Bekele, 2006).

Teff seedlings are also susceptible to Damping-off caused by Drechslera poae and Helminthosporium poae (Baudys) Shoemaker, when sown too early (Ketema, 1991). Insect pests of teff in Ethiopia are Wello-bush cricket, Decticoides brevipennis, red teffworm, Mentaxya ignicollis, teff epilachna, and teff black beetle. Since teff has been limited to small zones in the United States a few disease and insect problems have been observed. However, a serious problem was observed in South Dakota where the stem boring wasp, Eurytomocharis eragrostidis (Howard) reduced forage yields by over 70% (ketema, 1997). Although the insect problem was observed in only one out of the five years in research trials, the significant losses obtained could be a deterrent to commercial expansion of teff production.

Teff can adapt to a wide range of environment and it considered that very impervious to, insect pests. Teff seeds remain viable for several years if direct contact with moisture and sunshine is avoided (Gamboa, 2008). In comparison with other common cereals, teff grain is less prone to attacks by weevils and other storage pests (Tadesse, 1969). Thus, it can be safely stored under traditional storage conditions with no chemical protection.

Teff can be grown from near sea level to altitudes over 3,000 *m*. It is particularly valued for areas too cold for sorghum or maize. It has wider altitudinal range than other cereal. In Ethiopia; it is cultivated between 1,100 and 2,950 *m*. While teff has some frost tolerance, it will not survive a prolonged freeze. Teff tolerates temperatures (at its lower altitudinal range) well above 35°C.Teffs tolerance of soil types seems to be very wide. As noted, it performs well even on the black cotton soils that are notoriously hostile to crops and farmers (National Research Council, 1996).

The average annual rainfall in teff-growing areas is 1,000 *mm*, but the range is from 300 to 2,500-*mm*. Teff resists moderate drought, but most cultivars require at least three good rains during their early growth and a total of 200 to 300 *mm* of water (National Research Council, 1996). The plant can certainly be grown in many countries. Some has long been produced for food in Yemen, Kenya, Malawi, and India. In addition, the plant is widely grown as a forage for grazing animals in South Africa and Australia.

2.1.2 Production of teff

Ethiopia is the largest teff growing country in the world. In 2017, teff accounts for 24% of the grain area, followed by maize 17% and sorghum 15%. Amhara and Oromia are the two major regions, and collectively, the two regions account for 85.5% of the teff area and 87.8% of the teff production (Lee, 2018). Its production mainly depends on soil type, altitude and agro-ecologic climatic conditions. Because of high prospects on producing this crop by Ethiopian farmers, every year the volume of production shows an increasing trend with average annual growth rate of 15.75 percent during the period 2003/04 to 2007/08 (Table 2.1).

Table 2. 1: Average annual growth rate of production, cultivated area and yield of teff

Year (E.C)	Production in quintal	% change	Cultivated Area in hectare	% change	Yield in qui per hec	% change
2003/04	16,773,480.00		1,989,068.00		8.43	
2004/05	20,255,214.00	20.76	2,135,553.00	7.36	9.48	12.46
2005/06	21,755,977.00	7.41	2,246,017.00	5.17	9.69	2.22
2006/07	24,377,495.00	12.05	2,404,674.00	7.06	10.14	4.64
2007/08	29,929,235.00	22.77	2,565,155.22	6.67	11.67	15.09
Average growth rate	15.75		6.57		8.60	

2.1.2.1 Teff commercial farming in Ethiopia

In response to chronic food insecurity the Ethiopian government established the Agricultural Transformation Agency (ATA) in December of 2010. The goal of agency is to accelerate agricultural sector transformation by addressing "systemic bottlenecks for achieving growth and food security" (ATA, 2011). With the support of the Gates Foundation, the ATA conducted an extensive diagnostic study of Ethiopia's agricultural system and proposed improvements that would enable agriculture to become the driver of economic growth in Ethiopia (Berhanu, 2014). Opening large tracts of arable land for foreign agribusiness investment is one of the strategies for creating this kind of growth.

Given significant productivity improvements in recent years, the Government of Ethiopia is running a pilot program to allow limited exports of teff and establish an 'Ethiopian teff' brand internationally. This allows teff grown by 40 licensed commercial farmers to be exported (Table 2.2), started from 2016. The goal is to export a minimum of 25,000 *MT* of grain and processed teff by 2025 by a larger, carefully selected and licensed, group of commercial farmers (Ethiopian Agricultural Transformation Agency, 2016).

Table 2.2: List of licenced teff exporter commercial farm in Ethiopia

N°	Name of commercial farm
1	Bale Gololcha and Ginir Agri Dev't and Mechanization Service PLC
2	Balegreen Spice and Grain Dev't PLC
3	Bayih Mekonin Ersha Limat
4	Bulala Dinkity Agriculture Trade & Industry PLC
5	Ethio Agri-ceft PLC
6	Geraye Wenze Agro Industry
7	Girma Dina Dibera Coffee & Field Crops Dev't
8	Gombo Huruma Agro Industry PLC
9	Lambadina Integrated Agriculture PLC
10	Melkam Endale Crop Farm
11	Michael Seyfu Integrated Agri. Dev't
12	Mohamed Awel Farms
13	Mola Bishaw Seed Production
14	Nasir Haji Boka PC
15	NINT Agri PLC
16	Nono Agri. Dev't PLC
17	Sofiya Ommer Farms
18	Tadele Jarra
19	Tamehe General Trading PLC
20	Tamene Gobena
21	Tuga Agro Industry PLC
22	Yebeletal Mekuriaw Agricultural Investment
23	Yemekel General Trading PLC
24	Amanuel Abraha Farm
25	Ashenafi and Eyerusalem Agri Dev't PLC
26	Anno Agro-Industry
27	Chilallo Enterprise PLC
28	Chombe Seyoum Adano-Inchini Farm
29	Elena Digrande Farm
30	Gash Reda Lema
31	Hibret Bogale Crop Production
32	Jeju Horticulture PLC
33	Kalibso Agricultural Trading PLC
34	Mekiya Enterprise
35	Melaku & Friends
36	Meychel Agricultural Production PLC
37	Real Teff
38	Savanna Farming PLC
39	Smur Agricultural Farm PLC
40	Soma Tipan PLC

2.1.3 Nutritional composition of teff grain

The nutrition composition of cereals varies widely and depends on the environmental conditions, soil, variety and fertilizer. Teff have recently been receiving global attention particularly as a "healthy food" due to the absence of gluten and gluten-like proteins, making it suitable for celiac disease patients (Spaenij-Dekking *et al.*, 2005). This super grain is currently emerging as healthy alternatives to gluten-containing grains in the gluten-free diet. Teff has dietary advantages such as slow-release of carbohydrate constituents that are useful for diabetic patients (Bultosa & Taylor, 2004a). Recently, the use of teff as a food is gaining popularity as both a naturally gluten-free alternative to wheat products and a nutrient-rich ingredient in the baby food industry (Curtis *et al.*, 2008; Hopman *et al.*, 2008). Rich source of the fiber is the other common factor, which make it dietary choice for the entire world.

Teff contains substantial levels of Vitamins A and C, as well as niacin, and their amount is generally increased by the yeast fermentation process involved in the production of injera (National Research Council, 1996).

2.1.3.1 Carbohydrates

Reported that complex carbohydrates make up 80 percent of teff grain. Teff has a starch content of approximately 73 percent, making teff a starchy cereal. The amylose content of 13 teff varieties tested that ranged between 20 to 26 percent, comparable to other grains such as sorghum (Baye, 2014).

National Research Council of Washington DC reported that carbohydrate content is found to be 73*g*/100*g* in teff flour, which is like other cereal grains, such as white wheat flour 75*g*/100*g*, rye flour 76*g*/100*g* but lower than maize flour (92 *g*/100*g*) and higher than soya flour (28g/100g) and brown wheat flour (69*g*/100*g*). (National Research Council 1996).

Using a scanning electron microscope, the size of teff starch was found to be 2-6 μm (Girma *et al.*, 2013). This makes teff starch granules smaller than those of wheat (A type 20-35 *μm*), sorghum (20 *μm*) and maize (20 *μm*). Given their larger surface area, smaller starch granules are more susceptible to enzymatic attack (Baye, 2014). In comparison to wheat which has larger starch granules, the in vitro starch digestibility of teff was found to be significantly lower

(Wolter *et al.*,2013). In line with this, the predicted glycemic index of teff, which is 74 was significantly lower than that of white wheat (100) but comparable to that of sorghum (72) and oats (71) (Wolter *et al.*, 2013).

2.1.3.2 Protein

The average crude protein content of teff is in the range of 8 to 11 percent, like other more common cereals such as wheat. Teff fractional protein composition suggests that glutelins (45 percent) and albumins (37 percent) are the major protein storages, while prolamins are a minor constituent approximately 12 percent (Baye, 2014). Teff's amino acid composition is well balanced. High concentration of lysine amino acid is found in teff. Similarly, compared to other cereals, higher contents of isoleucine, leucine, valine, tyrosine, threonine, methionine, phenylalanine, arginine, alanine and histidine are found in teff.

2.1.3.3 Fiber

In case of fiber content in teff is 3 % (dry base) particularly high and exceeds that of most other cereals, such as wheat (2%), rye (1.5%), rice (0.6–1.0%) and sorghum (0.6%) dietary fiber in dry basis (Gebremariam *et al.*, 2014). Contrasting soybean and peas, teff grain is not a significant source of soluble fiber. The total dietary fiber is close to the value found in cereals (7 to 9.7% dry base), wherein the embryo contains higher levels than those in perisperm. The soluble fiber content is reported ranging from 1.3% to 6.1% (dry base). Higher fiber content in teff because it is always consumed in the whole-grain, since it is impossible to perform any fractionation during the milling process due to the small size of teff grains (Bultosa & Taylor, 2004).

2.1.3.4 Minerals

The difference in mineral content between and within teff varieties is wide ranging. Red teff has a higher iron and calcium content than mixed or white teff (Baye, 2014). On the other hand, white teff has a higher copper content than red and mixed teff (Table 2.4). The most recent study investigated iron content in selected teff grains and their findings showed that teff contained almost 38mg/100g, more than 150*mg*/100*g* of iron in locally purchased white, and red teff grains varieties respectively (Baye, 2014). Bultosa (2007) reported that the ash content of 13 teff varieties ranged from 3.16 to 1.99%, this grain has the ash content greater than rice (0.5%),

wheat (1.8%), and other cereals, whereas quinoa has slightly higher ash than the teff. In addition to providing protein and calories, teff is a good source of minerals, particularly Fe resulting in a mineral content approximately two to three times that of wheat, barley, and sorghum (Mengesha, 1966).

Table 2.3: Mineral content of teff grain in *mg/100g* (Baye *et al.*, 2014)

Minerals	White teff	Red teff	Mixed teff
Iron	9.5-37.7	11.6- >150	11.5- >150
Zinc	2.4-6.8	2.3-6.7	3.8-3.9
Calcium	17-124	18-178	78.8-147
Copper	2.5-5.3	1.1-3.6	1.6

2.2 Mycotoxin

The term mycotoxin was initial used in the 1960s to describe the toxin associated with infected peanuts in animal feed and the loss of turkeys in England or Turkey-X-disease. This mycotoxin was later identified as the Aspergillus flavus toxin aflatoxin B1. Mycotoxins are toxic secondary metabolites produced by many species of microscopic filamentous fungi occurring on field cereals, including barley and these fungi evoke a toxic response when introduced in low concentrations to higher vertebrates and other animals by a natural route (Goldblatt, 2012). Several fungi produce mycotoxins, particularly by many species of Aspergillus, Fusarium, Penicillium, Claviceps, and Alternaria. They comprise a group of several hundreds of chemically different toxic compounds. (Huwig, 2001).

