ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL SCIENCE

CENTER FOR FOOD SCIENCE AND NUTRITION

STUDY ON *ASPERGILLUS* SPECIES AND AFLATOXIN CONTAMINATION OF PRE AND POST- HARVEST MAIZE GRAIN IN WEST GOJAM, ETHIOPIA.

BY: Masresha Ahmed

ADVISOR: Dr. Ashagrie Zewdu

A Thesis submitted to the School of Graduate Studies, Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Food science and Nutrition.

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LIST OF ABBERVATIONS AND ACRONYMS

AAU: Addis Ababa University

ACN: Acetonitrile

AF: Aflatoxins

AFB1: Aflatoxin B1

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2

AFBO: AFB1-8, 9-epoxide

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

AFs: Aflatoxins

ANOVA: Analysis of Variance

AOAC: Analysis of official Analytical chemist

CSA: central static‟s Agency

DON: Deoxynivalenol

EATA: Ethiopian Agricultural Transformation Agency

ECEA: Ethiopia Commodity Exchange Authority

EFSA: European Food Safety Authority

ELISA: Enzyme Linked Immunosorbent Assay

EU: European Unions

FAO: Food and Agricultural Organization

GAP: Good Agricultural Practices

GMO: Genetically Modified

HACCP: Hazard Analysis Critical Control Point

HPLC-FLD: High Performance liquid chromatography-fluorescence detector

IFPRPI: International Food Policy Research Institute)

LSD: Least significant Difference

PDA: potato dextrose agar

Ppb: Parts per billion

RATES: Regional Agricultural Trade Expansion support

TFA: Triflouroacetate acetic acid

µg/kg: Microgramme/kilogramme

ABSTRACT

Maize is an important crop for overall food security and for economic development in Ethiopia. However, the grain is vulnerable to degradation by mycotoxigenic fungi which include *Aspergillus, Fusarium and Penicillium.* Aflatoxins, a group of mycotoxins mainly produced by *Aspergillus flavus* and *Aspergillus Parasiticus*, have adverse health effects on humans and livestock that ingest aflatoxin contaminated food products and feeds. Therefore, the present study was designed to assess the level of *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize from West Gojam. A study was carried out from September 1, 2014 to May 1 2015. Pre-validated structured questionnaire was used to obtain information on maize production practices. *Aspergillus* species were isolated from Maize kernels on potato dextrose agar medium. Aflatoxin contaminations in Maize kernels were analyzed by High Performance liquid chromatography fluorescence detector. The results indicated that from fifteen pre- and fifteen post-harvest maize samples 77.7 % of pre harvest with level range from 3.13 to 63.66 µg/kg and 80 % of post-harvest sample with level range from 9.02 to 139.8 µg/kg were contaminated by total aflatoxin. The mean total aflatoxin was 18.38 µg/kg for pre harvest and 43.36µg/kg for post-harvest. Average aflatoxin concentrations in the samples were (AFG2= 2.10 µg/kg, AFG1= 10.10 µg/kg and AFB2= 1.17µg/kg) in pre harvest sample and (AFG2= 8.14 µg/kg, AFG1= 18.11 µg/kg and AFB2= 7.2 µg/kg) in post-harvest sample. In addition, the pre and post maize sample was also showed that high mean aflatoxin B1 level of 5.00 µg/k with 66.7% in pre harvest maize and 9.86 µg/kg with 87.7% in post-harvest maize. To be precise, Paired t-test statistical analysis for mean of total and aflatoxin B1 in pre and post- harvest maize samples was showed that both total and aflatoxin B 1 increased significantly from pre harvest to post - harvest maize ($p< 0.05$). 33.3 % of pre harvest and 73.3 % of post-harvest maize samples were exceeded the US Food and Drug Administration and the World Health Organization (tolerance limit of 20µg/kg). About 66.7 % pre harvest and 86.7% post-harvest of this study sample were exceeded the acceptance limit of total Aflatoxin recommended by European Union maximum limit (4 µg/kg). About 66.7 % pre harvest and 86.7% post-harvest of this study sample were exceeded the acceptance limit of Aflatoxin B1 recommended maximum limit (2 µg/kg) of European Union. This research has also shown that high *Aspergillus* species contamination in pre and post-harvest maize, in pre harvest 53.3 % of s maize samples were contaminated by *Aspergillus* species (26.7% = A.flavus, 13.3%= *A. parasiticus and 13.3%= A. niger) and* in post-harvest

79.9% of maize samples were contaminated *by Aspergillus* species *(*46.6 % = *A.flavus, 20.0 %= A. parasiticus and 13.3%= A. niger*) species. In conclusion, the results of the present study revealed that although it was expected that pre harvest maize to have minimal *Aspergillus* and aflatoxin contamination, the contamination was high in pre harvest and significantly increase from pre harvest to post harvest. Therefore, prevention through pre, harvest and postharvest control management practice should be done to ensuring a safe final product.

Key words: Mycotoxin, *Aspergillus* species, Aflatoxin, pre and post- harvest, Maize

1. INTRODUCTION

1.1. Background of the study

Maize is the third most important crop after rice and wheat cultivated in the world. It is a food that is part of the staple diet in Sub-Saharan Africa (Muthomi *et al.,* 2012). Maize is Ethiopia‟s leading cereal in terms of production, with 6 million tons produced in 2012 by 9 million farmers across 2 million hectares of land. Over half of all Ethiopian farmers grow maize, mostly for subsistence, with 75 % of all maize produced being consumed by the farming household. Currently, maize is the cheapest source of calorie intake in Ethiopia, providing 20.6 % of per capita calorie intake nationally .Maize is thus an important crop for overall food security and for economic development in the country (EATA, 2012). However, the grain is vulnerable to degradation by mycotoxigenic fungi which include *Aspergillus, Fusarium and Penicillium (*Muthomi *et al., 2012).*