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, 2014). Toxic syndromes resulting from the intake of mycotoxins by man and animals known as mycotoxicosis.

The prompting 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 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. 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 (Darwish, 2014).

2.2.1 Aflatoxin

Aflatoxins (AFs) are the best known and most widely studied mycotoxins. They were first isolated in the early 1960s when 100,000 turkey pouts died after consuming aflatoxin contaminated peanut meal in the UK (the so-called Turkey X disease); this event was followed by proliferation in research on fungal toxins contaminating food and feeds. AFs were found to be the most potent naturally formed carcinogen, and researchers started their investigating on factors that influence this production. AFs are highly toxic, mutagenic, and carcinogenic compounds (Lee *et al.*, 2004)

They are secondary metabolites that are produced mainly by *Aspergillu sparasiticus* and *Aspergillus flavus*; in fact, the name ''aflatoxin" is derived from the first letter in Aspergillus, and the first three letters in flavus. These fungi are found in many countries, especially in tropical and subtropical regions, where the temperature and humidity conditions are optimal for the growth of molds and the production of toxic. AFs are natural contaminants of several agricultural products, such as: corn, peanuts, cotton seed, and other grain crops. Food is the major way through which humans as well as animals are exposed to these mycotoxins. (Iqbal *et al.*, 2006)

Structurally, Aflatoxins are difurocoumarin derivatives that fluoresce under ultraviolet light. Depending upon colour of the fluorescence, AFs are divided into aflatoxin B1 and B2 (AFB1, AFB2) for blue, and G1 and G2 (AFG1, AFG2) for green (Figure 2.1). AFB1 is the most common produced mycotoxin and the most potent; it has been reported to be the most powerful natural carcinogen in mammals (Creppy, 2002).

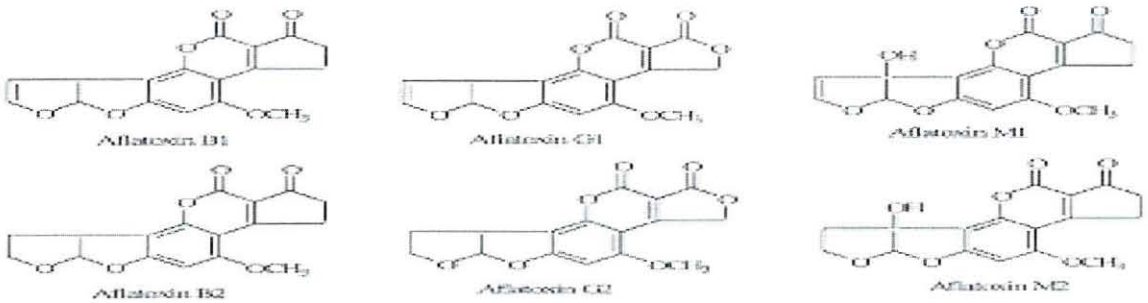

Figure 2.1: Chemical structure of the main aflatoxins.

Aflatoxin can be detoxified by treating the products with NH_4OH and H_2O_2 at the rate of 1% on a dry matter basis. Aflatoxin may be prevented by packing the dried products in polythene or propylene bags. Antoxigenic strains may be used for the prevention of Aflatoxin production (Iqbal *et al.*, 2006).

2.2.2 Aflatoxin in cereal

Aflatoxin contamination of food and feeds is a serious problem worldwide. Studies focusing on AF contamination in food stuffs have in detail been reported in many countries, especially those in tropical and subtropical regions, such as Asia and Africa (Bankole *et al.*, 2010).

Aflatoxin contamination can develop both in the pre-harvest and post-harvest periods, but the highest levels are usually associated with post-harvest spoilage of food commodities, stored under inappropriate high moisture content and high temperature conditions which facilitate the rapid growth of molds; the level of contamination depends on the plant stress, temperature, water activity, genotype, culture and storage conditions, but appropriate postharvest treatments, under dry cool settings, should control this source of contamination (Moss, 2002).

As far as pre-harvest, is concerned, aflatoxigenic fungi have a complex ecology. The spores of A flavus and A parasiticus can germinate on the stigma surfaces of plants, and the germ tube can penetrate the developing embryo in a manner which mimics pollen germ tubes. The mycelium can establish an endotrophic relationship, which is not harmful to a healthy plant, while if the plant is stressed (e.g. drought), significant levels of aflatoxin may be produced during field growth. Under these circumstances food commodities may already be contaminated at harvesting

and, even though the concentrations are never as high as those formed in stored commodities, they can be economically significant, and this field contamination is much more difficult to control than post-harvest spoilage (Moss, 2002).

Although a wide variety of foods are susceptible to aflatoxin contamination, it has most commonly been associated with peanuts, maize, pistachio, dried fruit, nuts, spices, figs, vegetable oils, cocoa beans, corn, rice and cotton seeds (Bankole *et al.,* 2010).

Among the agricultural commodities usually infected by aflatoxigenic fungi some are food sources while others are used as animal feeds: the greatest difficulty is that aflatoxin affects the health of the humans and the livestock that consume these commodities and the related products. Zinedine reported that AFB1 and OTA are amongst the most frequently observed combinations of mycotoxins in different plant products. According to other authors, cereals, olivesand dried vines are other commodities which could support aflatoxigenic and ochratoxigenic mold growth and OTA and AFB1 production (Zinedine, 2006).

While aflatoxin B1 is frequently found in contaminated feeds, aflatoxin M1, its hydroxylated metabolite, is normally not present in food, except though carry-over from animal feed (Fallah, 2010) following the ingestion of contaminated feedstuffs by lactating dairy cows, AFB1 is biotransformed, by hepatic microsomal cytochrome P450 into AFM1, and is then excreted into the milk. Moreover, the AFM1 content in milk is closely correlated to the level of AFB1 in the raw feedstuffs. AFM1 can be detected in milk 12–24 *h* after the first ingestion of AFB1; generally, it is deemed that approximately 1–3% of the aflatoxin B1 present in animal feeds appears as AFM1 in milk, depending on the animal, time of milking and many other factors. When the intake of the contaminated source is stopped, the concentration of the toxin in the milk decreases to an undetectable level within 72 *h* (Gürbay, 2006).

Additionally, when specific conditions during feed storage are prevalent for the growth of aflatoxigenic species, an additional production and accumulation of AFB1 may occur; this in turn leads to the accumulation of additional AFM1 in the milk. Aflatoxin M1 can survive pasteurization and has even been reported in UHT milk (Fallah, 2010).

2.2.3 Conditions for aflatoxin contamination

The production of mycotoxins within the fungus depends on food sources and the enzymes of the fungus and other environmental factors (Sanchis *et al.*, 2004). The growth of aflatoxicogenic fungi is directly related with the production of aflatoxin, so that situations suitable for these fungal growths is favourable for aflatoxin production. The main factors influencing fungal growth in stored food products are the moisture content (more precisely, the water activity) and the temperature of the food. Food grains are normally harvested at higher moisture content and then dried to bring down the moisture content up to safe level before storage. Thus, delay in drying to safe moisture levels increases risks of mould growth and mycotoxin production (Chulze, 2010). Aflatoxin infection occurs in crops prior to harvest and once the grain reaches storage. It can be produced when maturing teff is under drought and insect stress with prolonged periods of hot weather. Post-harvest contamination can occur if crop drying is delayed.

According to Schmidt, (2013) moulds produce aflatoxins under a wide range of conditions and, therefore, the potential for a challenge should always be considered with plant stress, harvest stress, storage stress and feedout problems. Aflatoxin contamination is also promoted by stress or damage to the crop due to drought before harvest, the insect activity, a poor timing of harvest, the heavy rains during and after harvest, and an inadequate drying of the crop before storage (Lizarraga-Paulin *et al.*, 2011). Aflatoxin production in the grain can happen in the field in the storage conditions between 20 and 40° c with 10-20% of humidity and 70-90% relative humidity in the air (Paterson, 2010). flavus has relatively high moisture requirements among storage fungi (Amare,2014). Hence, aflatoxin contamination of grains is aggravated by high seed moisture. Aflatoxin contamination is a perennial risk between 40°N and 40°S of the equator (Schmidt, 2013).

2.2.4 Prevention strategies of aflatoxins in cereal

Aflatoxin contamination may occur in the field before harvest, during harvesting, or during storage and processing, thus methods for the prevention of contamination can be divided into preharvest, harvesting and post-harvest strategies. Whereas certain treatments have been found to reduce aflatoxin formation in peanuts, the complete elimination of aflatoxin is currently not realistically achievable. Current management practices that reduce the incidence (Torres *et al.*,

2014) of aflatoxin contamination in the field include timely planting, maintaining optimal plant densities, proper plant nutrition, avoiding drought stress, controlling plant pathogens other than Aspergilli, weeds and insect pests and proper harvesting (Bruns, 2003) and, in post-harvest drying, storage and processing. Codex Alimentarius have developed code of practice for the prevention and reduction of aflatoxin in cereal.

The recommendations for the reduction of aflatoxins in cereals are separated into two parts: recommended practices based on Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP); a complementary management system to consider in the future is the use of Hazard Analysis Critical Control Point (HACCP) (Kabak, Dobson, & Var, 2006). HACCP system involves a science-based analysis of potential hazards involved in the production of foods, determination of where the hazards can occur in processing techniques, institution of preventives measures, and corrective actions if they do occur. This system is designed to critically evaluate the effectiveness of controls at each major step involved in processing food. The implementation of HACCP principles to minimize aflatoxins contamination has been successfully applied in Southern Africa in commercially produced peanut butter (Torres *et al.*, 2014).

2.2.4.1 Pre-harvest cropping system

The pre-harvest control of aflatoxin contamination of cereal must take into consideration all the varied environmental and agronomic factors that influence pod and seed infection by the aflatoxin-producing fungi, and aflatoxin production. These factors can fluctuate considerably from one location to another, and between seasons in the same location. However, using proper agricultural practices, including crop rotation, tillage, planting date, and management of irrigation and fertilization, should reduce aflatoxin contamination in cereals (Torres *et al.*, 2014).

2.2.5 Regulation of aflatoxin

When it became evident that aflatoxin exposure caused cancer in many species, most countries, established various regulations for aflatoxin levels (either total aflatoxins or for AFB1) in food and/or feed to limit exposure to this group of mycotoxins (Van-Egmond *et al.*, 2007). Many countries have set a limit for a maximum tolerable level of aflatoxin in food and food stuffs and

restrict the import of contaminated products to their country. Aflatoxin is becoming a major impediment to the global exchange/trade of plant and plant products. Aflatoxin regulation creates a demand for aflatoxin safe food. Different countries have different regulations for aflatoxin to protect consumers from the harmful effects of mycotoxins that may contaminate foodstuffs, as well as to ensure fair practices in food trade.

The hazardous nature of aflatoxin to humans and animals has forced the need for establishment of control measures and tolerance levels by national and international authorities. The number of countries regulating aflatoxins has significantly increased over the years. Such lower limits for aflatoxin had an enormous impact on the ability of developing countries in Africa like Ethiopia to export goods. Aflatoxin is more problem for developing nations than developed countries. In the developing countries, where food supplies are already limited, legal measures may lead to lack of food and to excessive prices.

According to Hell and Mutegi (2011), aflatoxin research in Africa is necessary to get policymakers in the Sub-Saharan region to recognize that the increased implementation of pre- and post-harvest interventions is important for increasing food security and ensuring food safety to protect the short and long-term health of the population. For example, a research by Dejene et al. (2012) from Northern Ethiopia on groundnut revealed that, from the total samples analyzed, 83.9% were unsafe for direct human consumption as per the EU MTIL and 46.6% were unfit for export to EU counties (as per the EU safe limit for import of groundnut); and on the basis of the FAO MTL, 16.6% of the samples exceeded the 30 ppb limit. The average concentration for the total samples had 10 times greater than the recommended maximum aflatoxin level. The maximum level of 8 *mg/kg* of AFB1 has been established in food subjected to sorting or physical treatment before human consumption, and the corresponding 2 *mg/kg* of AFB1 for direct human consumption.

Although aflatoxins are regulated in more than 80 countries, their legislation is not yet completely harmonized at the international level. Several institutions around the world have classified and regulated aflatoxins in food. The European Union (EU) has the most rigorous regulations concerning mycotoxins in food. The maximum residue levels for total AFs and for the most toxic of them (AFB1) according to the EU Commission Regulations are 2 and 4 *μg/kg*,

respectively. The maximum legal limit for AFM1 in milk is set at 0.05 *μg/kg* (50 ppt) for all EU Member States, and 25 *ppt* for baby food. The European Committee Regulations (ECR) has established the maximum acceptable level of AFB1 in cereals, peanuts and dried fruits for direct human consumption in 4 *ng/g* for total aflatoxins (AFB1, AFG1, AFB2, AFG2) and 2 *ng/g* for AFB1 alone. The International Agency for Research on Cancer (IARC) classified aflatoxins as Group 1 of human carcinogens (Park, 2004). In USA, the U.S. Department of Agriculture and the U.S. Food and Drug Administration (FDA) have established an "actionable" level of 15-20 ppb of AFs in animal feed products

Although Ethiopia has aflatoxin and other mycotoxin regulation their legislation is not yet completely harmonized (PACA, 2014). This increases the exposure of humans and animals from aflatoxin contamination. However, aflatoxin regulation is not the mandatory case in Ethiopia, because almost all the effect is from indigenous contamination of commodities. Hence, more emphasis should be given for control of the toxin. Aflatoxin remains largely unregulated throughout Africa. As of 2003, aflatoxin regulations existed for five countries in Africa. However, 99 % of Europe's populations are protected from aflatoxin contamination

2.2.5 Mycotoxin contamination in Ethiopia

Darwish *et al,* (2014) reported that the occurrence of mycotoxins in Barley, sorghum, teff and wheat of Ethiopia and other African country. Table 2.4 shows the concentration of mycotoxin in different agricultural crop in some African country. In Ethiopia, AF contamination of Shiro and ground red pepper samples collected from open markets in Addis Ababa was investigated. From 60 samples, each of ground red pepper and Shiro, 8 (13.33%) and 5 (8.33%) were positive for AFs, respectively. AF levels in Shiro and ground red pepper positive samples ranged from 100–500 *μg/kg* and 250–525 *μg/kg*, respectively (Ayalew *et al.*, 2006).

In another study, the occurrence of mycotoxins in barley, sorghum, teff and wheat was examined, AFB1 and OTA were detected in samples of all four crops. 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 analyzed. FUM and ZEA occurred only in sorghum

samples with low frequencies at concentrations reaching 2,117 and 32 *μg/kg*, respectively (Fufa and urga, 1996).

2.2.5.1 Level of aflatoxin in Ethiopian food

In Eastern Ethiopia, aflatoxin levels ranging from 5 to 250 *μg/kg* were detected in groundnut samples (Tadesse *et al.*, 2006), another study shows that 4.1*μg/kg* of aflatoxin was detected from maize in Ethiopia. Assefa *et al.*, (2012), identify two major aflatoxin-producing fungi namely, A. *flavus* and A.*nigur* from groundnut at Northern Ethiopia. Similarly, Assefa *et al.*, (2012) also reported that all the samples of sorghum and finger millet from Ethiopia were contaminated with Aspergillus species. Early in 2000 Wubet *et al.*, A. *flavus* was isolated from 70% of the maize samples and reported that 80% of the isolates can produce aflatoxin (Wubet *et al.*, 2000). A survey by Assefa *et al.*, (2012) from Northern Ethiopia indicates that there is 100% positive for Aspergillus fungi from samples of groundnut. The presence of aflatoxins in food means a risk for both animals and human beings. Another survey by Amare (2010) from Ethiopia discovered that Aspergillus species has arose from 94% of samples from all sample areas and aflatoxin was detected from 88% of the samples. Risk of aflatoxins exposure in Ethiopia is very high. Aflatoxin has impact on agriculture, health, trade, economy and food security. A survey by Ayalew, at different locations of Ethiopia from maize indicates that a Species of Aspergillus, Fusarium and Penicillium occurred in 94%, 76.5% and 64% of the samples, respectively. Aflatoxins were detected in 88% of the samples at 27 *μg/kg* in one sample and less than 5 *μg/kg* in others (Ayalew, 2010). Hence, this paper provides an overview on the impacts of aflatoxin on human an and economy (trade) in Ethiopia teff.

Table 2.4: Incidence of mycotoxins in the agricultural crops and foodstuffs in Ethiopia

Mycotoxin	Food stuffs	Concentration (ppb)	Reference
AFs	Shiro and ground red pepper	100-525	
AFs	Sorghum, barley teff and wheat	0-26	Fufa and urga, 1996
OTA	Sorghum, barley and wheat	54.1-2	
DON	Sorghum	40-2,340	Ayalew *et al.*, 2006
FUM	Sorghum	2,117	
ZEA	Sorghum	32	

2.2.6 Socio-economic impacts of aflatoxin contamination

Aflatoxins are among the main food contaminants with significant negative impact on health, food and nutritional security and incomes at the household, community and national levels. Food contaminated with aflatoxins may result in fatal aflatoxicosis and chronic mutagenic and carcinogenic effects with long latency periods. Aflatoxins also are associated with exacerbation of the protein malnutrition syndrome Kwashiorkor in human children. Estimating the human health effects of aflatoxins in terms of primary liver cancer, requires data on human exposure to aflatoxins. In developing countries, many individuals are not only malnourished but also are exposed chronically to high levels of this mycotoxin in their diet (Coulibaly., 2008).

The economic impact of aflatoxin contamination depends on the influence that the susceptible commodity makes to a country's consumption and income. It depends on the commodity's share in the nutrient requirements for the household, its share as a source of income derived via domestic and international trade, and the extent of awareness about the problem within households and markets (Wu *et al.* ,2010).

If there is general awareness of aflatoxin in a country and there are supporting regulations and institutions, then the human health impact of aflatoxin contamination will be low, but market impact will be high. This is because producers will have to bear the burden of reduced revenues from discarded grains or costs borne for prevention and control strategies. On the other hand, if

awareness is low and there are inadequate regulations to control it, aflatoxin-contaminated grain will trade freely, in which case the health impacts will be high, this is largely true in Ethiopia. Most of maize production in Africa is used for a producer's own consumption, implying that the human health impact will be the greatest if there is lack of awareness about aflatoxin. Aflatoxins disproportionately impact the poor. Food-insecure households are more likely to consume contaminated food rather than sell it at lower prices or discard it. The poor may also not be able to adopt costly control strategies. A well-meant awareness campaign can reduce prices for aflatoxin-contaminated food, resulting in direct market losses for the poor and more severe health impacts because of farmers' own consumption of low-price-yielding, contaminated grain. Therefore, policies and regulations to control aflatoxins require care in accounting for the distributional impact (Coulibaly., 2008).

2.2.5 Methods for detection and quantification of aflatoxins

Several methodologies for detection and quantification of AFs have been developed. The principal immunochemical based assay is the widespread enzyme linked immunosorbent assay (ELISA). Other methodologies base their performance upon electrochemical and optical principles such as: chromatography, UV-absorption, spectrometry, fluorescence and immunochemical assay tests. The methods require well equipped laboratories, trained personnel, harmful solvents and several hours to complete an assay. New methods for detection of aflatoxins try to avoid these disadvantages. Among such novel methods, it can be found: biosensors, electrokinetics, electrochemical transduction, amperometric detection, and adsorptive stripping voltammetry. Each of the methodologies has its own advantages and limitations according to sensitivity, easiness of use and cost-effectiveness (Vosough *et al.*, 2010)

2.2.5.1 Chromatography method

Chromatography is one of the most popular methods to analyse mycotoxins such as aflatoxins. The most common techniques of chromatography are Gas chromatography (GC), liquid chromatography (LC), High performance liquid chromatography (HPLC) and Thin-layer chromatography (TLC). From these methods, LC and HPLC are the most used. In many cases, they are followed by fluorescence detections stage. LC, TLC and HPLC are the most used quantitative methods in research and routine analysis of aflatoxins (Vosough *et al.*, 2010); these

techniques offer excellent sensitivities, but they frequently require skilled operators, extensive sample pre-treatment and expensive equipment.

2.2.5.1.1 High Performance Liquid Chromatography (HPLC)

HPLC is the most common methods to detect and quantify aflatoxins in food. It has been used jointly with techniques such as UV absorption, fluorescence, mass spectrometry and amperometric detectors. High Performance Liquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). The sample is carried by a moving carrier gas stream of helium or nitrogen. HPLC can separate and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Because of this versatility, HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals.

Sample retention time will vary depending on the interaction between the stationary phase, the molecules being analyzed, and the solvent, or solvents used. As the sample passes through the column it interacts between the two phases at different rate, primarily due to different polarities in the analytes. Analytes that have the least amount of interaction with the stationary phase or the most amount of interaction with the mobile phase will exit the column faster.

Elizalde-González *et al.*, (1998) analyzed aflatoxins B1, B2, G1 and G2 based on HPLC and amperometric detection, and report that it is possible to detect 5 *ng* of all four aflatoxins. This proposed method is recommended for detection and quantification of the less toxic aflatoxin B2, which is presented in grains. Quinto *et al.*, (2009) proposed a new method for determine aflatoxins B1, B2, G1, and G2 in cereal foods. This method is based on solid phase microextraction coupled with HPLC and a post-column photochemical derivatization to improve the fluorescence of analytes and fluorescence detection. Such method is fast compared with the complete analytical process that uses Immunoaffinity column. However, its sensibility is below the legal limits.

There are several techniques that use chromatography for aflatoxin analysis in food (principally in milk, cheese, corn, peanuts, nuts). Commonly the quantification of the aflatoxins is made by a fluorescence detector that takes advantage of fluorescence properties of aflatoxins under determined wavelength. As a result, researchers have been focused on improving these fluorescence properties to develop more sensitive methods than the commonly used so far. Currently techniques such as pre-column derivatization and post column derivatization are commonly used to improve aflatoxins fluorescence properties. They also have a clean-up stage to obtain a purer sample, permitting a better quantification. Some of the common methods used in the clean-up stage are: immunoaffinity column and solid phase extraction.