*.Aspergillu*s species are the most common toxigenic species in various grains, legumes, oil seeds and foods and feeds. The two most agriculturally important species are *Aspergillus flavus and A. parasiticus,* which are found through-out the world, being present in both the soil and the air (Abbas *et al*., 2009). And they are the predominant fungi responsible for aflatoxin contamination of crops prior to harvest and during storage. Generally, tropical conditions such as high temperatures and moisture, monsoons, unseasonal rains during harvest, and flash floods lead to fungal proliferation and mycotoxins. Poor harvesting practices, improper storage, and less than optimal conditions during transport and marketing can also contribute to fungal growth and proliferation of mycotoxins (IFPRPI, 2003). Contaminated maize may appear normal without any visible signs of fungal infection (Muthomi *et al.,* 2012). Maize contamination by fungi does not only reduce its quality through discolouration and reduction of nutritional value but also lead to mycotoxin production (Bennet *et al.,* 2003; Harish *et al.,* 2013).

Mycotoxin poisoning in humans and animals occurs through ingestion, inhalation and absorption through the skin. Humans can be exposed to aflatoxins by the periodic consumption of contaminated food, contributing to an increase in nutritional deficiencies, immunosuppression and hepatocellular carcinoma (IFPRPI, 2003; Harish *et al.,* 2013). Mycotoxins that pose human health risks include aflatoxins, deoxynivalenol (DON), fumonisins, ochratoxins, and ergot

alkaloids. Some are produced before harvest (DON, ergot); some during and immediately following harvest (fumonisin, ochratoxin); and a few predominantly during storage (aflatoxin) (IFPRPI, 2003; Kumar *et al.,* 2000).

Aflatoxins are polyketide secondary metabolite produced by a very common food contaminating species *Aspergillus flavus* and *A. parasiticus*. There are 14 known aflatoxins, most of which are metabolites formed endogenously in animals administered by one major toxin, i.e., aflatoxin B1, B2, G1 and G2. *A. flavus* produces B1 and B2 while *A. parasiticus and A. nomius* produces all of these four major toxins (Peraica *et al.,* 1999). Among the 18 different types of aflatoxins identified, the major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2). Aflatoxin B1 is found widely and in greater concentrations than other naturally occurring forms of aflatoxin throughout the world in foods such as maize, peanuts and peanut products, cotton seed and its extractions, and, to some extent, chillies, peppers, and pistachio nuts (Ayyathurai *et al*.,2009). AFs are soluble in methanol, chloroform, and acetone. Its order of toxicity is B1 > G1 > B2 > G2. Letters „B‟ and „G‟ refer to its blue and green fluorescence colours produced by these compounds under UV light. Numbers 1 and 2 indicate major and minor compounds, respectively (Fadia *et al.,* 2014; Harish *et al.,* 2013).

1.2. Statement of the problem

The safety of food and feed for human and animal consumption should be of top most priority with regards to the regulation of agricultural and food industries (Bankole *et al.,* 2003). Mycotoxin attracts worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and trade (Harish *et al.,* 2013). Aflatoxin contamination is a serious food safety problem throughout the world (Abbas *et al.,* 2005).

Mycotoxins have been found in homes, agricultural settings, and food. Maize is one of the richest substrates for aflatoxin elaboration and even standing crop get high degrees of infestation (Wagach *et al.,* 2008).

Mycotoxins may have toxic effects ranging from short-term mucous membrane irritation to suppression of the immune system and cancer. The health effects of ingesting moldy foodstuffs might include acute (immediate) and chronic (long-lasting) damage to the liver, kidneys and gastrointestinal tract (Fadia *et al.,* 2014). The U.S Food and Drug Administration has set an aflatoxin limit of 20 parts per billion (ppb) for foods and for most feeds and feed ingredients. The European Union has enacted very severe aflatoxin tolerance level of 2µg/kg aflatoxin B1 and 4µg/kg total aflatoxins in nuts and cereals for human consumption (Ayyathurai *et al*., 2009) High-level exposure may cause instant death while long-term chronic effects include cancer, mutagenicity and nervous disorders (Muthomi *et al.,* 2012). Chronic exposure to aflatoxins is associated with impaired immunity, malnutrition and liver cancer which is the third most common cause of death from cancer in Africa. About 250,000-hepatocellular carcinoma related deaths occur annually in parts of sub-Saharan Africa due to aflatoxin ingestion alone (Wagacha *et al.,* 2008).

Aflatoxin contamination is not only a potential source of health hazards but is also involved in the spoilage of agricultural commodities (Kumar *et al.,* 2000). The regulations on the import and sale of aflatoxin contaminated food products results in huge losses each year to the agriculture and feed industries (Ayyathurai *et al*., 2009). Although aflatoxins are frequent contaminants of a wide variety of cereal grains and groundnuts, contaminated maize staples are the main source of dietary aflatoxins consumed daily, especially in developing countries (Nyandieka *et al.,* 2009).Maize is one of the major cereals crops of global importance, and has always been an important commodity to be traded overseas as food, feed and an industrial grain crop in several

countries. Unfortunately, it is also vulnerable to the growth of aflatoxigenic fungi, resulting into subsequent aflatoxin production which causes major yield and economic losses (Harish *et al.,* 2013). The Food and Agricultural Organization (FAO), estimates that between 25% and 50% of agricultural crops worldwide is contaminated by mycotoxins (Nyandieka *et al.,* 2009). The estimated value of maize lost to aflatoxin is $225 million per year, out of the $932 million due all the mycotoxins in the United States (Harish *et al.,* 2013).