CHAPTER THREE

MATERIALS AND METHOD

3.1 Teff sampling area

According to Ethiopian agricultural transformation agency 40 teff commercial farms are given licence to export teff. From 40 licenced commercial farms 11 were selected purposefully, which is, four samples from West Gojam, two samples from East Gojam, two samples from Raya zone, two samples from Nazerit and one sample from Guraga zones, of Ethiopia (Table 3.1). The criteria for selection is from the 40 farms the 11 farms are already harvest and they are searching for export market. Experiments were carry out from December 2017 to April 2018 in Addis Ababa University Centre for Food Science and Nutrition, Food toxicology Laboratory.

Table 3. 1: location of selected commercial farms

Farm Sample code	location	No people for KAP
TF100A	West Gojam	6
TF100B	West Gojam	6
TF101	West Gojam	6
TF102	East Gojam	5
TF103	East Gojam	5
TF104	West Gojam	5
TF200	Guraga Zone	6
TF300	Raya	5
TF301	Raya	6
TF400	Nazerit	5
TF401	Nazerit	5

3.2 Methodology for the study of knowledge, attitude and practice (KAP) towards aflatoxin contamination

A total of 60 person who were involved in the production of teff, from commercial farms wear selected for this study. where 12 are owner and 48 of them are employers of the farms. These people were randomly selected from the list of farms by the researcher and, the number of participant from each farm is listed in table 3.1.

3.3 Method for measuring storage temperature and RH

Dailey storage Temperature and RH was measured for consecutive 7 days and the average temperature and RH was calculated. Aflatoxin content of the sample as a function of weekly average storage temperature and RH was constructed and discussed

3.4 Sample collection

The weight of the incremental sample taken from a lot was about 100 grams, 'incremental sample' means quantity of material taken from a single place in the lot or sublot (Table 3.2). The following formula (Eq (1)) was used as a guide for the sampling of lots traded in individual packs, such as sacks. Sampling frequency (SF): every nth sack or bag from which an incremental was taken (decimal figures was rounded to the nearest whole number). (Official Journal of the European Union, 23 February, 2006).

$$\textbf{Sampling frequency (SF)}n = \frac{\text{Weight of the lot} \times \text{Weight of the incremental sample}}{\text{Weight of the aggregate sample} \times \text{Weight of individual packing}} \quad \textbf{Eq (1)}$$

Where: -

Weight: in kg

Table 3. 2: Number of incremental samples taken from each farm

Farm Sample code	Lott weight (kg)	Weight of the incremental sample	Weight of the aggregate sample	Weight of individual packing	Sampling frequency (SF) n
TF100A	200000	0.1	10	100	20
TF100B	200000	0.1	10	100	20
TF101	195000	0.1	10	100	20
TF102	80000	0.1	10	50	16
TF103	50000	0.1	10	50	10
TF104	150000	0.1	10	50	30
TF200	30000	0.1	10	50	6
TF300	50000	0.1	10	50	10
TF301	25000	0.1	10	50	5
TF400	70000	0.1	10	50	14
TF401	15000	0.1	6	50	5

Table 3.2 and annex B shows how the sampling was done. Samples were properly labelled with the name of the commercial farm, code and sample collection date and properly wrapped with zipper freezer bags (Falcon®) to preserve from physical and microbial damage (figure 3.1). During the survey, data were collected to assess knowledge, practice and attitude of the farmers on food processing, handling and storage.

Figure 3.1: Sample collection

3.5 Moisture content determination and sample preparation

The collected teff sample was initially analysed for moisture content according to weight loss after drying method. Moisture content was determined by AOAC 2010 Method 925.10. The Aluminium dishes used for the moisture determination were dried at 130 ^oC for 1hr using an air oven (Memmerty®). The dishes were removed and kept in desiccator for about 30 minutes. The mass of empty dishes has been measured as M_1. This was continuing until constant weight was obtained. About 5 gm of the sample was weighed using analytical balance in to the dish and record as M_2. The sample was mixed thoroughly and dried at 100 ^oC for 6 hrs. After 6 hours it was kept in a desiccator to cool. After cooling the weight was taking as M_3. Then keep in oven for another 15 minutes. Then it was allowed to cool in a desiccator until constant weight was recorded. Then, the moisture content has been calculated based on the mass difference. Moisture content was expressed as Eq (2).

$$\% \textbf{ Moisture content} = \frac{(M1-M2) - (M\,3 - M1)}{(M1-M2) - (M\,3 - M1)} \times 100 \qquad \textbf{Eq (2)}$$

Where: -

M_1 is mass of empty dish

M_2 is mass of teff before drying

M_3 is mass of teff and dish after drying

After moisture determination the sample was dried and milled for 15 minutes with mill to medium sized powder and passed through 20µm mesh size sieve.

3.6 Procedure for Aflatoxin analysis

3.6.1 Sample Extraction Clean Up

About 20 grams of dried ground sample was placed into a 1 litter 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 5 minutes at

high speed and then the extract was filtered through a filter paper (Whatman® No.4) and 10 *ml* of clear filtrate was transferred to a beaker and 40 *ml* of PBS solution was added. And it passed through 0.45 *µl* syringe to remove residual turbidity. Immunoaffinity column (Aflaprep®,1ml), with a capacity greater than 100 *ng* for aflatoxin B1 and recovery (80%-120%), were placed at ambient temperature for conditioning. The conditioned Immunoaffinity column was filled with 1 *ml* of the diluted extracts then about 25 *ml* of the extracted was passed slowly and continuously through the columns (approximately 2*ml/min*).

Figure 3.2: Sample Preparation and Clean Up for Aflatoxin Analysis

Then the column was rinsed by 10 *ml* of deionized water and some air was pressed through the column to make sure that all the residual fluids was removed from the columns. Then 1 *ml* of 100 % HPLC grade methanol was added to the column and waited for 10 minutes until the aflatoxin was dissolved. Then aflatoxins have been eluted (at flow rate of approximately 1 *drop/sec*) in 2*ml* amber glass vial. All eluted residues were collected by pressing air thoroughly through the column

3.6.2 Standard Preparation

Aflatoxin standards was obtained from Sigma Aldrich (St. Louis, MO, USA). For each aflatoxin, a stock solution of $5mgml^{-1}$ was prepare in benzene-acetonitrile (98: 2, *v/v*, 2 *ml*) and store at $20^{O}C$. The solution was calibrated spectrophotometrically at 350 *nm* (AOAC International, 2005). The working standard solution was prepared as follows: an aliquot (100 *ml*) of each aflatoxin stock solution transfer into a 10 *ml* calibrated flask, evaporate under nitrogen and re-

dissolved in chloroform by ultrasonication. An aliquot (100 *ml*) of this solution was evaporate under nitrogen and re-dissolved by the mobile phase (0.5*ml*), to obtain calibrant solutions at concentrations between 0.2 and 2 *mg* for AFB1 and, AFG1, and between 0.1 and 1 *mg* for AFB2 and AFG2.

3.6.3 Determination of Aflatoxins in Teff

Analysis of aflatoxins (B1, B2, G1 and G2) in the teff samples was carried out by the method of Scott and Lawrence (1997) with some modifications and method validations. The toxin was quantified using HPLC-fluorescence detector (Shimadzu LC RF-20A prominence L20495273405, US), equipped with a reverse phase C_{18} column (Hichrom C_{18}, 100 m*m* x 4.6 *mm*, 5μm, UK). The mobile phase was a mixture of a de-ionized water, methanol and - acetonitrile (60:25:15, *v/v/v*), vacuum filtered and sonicated for 30 minutes to degas. For better resolution isocratic method was applied throughout the analysis. Distilled and ultrapure water was used throughout mobile phase preparation.

After sample clean-up process 20μl elute was injected in to the HPLC and the operating condition were as follows: The Column temperature was 25°*c*; The flow rate was 1.0*ml* min^{-1} isocratic; and the running time were 20 minutes per analysis. The detection wavelength: excitation wavelength was 365 *nm*, and emission wavelength was 440 *nm*.

3.7 Quality assurance and quality control

The validity of the method was assured and controlled by determining and conducting, Identification of aflatoxin retention time, limit of Detection and Quantification (LOD and LOQ), linearity, accuracy, recovery, precision and working rang analysis result.

3.7.1 Identification of aflatoxin retention time

Peak identification of Aflatoxins was determined by injecting 10*ppb* individual and 2.*ppb*,20*ppb*,5*ppb*,20*ppb* mixed aflatoxins (AFG2, AFG1, AGB2, and AFB1) standard at the same condition. Then retention time precision was determined by percent relative standard deviation (%RSD), must be less than 2% (FDA, 2000).

3.7.2 Limit of detection and quantification

Limit of detection was determined by injecting (0.05, 1.5, 0.02, and 0.5) *ppb* individual aflatoxins standards (AFG2, AFG1, AFB2 and AFB1) to get the lowest amount of analyte greater than three times of noise level S/N > 3. In the same way LOQ was determined by injecting low concentration (0.1, 3, 0.1, and 1) *ppb* of individual aflatoxins standards (AFG2, AFG1, AFB2 and AFB1) to obtain the lowest amount of analyte, which can be reproducibly quantitated above the baseline noise, that gives S/N >10.

3.7.3 Linearity

Linearity between peak area and aflatoxin concentration was studied by selecting seven (2, 5, 10, 20, 30, 40 and 50) *ppb* standards and a regression equation was found by poling pick area (y) versus aflatoxin concentration(x) expressed in *ppb* and regression coefficient (r^2) Was cheeked if it is greater than 0.997 which is acceptable by FDA (FDA, 2000).

3.7.4 Accuracy and Recovery

The accuracy of this analytical method was cheeked based on the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used because it is not possible to prepare a blank sample matrix without the presence of the analyte. Accuracy criteria for an assay method (FDA), the mean recovery should be 100 ± 20% at each concentration over the range of 80-120% of the target concentration (FDA, 2000).

3.8 Calculation and interpretation of results

Concentrations of aflatoxins were quantified with reference to AFs standards. The calibration curve for the standards was established with a concentration range from (2 *μg/Kg* -50 *μg/Kg*). The toxin level was calculated with the formula Eq (3).

$$\mathbf{AFT}\left(\frac{\mu g}{kg}\right) = \frac{A \times T}{I \times W} \qquad \textbf{Eq (3)}$$

Where: -

A= *μg/kg* of aflatoxin as eluate injected

T= final test solution eluate volume (*ml*)

I= volume eluate injected into LC (*ml*)

W= mass (*g*) of commodity represented by final extract

3.9 Statistical Analysis

All HPLC readings were conducted in triplicate and the data was averaged and expressed in the form of mean plus or minus standard deviation. For statistical analysis, IBM SPSS statistics version 21 software were used. In the SPSS method, one - way analysis of variance (ANOVA) and paired samples T-test was performed to evaluate the level of total aflatoxin mean comparison between the study sites. Descriptive statistics and Microsoft excel was also used to summarize the data.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 KAP

4.1.1 General Information

This study assessed knowledge, attitude, and practices (KAP) of the employees and owners of teff commercial farms of Ethiopia, and 82 % of the respondents are employees, where the sample were collected and 18% were owner of the farm, on issues related to molds in teff. A survey was involving structured questionnaire with 60 respondents from different farm. Descriptive statistics and Weighted mean were calculated according to Eq 3, and the results of the study underline the need to raise farmer's knowledge about health risks associated with spoilage molds in food and prevention and management options.

$$\text{Weighted mean} = \sum \frac{\text{respond} * \text{no peopel respond}}{\text{total no of peopel}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Eq (3)}$$

4.1.2 KAP towards aflatoxin contamination

Table 4.1 revealed mean scores of the eight statements related to the food safety knowledge of aflatoxins contamination. Majority of the farmers (76.7%) had general knowledge about storage condition in which the statement on ". Storage of processed teff should be cleaned, dried, waterproof, free from infestation, and sealed to prevent water, rodents or insects from reaching?" revealed the highest mean score of 2.76. About 53.3% of the respondents responded to the statement on "Do you know that some climatic conditions (temperature, rain or humidity, rought, etc.) are conducive for the proliferation of poison forming molds in crops?" in which the mean score accounted for 2.38. Meanwhile, the lowest mean score (1.5) of the knowledge of aflatoxins contamination indicated that about 66.7% of the respondents responded to the statement on "Do you know that intake of teff with aflatoxins have adverse health implications?" The overall mean

score of 1.975 as revealed in Table 4.1 showed that most of the manufacturers had medium knowledge about food safety towards aflatoxins contamination in teff production. Another study f found that about 67.8% of farmers in Gujarat, India were in medium category towards knowledge of aflatoxins management in cereals.