In recent years, data on mycotoxins of maize in Africa have begun to accumulate with reports, for instance, from Kenya (Muthomi *et al.,* 2012), Nigeria (Ubwa *et al.,* 2012) and Benin (Sétamou *et al.,* 1997). In Ethiopia there are some reports but they have limited information on the occurrence of *Aspergillus* species and aflatoxins in pre and post-harvest maize, Habtamu Fufa (2001) has done on most commonly consumed agricultural commodities in some part of Ethiopia which was not more specific and far from my study area and Amare Ayalew (2010) has done some work on mycotoxins and surface and internal fungi of maize but the study was limited to the three cities (Dire Dawa, Adama and Ambo) which were far from my study area and they are not major maize producers. Research has also been done on prevalence of *A.flavus* in cereal in 1985 (Dawit *et al.,* 1985). In general there is limited works has been done on level of *Aspergillus* and Aflatoxin contamination in pre harvest and post-harvest maize in the world, especially from the same farmer. Therefore the present study is designed to assess the level of *Aspergillus* and Aflatoxin contamination in pre and post-harvest maize products in west Gojam Ethiopia.

1.3. Significance of the study

Aflatoxin contamination is not only a potential source of health hazards but it is also involved in the spoilage of agricultural commodities. This then calls for assessment of the occurrence of the level of *Aspergillus* and Aflatoxin contamination in pre harvest and post-harvest maize products in west Gojam Ethiopia. West Gojam zone is the first maize producer in Ethiopia but this zone has not received appropriate attention in pursuit of the level of *Aspergillus* and Aflatoxin contamination in maize. Therefore, data generated from the study will give an insight in to level of *Aspergillus* and Aflatoxin contamination in pre harvest and post-harvest maize products, Researchers, students, teachers, and academicians of the field area can use the findings of the study as a reference material, for the public it will create awareness about the Aflatoxin problem and It will recommend the manufacturers and if the production, quality and safety of the product increase it could be a potential export commodity in the future. The results from this study may lead for the development of Strategies for mycotoxin control and prevention and development of maize value chain.

1.4. Objectives

1.4.1. General Objective

- To assess the level of *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize grain in west Gojam, Ethiopia.

1.4.2. Specific objective

- To determine the distribution and levels of *Aspergillus* species in pre harvest and post-harvest maize products in west Gojam, Ethiopia
- To determine the level of Aflatoxins (B1, B2, G1, G2) contamination in pre harvest and post-harvest maize products in west Gojam, Ethiopia.

2. LITERATURE REVIEW

2.1. Overview of maize

Maize or corn (*Zea mays*) is a plant belonging to the family of grasses (*Poaceae*). It is cultivated globally being one of the most important cereal crops worldwide. Maize is not only an important human nutrient, but also a basic element of animal feed and raw material for manufacture of many industrial products. The products include corn starch, maltodextrins, corn oil, corn syrup and products of fermentation and distillation industries. It is also being recently used as biofuel. Maize is a versatile crop grown over a range of agro climatic zones. In fact the suitability of maize to diverse environments is unmatched by any other crop. It is grown from 58^{o}N to 40^{o}S, from below sea level to altitudes higher than 3000 m, and in areas with 250 mm to more than 5000 mm of rainfall per year. The major reason maize has spread so widely is its ability to produce high yield of grain under a wide variety of climate conditions (Nadzger, 2010).

2.2. Overview of the maize in Ethiopia

Maize has been introduced to Ethiopia in the 1600s to 1700s. In Ethiopia, it grows under a wide range of environmental conditions between 500 to 2400 meters above sea level. Maize is Ethiopia‟s leading cereal in terms of production, with 6 million tons produced in 2012 by 9 million farmers across 2 million hectares of land. Over half of all Ethiopian farmers grow maize, mostly for subsistence, with 75 % of all maize produced being consumed by the farming household. Currently, maize is the cheapest source of calorie intake in Ethiopia, providing 20.6 % of per capita calorie intake nationally (Diriba *et al.,* 2011; EATA, 2012).

2.2.1. Importance of maize in Ethiopia

Maize is an important crop for overall food security. Maize is also used for making local beverages. Additionally, the leaves and Stover‟s are used to feed animals and the stalks are used for construction and fuel. A small quantity of the grain produced is currently used in livestock and poultry feed, and this is expected to increase with the development of the livestock and poultry enterprises in the country. The green fodder from thinning and topping is an important source of animal feed and the dry fodder is used during the dry season. Moreover, the crop has potential uses for industrial purposes, serving as a starch, a sweetener for soft drinks, an input for ethanol fuel production and oil extraction, etc. Ethiopia is already a significant maize producer in Africa, and this role could be further enhanced. Currently,

Ethiopia is the fourth largest maize producing country in Africa, and first in the East African region. It is also significant that Ethiopia produces non-genetically modified (GMO) white maize, the preferred type of maize in neighboring markets. This strategy envisions exports markets being a significant part of the demand sink for Ethiopian maize (RATES, 2003; EATA, 2012).

2.2.2. Major maize producing areas

Maize is mainly grown in the four big regions of the country: Oromia, Amhara, SNNP, and Tigray. Oromia and Amhara contribute to almost 80% of the maize produced in 2012 (CSA, 2011/2012). Ten zones found in the two regions contributed to more than half of the national maize production in 2012. Among the top maize producing zones are: West Gojjam (5.6 million q), East Wellega (4.3 million q), Kaffa (3.8 million q), East Shewa (3.1 million q), West shewa (2.9 million q), West Arsi (2.7 million q), Illubabor (2.7 million q), East Gojjam (2.2 million q), West Wellega (2.1 million q), and West Harerghe (2.1 million q). Other regions such as Benishangul Gumuz and Gambela also grow maize and have the potential to increase their current production level in the future.

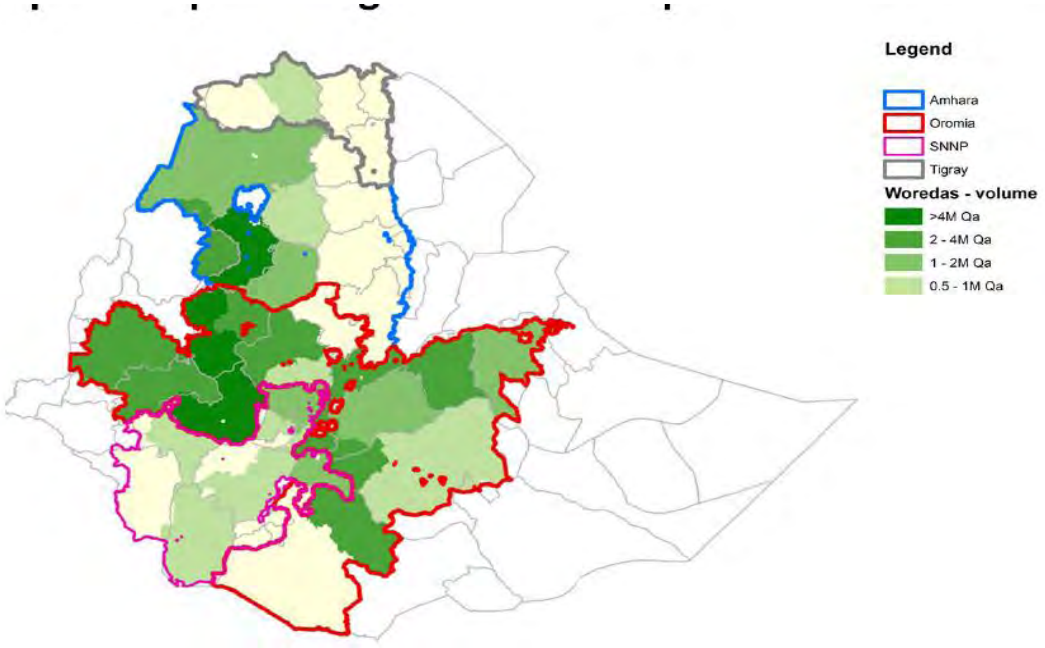

Fig 1: Top maize producer in Ethiopia (maize Ethiopia) (EATA, 2012).

2.2.3. Components of the maize value chain

To ensure all components of the maize sector in Ethiopia are addressed in a comprehensive and coordinated manner, a value chain approach is being followed. The core components identified within the Ethiopian context include: research and technology development; access to inputs; on-farm production; post-harvest processing & storage; trade, marketing and demand sinks. These value chain components are shown in Figure 2

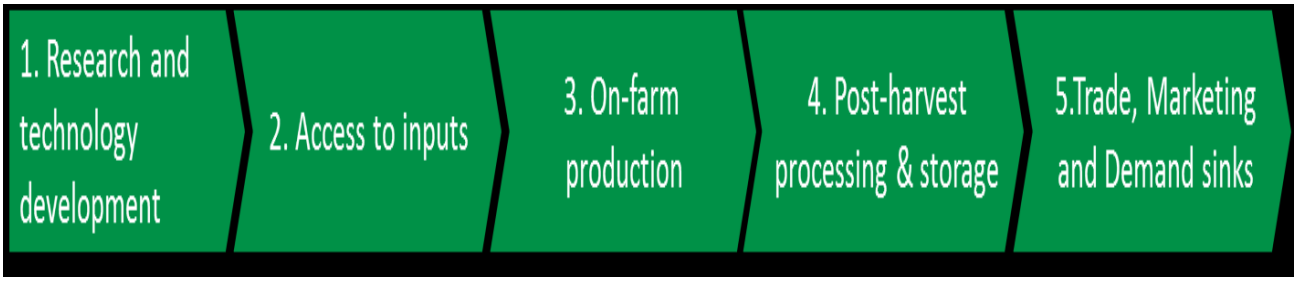

Fig 2: Maize value chain (EATA, 2012).

2.3. The genus Aspergillus

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. The first attempt to define the genus *Aspergillus* was made by Micheli in 1729. Although *Aspergillus* is a separate genus, it is closely related to *Penicillium* species in the fungal kingdom (Rahul *et al.,* 2014). The genus *Aspergillus* includes over 200 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man. Among these, *Aspergillus fumigatus* is the most commonly isolated species, followed by *Aspergillus flavus* and *Aspergillus niger*. *Aspergillus clavatus, Aspergillus glaucus* group, *Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus ustus*, and *Aspergillus versicolor* are among the other species less commonly isolated as opportunistic pathogens (Clement *et al*., 2013 and Rahul *et al.,* 2014). The two most agriculturally important species are *Aspergillus flavus* and *A. parasiticus*, which are found through-out the world, being present in both the soil and the air. When conidia (spores) encounter a suitable nutrient source and favorable environmental conditions (hot and dry conditions) the fungus rapidly colonizes and produces aflatoxin (Abbas *et al.2009*).

2.3.1. Transmission and life cycle *Aspergillus flavus*

Aspergillus flavus grows saprophytically in infected plant tissues such as maize kernels, cobs, and leaf tissue that remain in the soil and contribute to primary inoculum. *A. flavus* is capable of surviving and overwintering in plant residues as mycelium (hypha) or sclerotia. The sclerotia either germinate to produce additional hyphae or they produce conidia (asexual spores), which can be dispersed in the soil and air. The inoculum for *A. flavus* is spread through water, wind and also transmitted through insects and bird damage. During the growing season, infected plant tissues can serve as sources of secondary conidial inoculum, which colonize new non infected plant tissues (Abbas *et al.,* 2005 and Abbas *et al.*2009).