Table 4.2 Shows the results of the six statements related to the food safety attitude of aflatoxins contamination. Majority of the farmers (53.3%) strongly agreed to the statement on "I believe that processed teff should be stored in clean, dry, free from infestation, and sealed to prevent water, rodents or insects" which contributed to the highest mean score of 4.

Table 4.1: Teff producer Knowledge on aflatoxin contamination

Statement	Respond %			Weighted Mean
	1*	2*	3*	
1. Storage of processed teff should be cleaned, dried, weatherproof, free from infestation, and sealed to prevent water, rodents or insects from reaching.	0 (0)	23.4(14)	76.7(46)	2.76
2. Transportation of processed teff should be loaded and unloaded properly to protect from damage.	13.3(8)	65(39)	20(12)	2.03
3. Transport vehicles should be examined for cleanliness, insect infestation, dampness or unusual odours.	36.7(22)	46.7(28)	16.7(10)	1.8
4. Have you heard about aflatoxins?	70(42)	8.3(5)	21.7(13)	1.52
5. Poor storage conditions will promote the presence of aflatoxins in foods.	63.3(38)	15(9)	21.66(13)	1.58
6. Do you know that intake of teff with aflatoxins have adverse health implications.	66.7(40)	16.7(10)	16.7(10)	1.50
7. Do you know conditions that favour mold on cereals?	21.67(13)	33.3(20)	45(27)	2.23
8. Do you know that some climatic conditions (temperature, rain or humidity, drought, etc.) are conducive for the proliferation of poison forming molds in crops?	15(9)	31.7(19)	53.3(32)	2.38
Overall Mean Score (n=60)				1.975

*Note: *1= Do not know, *2 = Not sure, *3 = Know.*

The second highest response responded by 30% of the manufacturers to the statement on ". I believe that teff that have been processed should be transported in a proper manner to protect from damage or dampness", where the mean score was 4.21. While the lowest mean score on food safety attitude towards aflatoxins contamination was 3.12, in which about 65% of the manufacturers are neutral to the statement on "I believe testing by appropriate methods of sampling and examination can prevent a hazard to health". Hence, from the survey conducted, the results revealed that attitude of the farmers were favourable with the overall mean score was

3.80. Based on past studies from (Toh *et al.*, 2000) found that knowledge and attitude were influenced and associated the behavioural actions. If people perceive the problems, there will become more aware of that risk. After that, they will seek the related knowledge and information to develop an attitude that will foster proper action to minimize the effects of aflatoxins contamination.

Table 4. 2: Food Safety Attitude towards Aflatoxins Contamination

Statement	Respond % (n)					Weighted Mean
	1*	2*	3*	4*	5*	
1. I believe that processed teff should be stored in clean, dry, free from infestation, and sealed to prevent water, rodents or insects.	0(0)	0(0)	10(6)	36.7(22)	53.3(32)	4.27
2. I am willing to destroy the rejected teff from the sorting activity.	0(0)	16.7(10)	28.3(17)	33.3(20)	21.7(13)	3.60
3. I believe that teff that have been processed should be transported in a proper manner to protect from damage or dampness.	0(0)	0(0)	8.3(5)	61.7(37)	30(18)	4.21
4. I believe that testing by appropriate methods of sampling and examination can prevent a hazard to health.	5(3)	8.3(5)	65(39)	13.3(8)	8.3(5)	3.12
5. I know that stick-into, teff kernels is possible presence of mold.	0(0)	0(0)	23.3(14)	60(36)	16.7(10)	4.0
6. I think that while trashing the crops, it will be contaminated with the faecal matter of animals.	8.3(5)	5(3)	15(9)	61.7(37)	10(6)	3.58
Overall Mean Score (n=60)						3.80

Note: 1 = strongly disagree, 2 *= Disagree, 3* = Neutral, 4* = Agree, 5* = strongly agree*

Table 4.3 shows the nine statements related to the food safety and hygiene practices of aflatoxins contamination. Majority of the manufacturers (73.3%) indicated that they make sure that warehouse for teff storage does not contain any openings to prevent entrance of rodents or leaks from rain to avoid contamination particularly aflatoxins in which contributed to the highest mean score of 2.65.

Table 4. 3: Food Safety hygiene practices towards aflatoxins contamination

Statement	Respond % (n)			Weighted Mean
	1*	2*	3*	
1. I clean and disinfect container (sacks) before use and re-use it to prevent aflatoxins formation.	33.3(20)	55(33)	11.7(7)	1.78
2. I Check the storage temperature and humidity.	100(60)	0(0)	0(0)	1.0
3. I do have a program to disinfect regularly the store and premises.	73.3(44)	21.7(13)	5(3)	1.32
4. I make sure warehouse for teff storage does not contain any openings to prevent entrance of rodents or leaks from rain.	8.3(5)	18.3(11)	73.3(44)	2.65
5. I test the product for aflatoxin.	60(100)	0(0)	0(0)	1.0
6. Follow the Good Hygiene Practices (GHP) to minimize the spread of aflatoxins contamination	46.7(28)	40(24)	13.3(8)	1.67
7. I conduct hygiene and GMP training program for workers.	81.7(49)	6.7(4)	11.7(7)	1.3
8. I use the legs of animals on the ground to trashing the crop	38.3(23)	11.6(7)	50(30)	2.12
9. I do you have the practice of removing old seed heads, stalks, and other debris that may have served, or may potentially serve as substrates for the growth of aflatoxin-producing fungi?	20(12)	30(18)	50(30)	2.30
Over all mean (n=60)				1.68

*Note: *1=Never, * 2=seldom and* 3=Always*

About 50% of the farmers respond for the question, do you have the practice of removing old seed heads, stalks, and other debris that may have served or may potentially serve as substrates for the growth of aflatoxin-producing fungi, in which accounted for mean scores of 2.3. While the lowest mean score was 1.0 no one of the manufacturers responded that they never test the product for aflatoxin and they never measure the temperature and relative humidity for the storage to minimize the spread of aflatoxins contamination in their premises. As a result, the

overall mean score from this study was 1.68, indicating that most of the farmers do not followed food safety and hygiene practices towards aflatoxins contamination in teff.

According to the (Hell *et al.*, 2000), adoption through good sanitation practices, good hygiene practices, and safe food handling practices particularly when handling, processing, preparing, storing, and transporting the products we can minimize mycotoxin contamination. Besides, there have another study from (Essono, 2009) indicated that aflatoxins levels were significantly correlated ($p<0.01$) with the processing practices, storage facilities, and storage duration. Thus, knowledge related food safety and all good practices can guide food handlers to emphasize the hygiene and sanitation in every stage involved is important to avoid from aflatoxins contamination into the products.

4.2 Moisture content of teff sample

The moisture content of teff samples were ranging from 9.54–11.73% with mean 10.44% (Table 4.4). Gebremariam *et al.*, (2014) reported that the moisture content of raw teff is in the range of 9.5-14%, another study shows, the moisture had ranged 11.22-9.30% with mean 10.53% (Bultosa, G, 2007), which is in the normal range for field dried teff grain.

Table 4. 4: Moisture content of teff sample

Sample code	Moisture content (%)
TF100A	10.07 ± 0.10^{d}
TF100B	11.73 ± 0.37^{a}
TF101	9.72 ± 0.07
TF102	10.08 ± 0.08^{d}
TF103	9.79 ± 0.10^{e}
TF104	9.54 ± 0.03^{f}
TF200	10.50 ± 0.04^{c}
TF300	10.02 ± 0.09^{d}
TF301	11.52 ± 0.01^{b}
TF400	11.40 ± 0.05^{b}
TF401	10.47 ± 0.02

4.3 Quality control and assurance aflatoxin detection method

4.3.1 Identification of aflatoxin retention time

Peak identification results are shown in Table 4.5. The retention time of individual and mixed aflatoxins gives a good precession having a range between (0.02 - 0.27) % RSD, which is acceptable according to FDA standard, less than 2% RSD.

Table 4. 5: Statistics for aflatoxin retention time identification

Aflatoxin	Aflatoxin injection retention time (Min)		N	Mean	Std. deviation	% RSD
	For Single run (10ppb)	For mixed run (5, 20, 5, 20 ppb resp.)				
AFG2	4.397	4.392	2	4.395	0.004	0.08
AFG1	5.434	5.428	2	5.431	0.004	0.08
AFB2	6.310	6.286	2	6.298	0.017	0.27
AFB1	7.907	7.901	2	7.904	0.004	0.05

The elution order of individual aflatoxins was in the order of AFG2, AFG1, AGB2 and AFB1 with 4.392, 5.428, 6.286. and 7.901 retention times respectively. In addition to the retention time chromatographic result for Blank (diluent), individual (AFG2, AFG1, AGB2 and AFB1) and mixed aflatoxins (figure 4.1-4.6) demonstrate the qualitative aspect of identification test is more defined and acceptable.