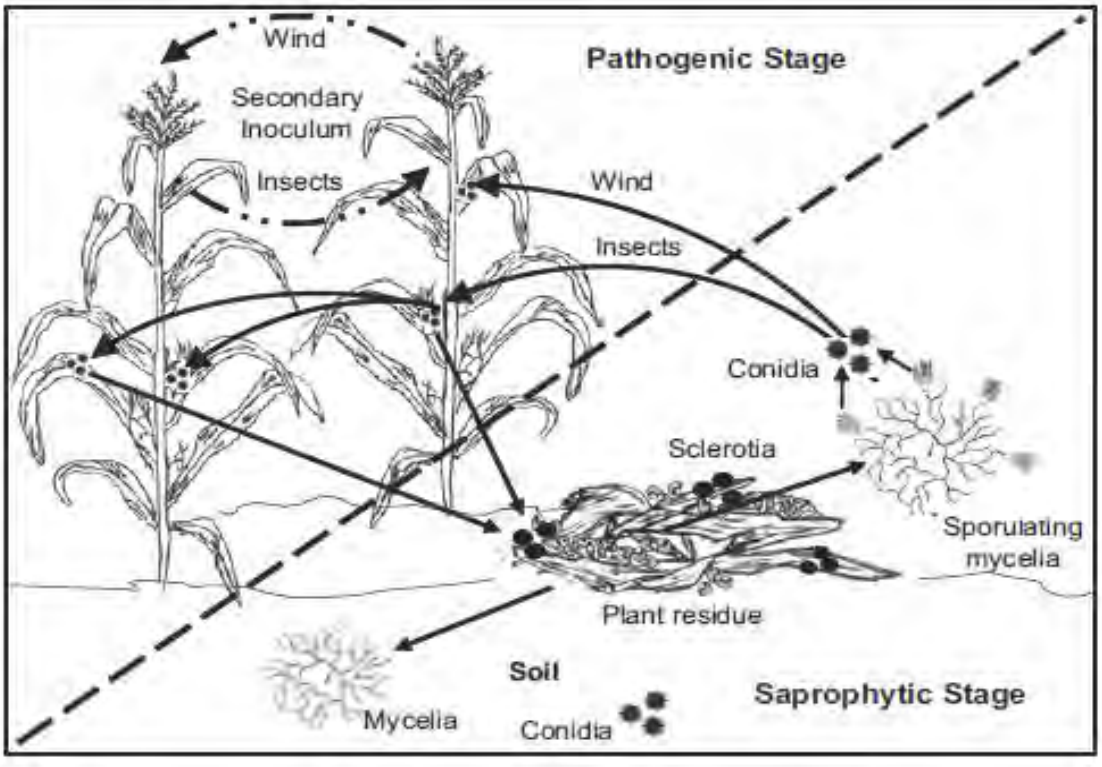

Fig 3: Life cycle of A. flavus in maize (Abbas *et al.*2009)

2.4. Aflatoxins

Aflatoxins are polyketide secondary metabolite produced by a very common food contaminating species *Aspergillus flavus* and *A. parasiticus*. There are 14 known aflatoxins, most of which are metabolites formed endogenously in animals administered by one major toxin, i.e., aflatoxin B1, B2, G1 and G2. *A. flavus* produces B1 and B2 while *A. parasiticus* produces all of these four major toxins (Peraica *et al.,* 1999). These toxins are usually found together with various foods and feeds in various proportions; however, aflatoxin B1 is usually predominant and is the most toxic. Aflatoxins G1 and G2 are formed only by *A. parasiticus.* When B1 and B2 are ingested by cattle, a portion of these aflatoxins is metabolized to M1 and M2 which may be found in the dairy milk. The produced aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) are difuranocoumarin derivatives and potent hepatic (Harish *et al.,* 2013). AFs are soluble in methanol, chloroform, and acetone. Its order of toxicity is B1 > G1 > B2 > G2. Letters „B‟ and „G‟ refer to its blue and green fluorescence colors produced by these compounds under UV light. Numbers 1 and 2 indicate major and minor compounds, respectively. A. flavus only produces B aflatoxins, while *A. parasiticus* and *A. nomius* also produce G aflatoxins (Fadia *et al.,* 2014; Harish *et al.,* 2013).

2.4.1. Chemical structures of aflatoxins

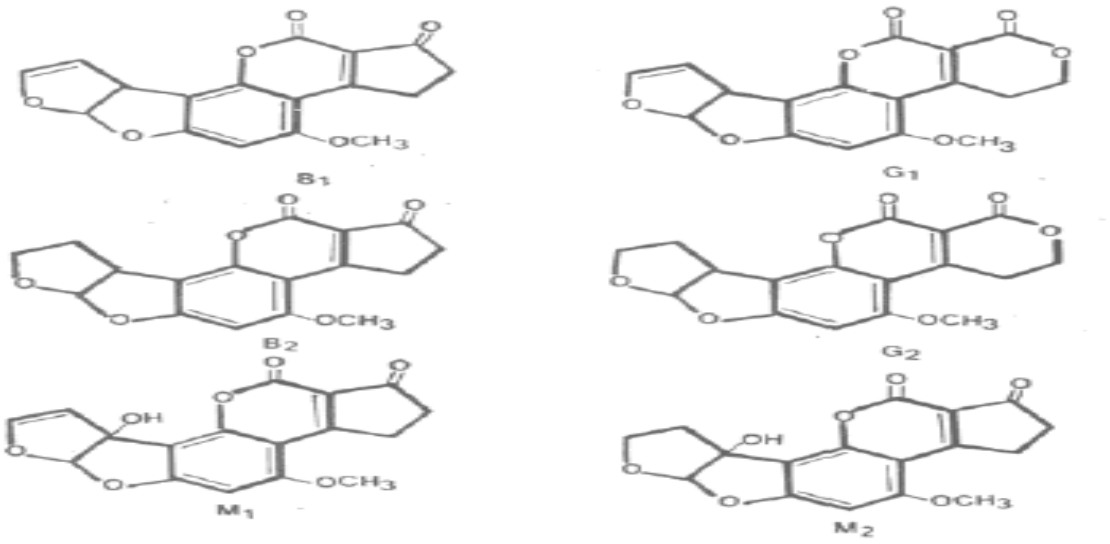

Fig 4: Structures of aflatoxin B1, B2, G1, G2, M1 and M2 (Harish *et al.,* 2013)

2.5. Review of related work done on *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize

Maize has been documented by several authors as an excellent substrate for mold growth and aflatoxin contamination.

A research conducted in India In order to understand the magnitude of aflatoxin contamination, a survey was conducted in different agro-ecological zones. About 242 samples consisting of pre- and post-harvest maize kernels, food products, poultry and livestock feeds were collected from farmers' fields, poultry farms, retail shops and supermarkets and analyzed for aflatoxin B1 (AFB1) contamination by enzyme- linked immunosorbent assay (ELISA) using antiserum raised against aflatoxin B1-Bovine serum albumin (AFB1-BSA). The results indicated that 61.3% of the maize kernel samples were contaminated with AFB1 and the levels of AFB1 in 26% of the pre- and post-harvest maize kernels exceeded 20µg/kg. The highest level of AFB1 (245µg/kg) was recorded in post-harvest maize kernel samples. In food products AFB1 was detected only in two samples out of 30 samples tested. Furthermore, the levels ranged from 0.6 to 3.7µg/kg. In poultry feeds, AFB1 was detected in 30 out of 53 samples and the levels ranged from 0.7 to 31.6µg/kg. Among the 40 livestock feed samples evaluated 29 samples were contaminated with AFB1 at level ranging from 1.8 to 244.9µg/kg (Ayyathurai *et al*., 2009).

Similar research conducted in India in 2012 on Occurrence of aflatoxin contamination in maize kernels and molecular characterization of the producing organism, *Aspergillus*. Pre- and post-harvest maize samples were collected from major maize growing areas in Tamil Nadu, India. Aflatoxin contamination was observed in 40.22% of the samples tested of which, 22.97% of pre-harvest and 53.93% post-harvest maize samples were found to be infected with AFB1 and 12.05% of the total samples exceeded WHO permissible limit of 20 µg/kg. AFB1 contamination ranged from 0 to 149.32 µg/kg (Muthusamy *et al.,* 2012)

A research conducted in West Iran on *Aspergillus* and Aflatoxin B1 Contamination of Stored Corn Grains in Western Iran in 2015. The result showed that One-hundred *Aspergillus* isolates were collected and identified in to six species *i.e*. *A. niger* (40%) followed by *A. flavus* (27%)*, A.*

ochraceus (15%), *A. fumigatus* (10%), *A. japonicus* (5%) and *A. sclerotiorum* (3%). They also determined the Aflatoxin B1 (AFB1) contamination status in the samples by enzyme-linked immune sorbent assay (ELIZA). Natural occurrence of AFB1 could be detected in 77% of samples were ranging from 0.046-10.776 µg/kg (Khosrow *et al.,* 2015).

Similar study was conducted in Kenya in order to assess A*spergillus* and Aflatoxin B1 contamination of maize and maize products during the 2008 and 2009 growing seasons. The result showed that higher frequency of *A. flavus* than samples from North rift region. Isolation frequency of *A. flavus* was higher in semi-processed than in whole maize grain and flour. Aflatoxin B1 was detected in maize and maize products at levels above the national tolerance levels of 10µg/kg. Samples from eastern region had higher aflatoxin B1 levels of up to 136.4 µg/kg in semi-processed maize, 77.4 µg/kg in whole grain and 40.9µg/kg in flour. The favorable conditions created by high temperatures and periodic drought could explain the higher fungal and aflatoxin contamination of samples from eastern Kenya. In addition, the unfavorable drying and storage practices may compound the problem. The author concluded and recommended that there is need for continued mycotoxin awareness campaigns among farmers, traders, transporters and processors (Muthomi *et al.,* 2012).

A survey was conducted on the incidence of fungi, and the natural occurrence of aflatoxins and fumonisins in preharvest maize from fields in south-western Nigeria. Mycological examinations revealed the predominance of F. verticillioides (Zea mays) (syn. F.moniliforme), occurring in 89.3% of samples with a mean kernel infection of 49.4%, while *Aspergillus* flavus was isolated from 65% of samples having a mean kernel infection of 6.8%. Aflatoxin B1 was detected in 18.4% of samples with a mean of 22 µg/kg, while aflatoxins B2, G1 and G2 were present in 7.8, 2.9 and 1% of the samples with mean levels of 10, 8 and 7 µg/kg, respectively, in contaminated samples. Total aflatoxins ranged from 3 to 138 µg/kg in positive samples, with a mean of 28 g/kg. Fumonsin B1 was the predominant toxin detected in terms of frequency (78.6% of samples) and quantity (concentration range 70–1780 µg/kg, mean 495 µg/kg). Fumonisin B2 was detected in 68 samples (66%) with a mean of 14 µg/kg. Fifteen samples were contaminated with both aflatoxins and fumonisins (Bankole *et al*., 2004).

Similar study in Benin, West Africa was also conducted on *Aspergillus flavus* Infection and Aflatoxin Contamination of Pre-harvest Maize. Eighty and sixty maize fields were sampled in

1994 and 1995, respectively. The result showed that in 1994, *A. flavus* was detected in more than 80% of the fields, whereas in 1995 the fungus was found in 60% of the fields. The percentage of Aflatoxin contaminated was higher in 1994 (42.5%) than in 1995 (30%) (Sétamou *et al.,* 1997).