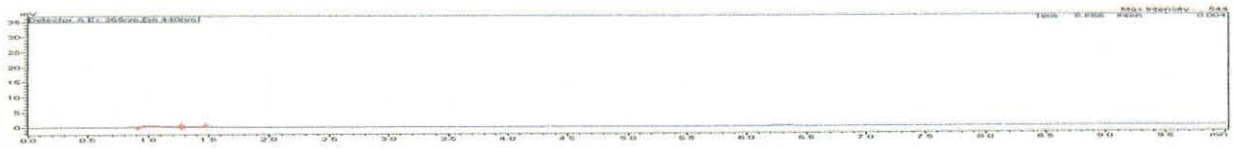

Figure 4.1: Chromatographic retention time for blank run

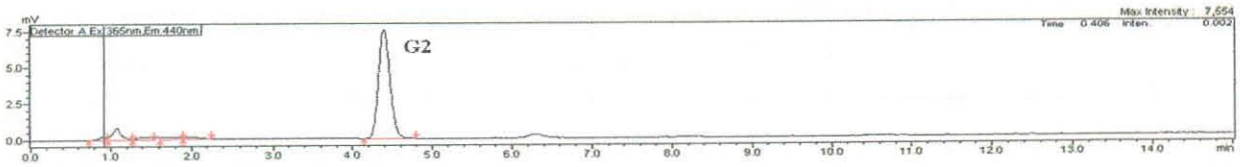

Figure 4.2: Chromatographic retention time for aflatoxin G2

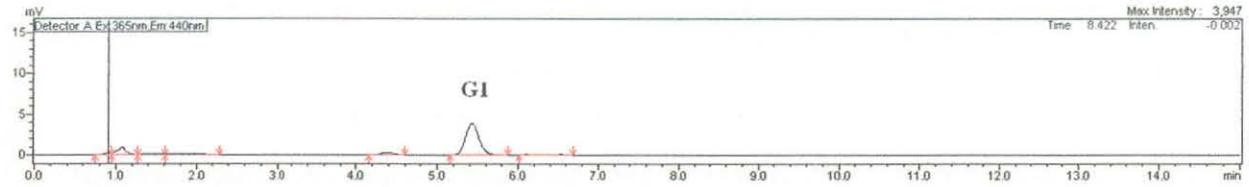

Figure 4.3: Chromatographic retention time for aflatoxin G1

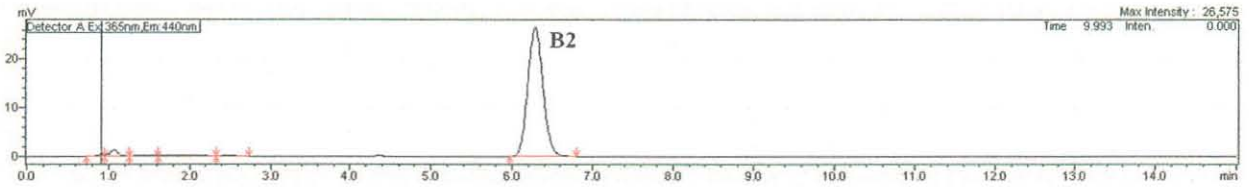

Figure 4.4: Chromatographic retention time for aflatoxin B2

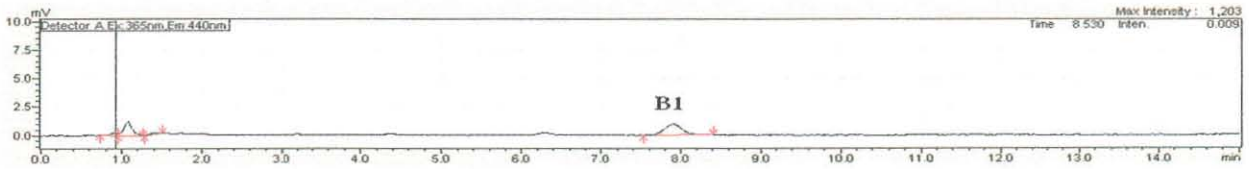

Figure 4.5: Chromatographic retention time for aflatoxin B1

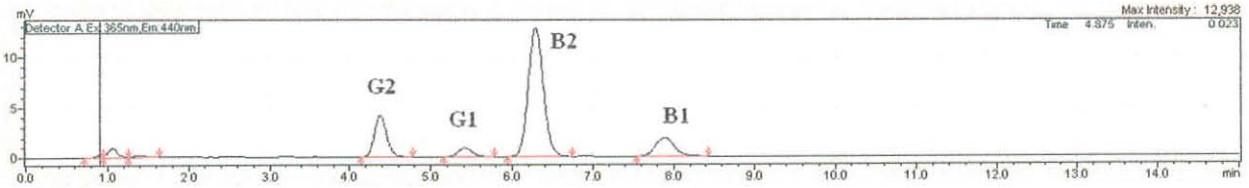

Figure 4.6: Chromatographic retention time for aflatoxin mixed standard

By overlaying individual aflatoxin retention time chromatogram over the mixed standard chromatogram (figure 4.7) it shows that it has a good relationship with each other.

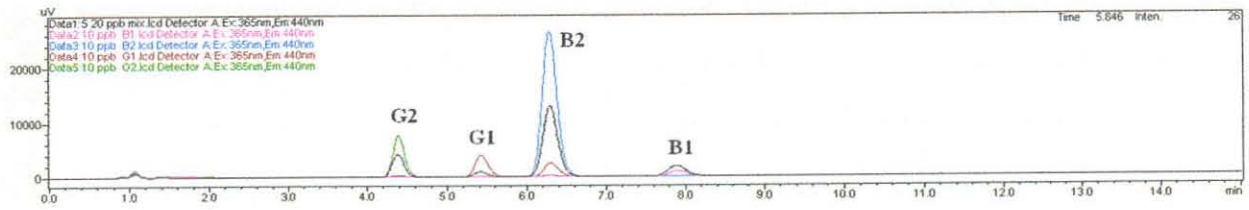

Figure 4.7: Overlay of chromatograms for mixed standards

4.3.2 Limit of detection and quantification (LOD and LOQ)

As shown in (Table 4.6) Limit of detection was determined by injecting (0.05, 1.5, 0.02, and 0.5) *ppb* of individual aflatoxins AFG2, AFG1, AFB2 and AFB1 respectively. And noise level S/N was 5.25,4.48,4.73 and 4.66 which, is the lowest amount of analyte greater than three times of noise level S/N > 3. In the same way LOQ was determined by injecting (0.1, 3, 0.1, and 1) *ppb* of individual aflatoxins AFG2, AFG1, AFB2 and AFB1 respectively to obtain the lowest amount of analyte, which can be reproducibly quantitated above the baseline noise, that gives S/N >10 it showed in table 4.2.2.

Table 4. 6: Statics for limit of detection (LOD) and limit of quantification (LOQ)

Aflatoxin Std	LOD		LOQ	
	Concentration in ppb	Signal to Noise Ratio, (S/N > 3)	Concentration in ppb	Signal to Noise Ratio, (S/N > 10)
AFG2	0.05	5.25	0.1	11.32
AFG1	1.50	4.48	3.0	10.47
AFB2	0.02	4.73	0.1	14.84
AFB1	0.50	4.66	1.0	10.13

4.3.3 Precision

As verified in (Table 4.7), the precision was evaluated through the repeatability of the method by assaying ten replicate injections of aflatoxin mixed standard of concentration (20 *ppb*), for AFG2 and AGB2 ,5*ppb* for AFG1 and AFB1 during the same day, under the same experimental conditions. It shows an acceptable percentage RSD which had a value of less than 0.5% and 3.0% for the retention time and peak area respectively. A precision criterion the instrument precision (repeatability) is normally expressed as the percent relative standard deviation for a statistically significant number of samples should be $\leq$ 5% RSD in FDA standard.

Table 4. 7: Statistics for precision check (Repeatability)

Aflatoxin	Injection Concentration (50 ppb)	N	Descriptive statistics of Peak area			Descriptive statistics of Retention time		
			Mean	STD	% RSD	Mean	STD	% RSD
AFG2	5 ppb	10	45573	1112	2.44	4.387	0.020	0.46
AFG1	20 ppb	10	11098	329	2.97	5.427	0.029	0.53
ADB2	5 ppb	10	174023	4485	2.58	6.302	0.026	0.42
AFB1	20 ppb	10	32494	827	2.54	7.903	0.039	0.49

4.3.4 Linearity

To confirm if there is linear relationship between concentrations (2, 5, 10, 20, 30, 40 and 50) *ppb* and peak area, a linear regression equation was plotted (Appendix A). The analysis was done in triplicate standard sample and the average was taken to construct calibration curve (Table 4.8).

Table 4. 8: Statistics for calibration data for each aflatoxin groups for linearity cheek

flatoxin G2			Aflatoxin G1			Aflatoxin B2			Aflatoxin B1		
					Peak Area						
al. I	Cal. II	Mean	Cal. I	Cal. II	Mean	Cal. I	Cal. II	Mean	Cal. I	Cal. II	Mean
42	3001	3072	453	548	501	10543	10121	10332	1140	1266	1203
97	6321	5959	1010	1095	1053	19778	22430	21104	2864	3125	2995
486	9997	10242	1948	2000	1974	37066	35755	36411	5923	5613	5768
943	18908	18926	3806	3916	3861	69347	68331	68839	11079	11231	11155
607	27559	27083	5434	5731	5583	98168	99962	99065	16007	16644	16326
827	36540	36184	7472	7702	7587	133262	133889	133576	21664	22330	21997
260	44942	45101	9498	9625	9562	168345	168354	168350	27440	27351	27396

The equation (Table 4.9) shows that individual aflatoxin had excellent relationship, and the respective coefficient of variation (R^2) was 0.9998, 0.9996, 0.9995 and 0.9999 for AFG2, AFG1, AFB2 and AFB1.According to FDA R^2 greater than 0.997 was found to be acceptable and had excellent relationship.

Table 4. 9: Statistics for calibration data for each aflatoxin groups and linearity cheek

Aflatoxin	N (Point)	Calibration Curve Equation	R^2
AFG2	7	y = 2,172.2x + 1,450.1	0.9998
AFG1	7	y = 469.1x + 94.52	0.9996
AFB2	7	y = 8,143.9x + 3,748.7	0.9995
AFB1	7	y = 1,357.2x + 229.8	0.9999

4.3.5 Working range

The range of this 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) obtained by the analytical method. As showed in table 4.10, the working range for G2 and B2 is from 0.2*ppb* to5 *ppb* for G2 and B1 is from 0.8*ppb* to 20 *ppb*.

Table 4. 10: Working range

Aflatoxin Sum (*ppb*)	Aflatoxin (*ppb*)			
	AFG2	AFG1	AFB2	AFB1
2	0.2	0.8	0.2	0.8
5	0.5	2.0	0.5	2.0
10	1.0	4.0	1.0	4.0
20	2.0	8.0	2.0	8.0
30	3.0	12.0	3.0	12.0
40	4.0	16.0	4.0	16.0
50	5.0	20.0	5.0	20.0

4.3.6 Accuracy and recovery

The accuracy of this analytical method was obtained by standard additions, which can also be used to determine recovery of spiked analyte. As illustrated in (Table 4.11), 8*ppb* and 40*ppb*

triplicate aflatoxin standard was spiked in teff sample and the mean percent recovery of response factor (area/concentration) was calculated. Accuracy criteria for an assay method (FDA, 2000), the mean recovery should be 100 ± 20%. The results of recovery and accuracy studies shown in the range between (80-103.5) % and it is evident that the method is accurate within the desired recovery range.

Table 4. 11: Statics for recover

Aflatoxins	Spiking Concentrations (*ppb*)		% Recovery				N	Mean	Std. Deviation	% RSD
	8 *ppb*	40 *ppb*	8*ppb*		40 *ppb*					
AFG2	2	10	98	99	98	104	4	99.8	2.87	2.88
AFG1	2	10	107	104	91	93	4	98.8	7.93	8.03
AFB2	2	10	70	68	89	93	4	80.0	12.83	16.04
AFB1	2	10	112	108	94	100	4	103.5	8.06	7.79

4.4 Level of aflatoxin in teff

For this study, teff samples were collected from 11 commercial farms of Ethiopia and 42 triplicate samples were analysed, including quality control samples. As shown in Table 4.12 from the samples analysed for AFB1, AFG1, AFB2, AFG2 all samples were found to be contaminated with a mean value of 2.56 *μg/Kg*. The highest AF concentration was 3.99 *μg/Kg* and the lowest concentrations was 0.5 *μg/Kg*. From the samples analysed, 13.88%, 0%, 8.38% and 77.73% were contaminated with quantifiable concentrations of aflatoxin G2, G1, B2 and B1 respectively. Aflatoxin G2 (13.88%) and B1 (77.73%) concentrations were relatively higher than the others. The European union commission regulation (EC) No 1881/2006 of 19 December 2006, states all cereals and all products derived from cereals, including processed cereal products, for aflatoxin B1 Maximum levels is 2 *μg/kg*, for the Sum of aflatoxin B1, B2, G1 and G2 Maximum levels is 4 *μg/Kg* (European Commission, 2006). when we see sample TF100A, TF100B, TF101, TF103 and TF301, aflatoxin B1 level 3.19,2.99,3.24 2.06 and 2.23 *μg/kg* respectively, which is above EU AFB1 limit.