In Ethiopia a research was conducted on mycotoxins and surface and internal fungi of maize in 2004/2005. The result showed that Species of *Aspergillus*, *Fusarium* and *Penicillium* occurred in 94%, 76.5% and 64% of the samples, respectively. *Fusarium verticillioides* and *Aspergillus glaucus* were among the dominant species. *A. flavus* was less frequent. The mean levels of total fungal density ranged from 2.9x103 cfug-1 in maize samples from Dire Dawa, 1.9x104 cfug-1 for samples from Adama to 3.8x105 cfu g-1 for samples from Ambo. Aflatoxins were detected in 88% of the samples at 27µg kg-1 in one sample and less than 5µg kg-1 in others. Fumonisins occurred in two samples from Dire Dawa at 700 and 240µg kg-1, and at 300µg kg-1 in one sample each from Adama and Ambo. Five samples contained DON at 50 – 700µg kg-1. NIV was detected at 50, 130 and 210µg kg-1. Aflatoxins were detected in 15 (88%) of the 17 maize samples; The mycotoxins analyzed played.a minor role in the contamination of maize of 2004/05 harvest in Ethiopia. The author conclude and recommended that further monitoring of mycotoxins in maize from different regions of the country is justified in order to conclusively determine the actual risks from mycotoxins and possibly low mycotoxin risk maize production areas (Ayalew, 2001).

A Survey of Aflatoxin contamination in Ethiopia was also conducted in 2001. Barley, wheat, maize, millet, sorghum, tef, pepper, peanut, broad beans and dry peas. Samples were collected from Southern Peoples Nations and Nationalities, Oromia and Harari Regional States. The result showed that Aflatoxin B1 was the predominant form; the incidence of samples containing it was 30% and then accompanied by aflatoxin G1 6%. The highest levels of aflatoxin B1, was observed in peanut and sorghum samples (738 and 692 µgkg-1, respectively). The highest level of aflatoxin G1 found was 201µgkg-1. Groundnut, sorghum and millet samples have been identified as high-risk commodities based on the incidence rate of aflatoxin contamination. Levels of total aflatoxin greater than 20 µgkg-1, were most frequently encountered in all aflatoxin positive samples of corn, sorghum, wheat, red pepper and peanut followed by barley (17%) and teff (13%) the result was also showed that 90% in in cereal and legume Samples is >20 µgkg-1 (Habtamu et *al.,* 2001).

A research was also conducted in Addis Ababa Ethiopia to assess of aflatoxigeinic *Aspergillus* species in food commodities from local market in 2013. The result showed that a total of 90 *Aspergillus* species (A. *flavus, A. parasiticus, A. niger and A. fumigatus*) were isolated from 108 samples of food commodities. The least number (5 isolates of *Aspergillus* species isolates were counted from the sample cookies while the highest (19 isolates) were counted from both the peanut and emmer wheat flour samples. From 33 *A.flavus* isolates 9 isolates (27.3%) were isolated from the peanut sample, while the fewest were isolated from the pea flour and cookies sample (n=3 (9.1%)). Six out of eighteen samples of peanut, emmer wheat flour, maize, and roasted barley were contaminated with *A. niger,* accounting (22.2%). A total of 21 (23.3%) isolates of *Aspergillus* species produced aflatoxin in vitro in SMKY broth culture out of 90 different *Aspergillus* species isolated from food commodities. Among the aflatoxigenic species isolated, *A. flavus* were responsible for the majority of cases (66.67%) followed by Aspergillus parasiticus (33.33%). None of the *A. fumigatus* isolates were found to produce aflatoxin in vitro on SMKY broth medium. Within species, 36.36% of *A. flavus* isolates were found to produce aflatoxin in vitro, while 18% of *A. parasiticus* isolates produced aflatoxin in vitro on culture media (Negero *et al*., 2014).

A research was also conducted on prevalence of *Aspergillus flavus* in Ethiopian Cereal grains in 1985 with prevalence of 88% (Dawit *et al*., 1985).

There are also some work have done by previous Addis Ababa University food science and nutrition MSc. students which have not published yet. These are Aflatoxin Content of Peanut (*Arachis hypogaea*) in Relation to Shelling and Storage Practices of Ethiopian Farmers, the result showed that 73.06% of peanut sample were positive for aflatoxin (Eshetu, 2010); Occurrence of aflatoxins in red pepper (capsicum annum L.): In relation with storage and quality grading in Ethiopia, the result showed that 100, 90, 80 and 70% of samples were contaminated by aflatoxin B1, G1, B2 and G2 respectively (Habtamu, 2014) and Aflatoxin and salmonella contamination in peanut butter and roasted peanut; the case of local and imported products from Addis Ababa Market, the result showed that 93 % of local peanut sample were aflatoxin positive with aflatoxin range from 3.92 to 547.85 µgkg-1 (Geresu, 2014)

2.6. Factors influencing fungi infection and aflatoxin development in maize

Mycotoxins are produced by fungal action during production, harvest, storage and food processing. Once the crop becomes infected under field conditions, the fungal growth continues with increasing at post-harvest and storage conditions. Genotypes, drought, soil types, and insect activity are important in determining the likelihood of pre-harvest contamination (Wagacha et al., 2008). Moisture content, relative humidity in the air and temperature of the environment are also important factors. Although the ideal temperature for mycotoxin production by many molds is in the range of 25 to 28°C. *A. flavus* is known to grow at temperatures as low as 10-15^{0}C. However, constant temperature (25°C) is generally accepted as the temperature near the optimum for aflatoxin production. The activities of molds are also governed by the relative humidity of surrounding air and moisture content of stored products. There is fairly defined relationship between water content in the grain and relative humidity of the surrounding atmosphere (Habtamu *et al.,* 2001). Other factors favoring mycotoxin contamination are stress factors during plant growth, late harvesting of crops, high ambient humidity preventing thorough drying, unscientific storage practices and lack of awareness (Wagacha et al., 2008).