Aflatoxin levels of teff samples collected from the commercial farms of Ethiopia were evaluated and compared against, Ethiopian teff flour standard and EU food safety guidelines. Accordingly,

the comparison reveals that from the total samples analysed, 91% contaminated by AFB1 and 45.45 % were unsafe for direct human consumption (2 *μg/kg)* tolerable intake level and, 27% of the samples very close to the 4 *μg/Kg* limit, which is greater than 3 *μg/kg* (table 4.12). Another research by Ayalew *et al.,* (2006), report that, the level of AFB1 was detected in 8.8% of the 352 samples analysed at concentrations ranging from trace to 26 μg/kg .similarly Aberra *et al.,* (1987),reported the positive numbers of white, mixed and red teff samples were significantly as low as 7 (9.8%),8(11.3%) and 10 (14.1%) *μg/kg*, respectively.

Table 4. 12: level of AF in teff sample

	Level of aflatoxin in *μg/kg (ppb)*				
Sample code	**AFG2**	**AFG1**	**AFB2**	**AFB1**	**Total AFs**
TF100A	0.37 ± 0.00^{e}	ND	0.20 ± 0.01^{e}	3.19 ± 0.22^{d}	3.76
TF100B	0.31 ± 0.02^{e}	ND	0.21 ± 0.00^{e}	2.99 ± 0.01^{d}	3.51
TF101	0.49 ± 0.01^{c}	ND	0.26 ± 0.01^{c}	3.24 ± 0.02^{d}	3.99
TF102	0.21 ± 0.00^{f}	ND	0.33 ± 0.15^{b}	1.53 ± 0.05^{b}	2.07
TF103	0.21 ± 0.01^{f}	ND	0.21 ± 0.01^{e}	2.06 ± 0.53^{b}	2.48
TF104	0.30 ± 0.01^{e}	ND	0.21 ± 0.01^{e}	ND	0.51
TF200	ND	ND	ND	1.89 ± 0.50^{b}	1.89
TF300	1.05 ± 0.03^{a}	ND	0.23 ± 0.02^{d}	1.66 ± 0.01^{b}	2.94
TF301	ND	ND	0.15 ± 0.00^{f}	2.23 ± 0.66^{c}	2.38
TF400	0.20 ± 0.01^{f}	ND	0.20 ± 0.01^{e}	1.52 ± 0.16^{c}	1.92
TF401	0.77 ± 0.00^{b}	ND	0.36 ± 0.02^{a}	1.58 ± 0.0^{c}	2.71
SUM	3.91	ND	2.36	21.89	28.16
Mean	0.36 ± 0.30		0.22 ± 0.09	1.99 ± 0.94	2.56 ± 0.99

ND: Aflatoxin is not detected, or it is less than the limit of detection, aflatoxin mean comparison *is along the column*

When we compare regionally samples from west Gojam have higher aflatoxin content (42 %) of the total aflatoxin level. East Gojam,Guraga zone, Raya and Nazerit the aflatoxin level was 16.2%,6.7%,18.9% and 16.4 respectively of the total aflatoxin.

Higher aflatoxin levels (3.99 *μg/kg*) were found at $25^{O}C$ average storage temperature and 65% store average relative humidity, and the moisture content was 11.73% which was higher than the others (table 4.13). This optimal temperature range is somewhat like that found by Schindler *et*

al, who obtained best production of aflatoxins between 25 and 35 ^{o}C. Considering the aflatoxin types, the highest contribution for the total level was from B1, B2, which was detected in most of the samples. This can be from different reasons such as, storage condition and moisture content.

Table 4. 13: Relationship between aflatoxin level, RH and storage temperature

Sample code	Storage Temperature (^{o}C)	RH of the store (%)	Aflatoxin Level ($\mu g/kg$)			
			AFG2	AFB2	AFB1	TAF
TF100A	31 ± 1.18	44 ± 0.40	0.77	0.36	1.58	2.71
TF100B	25 ± 0.90	65 ± 1.30	0.49	0.26	3.24	3.99
TF101	25 ± 0.01	45 ± 1.53[f]	0.21	0.33	1.53	2.07
TF102	29 ± 1.13	41 ± 0.92	1.05	0.23	1.66	2.94
TF103	25 ± 0.53	39 ± 0.95	0.21	0.21	2.06	2.48
TF104	28 ± 1.17	42 ± 0.20	ND	ND	1.89	1.89
TF200	30 ± 0.50	59 ± 0.65	0.20	0.20	1.52	1.92
TF300	29 ± 0.65	55 ± 0.25	ND	0.15	2.23	2.38
TF301	21 ± 0.26	64 ± 0.50	0.37	0.2	3.19	3.76
TF400	24 ± 0.83	62 ± 1.00	0.31	0.21	2.99	3.51
TF401	26 ± 0.74	47 ± 1.96	0.30	0.21	ND	0.51

***ND:** Aflatoxin is not detected, or it is less than the limit of detection*

The most important factor controlling the proportion of aflatoxin B1, B2, G1 and G2 produced by A.*flavus* appears to be temperature. Rabie and Smalley (1965) reported a temperature optimum of (20-26)^{0}C for aflatoxin (B1and B2). The optimum for (G1and G2) was between 29 and 30 ^{o} *C*. In this study sample TF102 and TF100A the level of aflatoxin and the storage temperature, which is examined is similar to Diener and Davis (1996) result, in which the level of aflatoxin G2 and B1 is almost in the ratio of 1:2 at 29 ^{o} *C* and 31 ^{o} *C* store temperature. whereas the ratio for TF100B, TF301 and TF400 is 1:7,1:9 and 1:10 and the temperature of the store was 25 ^{o} *C*, 21 ^{o} *C* and 24 ^{o} *C* respectively (Figure 4.8).

Sorenson *et al.,* (1967) found that at lower temperature (15 ^{o} *c* - 20 ^{o}*c*) essentially equal amount of aflatoxin B1 and G2 Were Produced on Rice by A. *flavus*. In this study at 25^{o}*c* storage temperature the ratio of B1 to G2 was about 2:1 and at 28 ^{o}*c* it was 5:1 and above. At higher temperature (32 ^{o}*c*) and above much less G1 and G2 was formed, and ratio was approximately 12:1 similar result was reported with cottonseed, rough rice, and peanuts.

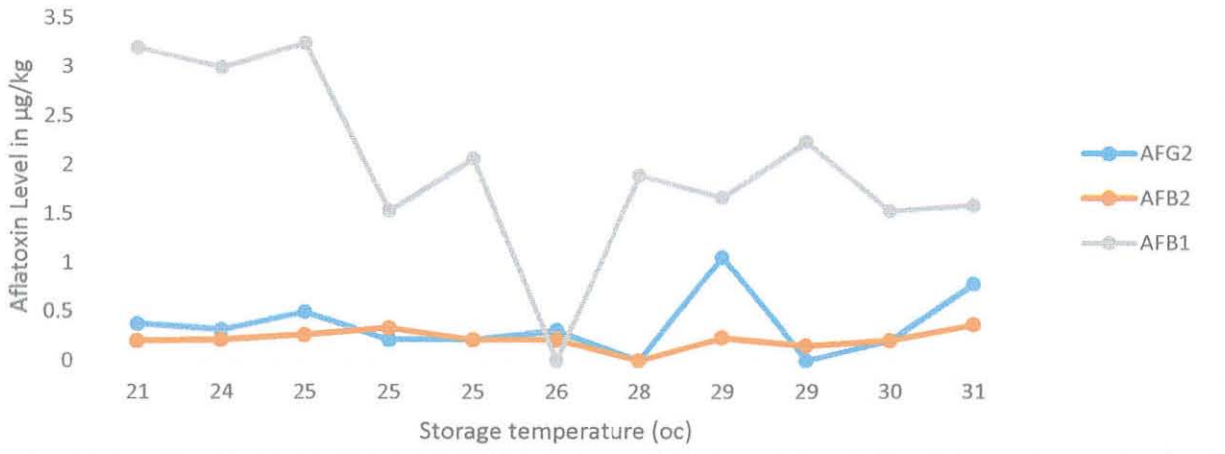

Figure 4.8:Aflatoxin content in teff as a function of weekly average storage temperature

The other factor which affect the aflatoxin type is maturity. Toxin was detected in some late planted cereals. Holbrook (2000) demonstrated in the laboratory that 1 year old cereals wear more readily invaded by A.*flavus* than Freshly dug immature and mature pods. A much higher percentage of A. *flavus* invasion occurred in overmatured cereals than in immature and mature cereals. However, this may leave a gap for future research for interesting researchers, to consider the effect of grain maturity in the level of aflatoxin in teff.

There was a significant positive linear relationship between aflatoxin B1 content in teff and moisture content (Figure 4.9). In the current study, there was a considerably high total aflatoxin content in sample TF100B ((3.24 ± 0.02) *µg/Kg*) at (11.73 ± 0.37) % moisture content. This result could be attributed to other confounding crop growth and environmental factors such as relative humidity. According to the fitted regression line (unstandardized $R^2 = 0.36$), moisture alone accounted for 36 % of the observed variations in aflatoxin content. Moisture content is an important variable influencing the colonization of pods by *Aspergillus flavus* and the subsequent aflatoxin contamination of grains.

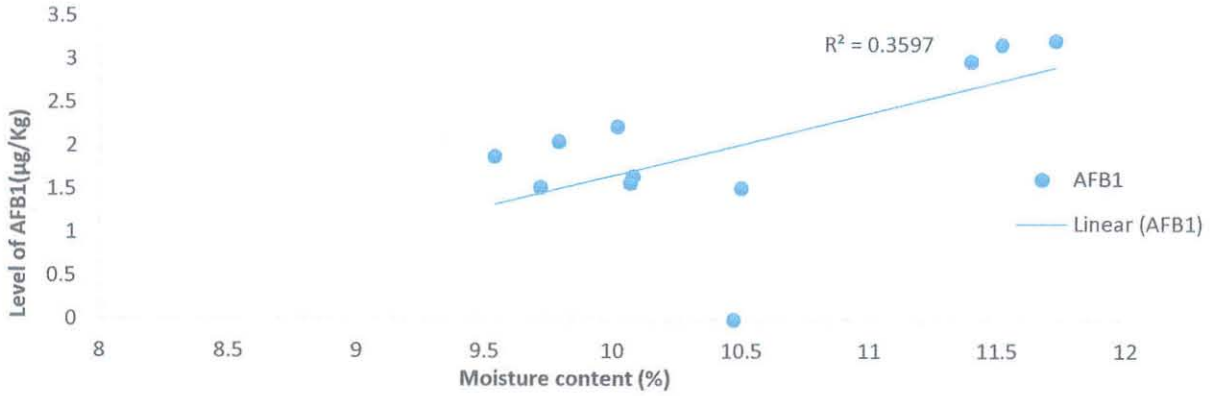

Figure 4.9: Aflatoxin B1 content in teff as a function of moisture content of teff

The other factor which affect the level of aflatoxin is storage relative humidity, in this study there was a positive linear relationship between aflatoxin B1 content in teff and storage relative humidity (Figure 4.10). There was a considerably high total aflatoxin content in sample TF100B (3.24 *µg/Kg*), TF 301 (3.19*µg/Kg)*, TF400 (2.99*µg/Kg*) at 65 %,64 %, 62 % relative humidity.

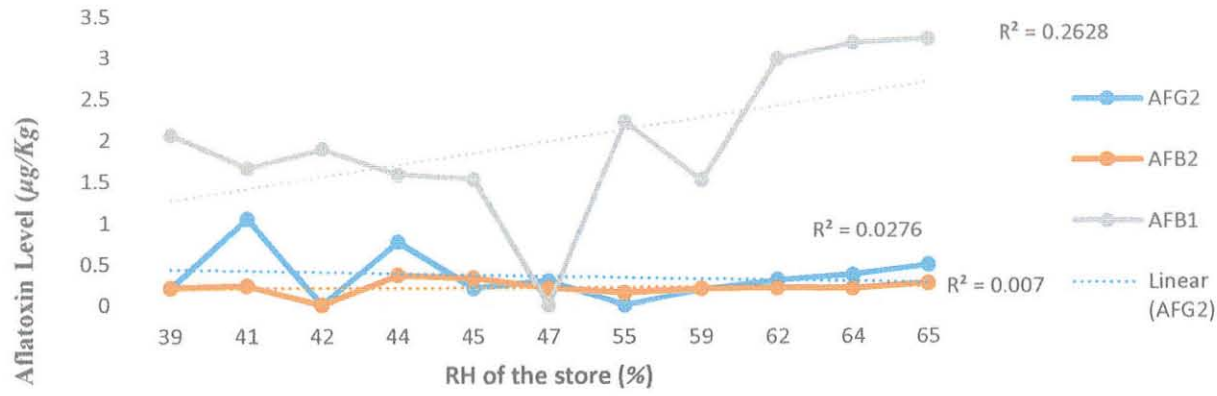

Figure 4. 10: Aflatoxin content teff as a function of weekly average relative humidity of the storage

According to the fitted regression line (unstandardized $R^2 = 0.26$), storage relative humidity alone accounted for 26 % of the observed variations in aflatoxin B1 content. However, this may leave a gap for future research for interesting researchers, to consider detailed study on the effect of relative humidity of the store in the level of aflatoxin in teff.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Teff is a comparatively good source of essential fatty acids, fiber, minerals (especially calcium and iron), and phytochemicals, such as polyphenols and phytates. Having a very good nutrient profile, recently teff become the new "super grain" of choice in Europe and North America and Ethiopia is ready to export teff for Europe and North America markets.

However, in the past decade, studies on the aflatoxin levels of teff is limited. The limited knowledge of teff's aflatoxin level with processing challenges faced in international market. This study measure aflatoxin level of teff produced by commercial farms and also evaluated knowledge attitude and practice of farmers towards aflatoxin contamination.

These studies have confirmed that among samples, which is collected from 11 commercial farms and analysed for AFB1, AFG1, AFB2, AFG2, all samples were below the maximum tolerable level (4 *μg/kg)* which is set by Ethiopian standard. But they are contaminated by AFB1 and it is unsafe for direct human consumption as per the Ethiopian and EU maximum (2 *μg/kg)* tolerable intake level

Although the amounts in tested samples were below the safe limit (4 *μg/Kg)*, as recommended by EU and Ethiopian standard, KAP study shows even though the commercial farmers have knowledge on mycotoxin related question their knowledge specific to AFs is inadequate. In addition to this, their practice towards reducing the contamination level of AFs is not adequate. But they have a positive attitude towards reduction of AFs level.

5.2 Recommendation

- In this study it has been shown that most of the samples it seems that they are not at risk which showed less than 4 *μg/Kg* but some preventive and detoxification measures of aflatoxins from cereals adopted by the joint WHO/FAO/UNEP conference on mycotoxins, should be recommended. During the cultivation, harvesting and storage periods of cereals all grains. and seeds must be protected from fungal contamination and aflatoxin formation.
- It is recommended that further analysis should be conducted on local producer and sample from market.
- All commercial farms should work in collaboration in minimizing aflatoxin contamination.
- Even though researches have been done about level of AFs in different cereals, this study shows that the problem on the occurrence of the toxin still persists. Therefore, as it is practiced in different food product the government should establish program to randomly take sample from export cereals and market places and cheek for AFs levels regularly and take corrective mitigation measures.

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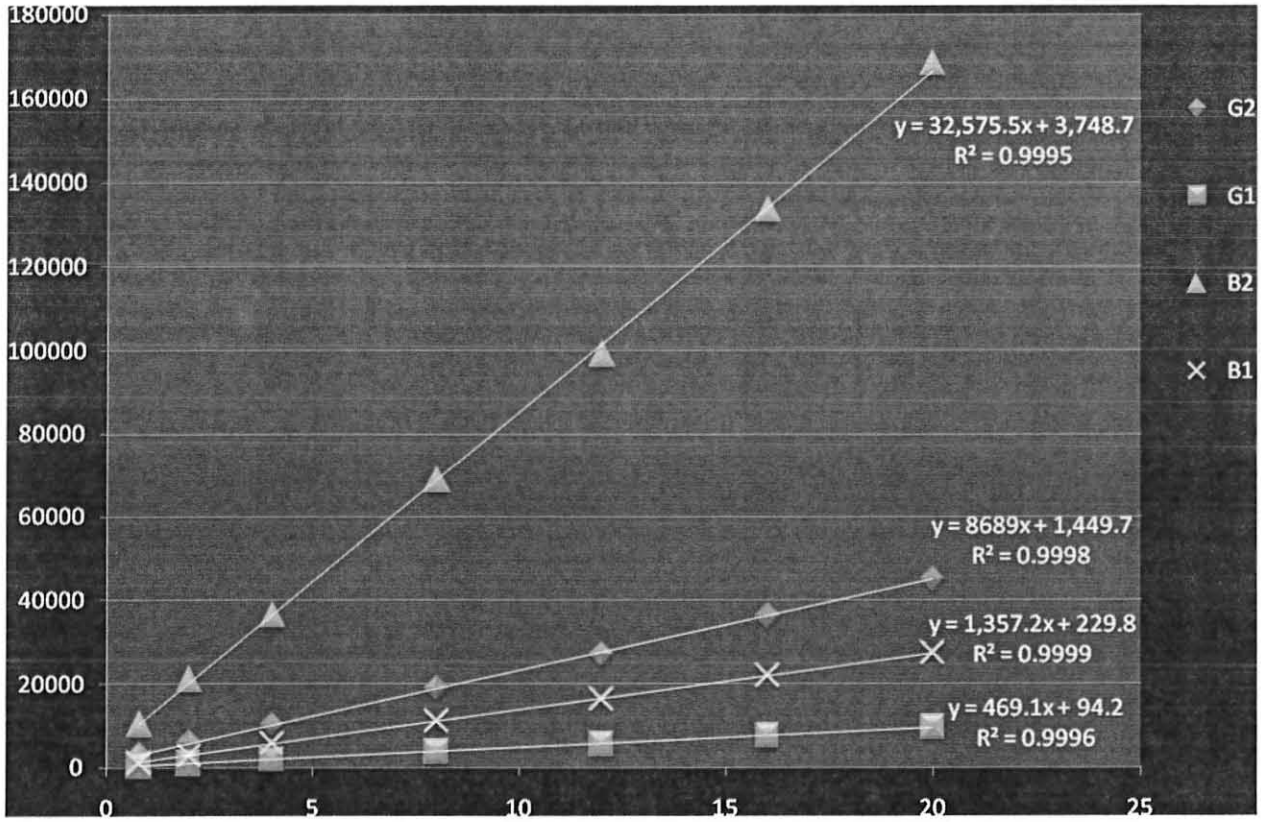
Appendix A

A. Linearity

Figure A.1: Calibration curves of four aflatoxins



Appendix B

B. Sampling

Table B. 1: Teff Sampling matrix

Lot weight (tonnes)	Number of incremental samples	Aggregate sample weight (kg)
≤ 0.05	3	1
>0.05 -≤ 0.5	5	1
>0.5-≤1	10	1
>1-≤3	20	2
>3-≤10	40	4
>10-≤20	60	6
>20≤50	100	10

Appendix C

D. Questionnaire for KAP analysis on Aflatoxin

Questionnaire for KAP analysis on aflatoxin

Dear respondent Good morning/Good afternoon. Thank you for your interest to talk this interview with me today. I am Haileab Negib who is a postgraduate student of Addis Ababa University center for food science and nutrition, conducting a study to determine the prevalence of aflatoxin in teff produced by commercial farms for export purposes in Ethiopia. The purpose of my visit today is to take information from you on the aforementioned issue. If you are willing to participate in the study, I will ask you few questions. Your honest answers to these questions will help me for a better understanding of the topic will eventually help in designing and implementing appropriate intervention to alleviate related problem. I greatly appreciate your participation in the study. I want to inform you that any information collected in this research will be utilized only for the research objectives
Date ----------
Are you willing to participate in the study? **Yes** ☐ **No** ☐
1.personal information
Name (if you are willing to give your name)
Address:
Telephone number
Job title
Educational background
Role in the commercial farm **Employee** ☐ **owner** ☐

nowledge on aflatoxins contamination				
tatement	Do not know	Not sure	Know	Comment
Storage of processed teff should be cleaned, dried, eatherproof, free from infestation, and sealed to prevent ater, rodents or insects from reaching.				
Transportation of processed teff should be loaded and loaded properly to protect from damage.				
Transport vehicles should be examined for cleanliness, sect infestation, dampness or unusual odours.				
Poor storage conditions will promote the presence of atoxins in foods.				
Do you know that intake of teff with aflatoxins have verse health implications?				
Do you know conditions that favour mold on cereals?				
Do you know that some climatic conditions mperature, rain or humidity, drought, etc.) are nducive for the proliferation of poison forming molds in ops?				

od safety Attitude towards aflatoxin contamination						
atement	Strongly Disagree	Disagree	Neutral	Agree	Strongly Agree	Comment
I believe that processed teff ould be stored in clean, dry, e from infestation, and sealed prevent water, rodents or ects.						
I am willing to destroy the ected teff from the sorting ivity						
I believe that teff that have en processed should be nsported in a proper manner to tect from damage or dampness						
I think that defective kernels uld be bagged separately and ged as unsuitable for human sumption						
I know that stick-into, teff nels is possible presence of ld						
think that while trashing the os, it will be contaminated n the fecal matter of animals						

gienic Practices towards Aflatoxin contamination				
tement	Never	Seldom	Always	Comment
I clean and disinfect container ore use and re-use it to prevent atoxins formation.				
I establish maintenance and itation procedures to ective control of aflatoxins.				
I do have a program to regularly infect the store and premises?				
I make sure warehouse for teff rage does not contain any openings prevent entrance of rodents or leaks m rain.				
test the product for aflatoxin?				
test the storage temperature.				
I conduct hygiene and sanitation ning program for workers.				
I use the legs of animals on the und to trashing the crop				
I do you have the practice of oving old seed heads, stalks, and er debris that may have served, or potentially serve as substrates for growth of aflatoxin-producing gi?				

Do you have any comments or suggestion for this research?----------
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---------- Thank you