2.7. Aflatoxins and their effects on humans and animal health

The toxic effect of mycotoxins on animal and human health is referred to as mycotoxicosis, the severity of which depends on the toxicity of the mycotoxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects of other chemicals to which the individual is exposed (Peraica *et al.,* 1999). Aflatoxins are of economic and health importance because of their ability to contaminate human food and animal feeds, in particular cereals, nuts and oilseeds. The toxins have adverse effects on plants, animals and humans. They are responsible for damaging up to 25% of the world‟s food crops, resulting in large economic losses in developed countries and human and animal disease in under-developed countries (Abbas *et al.,* 2005). Known for decades, aflatoxin contamination of maize has gained global significance due to the improved knowledge of the deleterious effects that contaminants have on human and animal well-being and the heavy reliance of smallholder populations on the two crops. Sufficient evidence that AFB1 and mixtures of B1, G1 and M1 are proven carcinogens has been provided by the International Agency for Research on Cancer

who classifies them as Group 1 carcinogens while M1 and B2 are designated to Group 2B. The deleterious pathway is as follows: AFB1 is metabolized (by the liver) to AFB1-8, 9-epoxide (AFBO) or to less mutagenic forms which then can either result in cancer, (toxicity or be excreted from the organism. The cancer is thus a result of formation of DNA-adducts by AFBO bonding with genetic material. Prolonged exposure to doses of 50 micrograms aflatoxin B1/kg/day has clinically significant effects. No animal species has been found to be immune to the effects of Aflatoxins (Murphy, 2006)

2.8. Strategies for mycotoxin control and prevention

Clearly the pre- or post-harvest prevention of mycotoxin contamination is the preferred strategy for minimizing mycotoxins in foods and feeds. Failure to prevent fungal invasion and toxin formation in the field or in storage will inevitably lead to an increased risk of adverse health effects and economic loss. However, if chemical monitoring is successful, maize consumption should not be a significant source of increased health risk from mycotoxins (Ronald *et al.,* 1999).

Several technologies have been tested in Africa to reduce aflatoxin risk. Field management practices that increase yields can reduce the risk of aflatoxin development. They include use of resistant varieties, crop rotation, well-timed planting, weed control, pest control especially control of insect pests and avoiding drought and nutritional stress through fertilization and irrigation. Measures to stop the infection process by controlling the aflatoxin causing fungi in the field are achieved through use of pesticides and aflatoxigenic fungi to competitively displace toxigenic fungi, and timely harvest. Postharvest interventions that reduce aflatoxin include rapid and proper drying, proper transportation and packaging, sorting, cleaning, drying, smoking. Post-harvest insect control, and the use of botanicals or synthetic pesticides as storage protectants. Another approach is to reduce the frequent consumption of „high risk" foods (especially maize and groundnut) by consuming a more varied diet, and diversifying the diet into less risky staples. Chemo-preventive measures that can reduce aflatoxin effect include daily consumption of chlorophyllin or oltipraz and incorporating hydrated sodium calcium alumino-silicates into the diet. Reduction and detoxification of aflatoxin is often achieved physically (sorting, physical segregation, flotation etc.), chemically (e.g. calcium

hydroxide, ammonia) and microbiologically by incorporating pro-biotics or lactic acid bacteria into the diet. Millers can use blending of less and more contaminated products to reduce the overall risk. Efficient monitoring and surveillance with cost-effective sampling and analytical methods also reduces the risk. Public education and awareness can sensitize the population on aflatoxin risk and its management (Kerstin *et al*., 2011; Bankole *et al.,* 2003)

2.9. Methods for Detection of Aflatoxins

Various analytical methods employed in analysis of aflatoxins in agricultural food crops and feeds have been explored. While chromatographic methods such as TLC and HPLC are considered the gold standard and are thus the most widely used techniques in aflatoxins analysis, they remain largely cumbersome, requiring extensive sample preparations, let alone very expensive equipment. This makes their routine use in analysis confined to laboratories. It is on the account of such limitations that it was necessary to develop more sensitive and better techniques for aflatoxins analyses (Alex *et al.,* 2014 and Braicu, *et al.,* 2008). Fluorescence detection is a very good alternative to the conventional techniques used today. It has a very high sensitivity, especially when is combined with other techniques as HPLC (Schaafsma et al., 1998). Analytical methods based on spectroscopy and immunochemistry have been added to the earlier chromatographic methods, of which immunoassays emerged as better alternatives for routine and on-site detection of aflatoxins. Improvement in analytical chemistry and recent advances in immunochemistry have led to more specific, sensitive, simple, and rapid immunoassays which have become the method of choice for on-site and routine analysis of mycotoxins in foods and feeds. It worth noting that although many sensitive methods have been described for analysis of aflatoxins, based on immunochemical format, most of them require labeling, as well as skilled and well trained operators. Therefore, the search for simple, label-free, and more rapid and sensitive tools that are based on immune-biosensor format appears to offer, for the near future, versatile, portable, sensitive, and accurate field use devices for aflatoxin detection (Alex *et al.,* 2014; Schaafsma *et al.,* 1998).

2.10. Method Validation

Validating bioanalytical methods includes performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given sample is reliable and reproducible for the intended use. Fundamental parameters for this include:

Linearity

The ability to induce a signal (response) that is directly proportional to the concentration of the given analytical parameter

Recovery

Detection of a known amount of an analytical parameter added to a specimen and included throughout the method of analysis. After deducting any detected content of the analytical parameter in question in the original specimen without addition, the recovery percentage can be calculated as a percentage of the amount added.

Precision

The general term "precision" is used to describe the magnitude of random (indeterminate) errors associated with the use of an analytical method. The sources of random error evaluated depend upon the range of conditions over which the data are collected. The following definitions are recommended

Repeatability (of results of measurements) - closeness of agreement between the results of successive measurements of the same measure and carried out under the same conditions of measurement. These conditions are called repeatability conditions. Repeatability conditions include the same measurement procedure, observer, measuring instrument (used under the same conditions), location, (and) repetition over a short period of time. Repeatability may be expressed quantitatively in terms of the dispersion characteristics of the results.

Reproducibility (of results of measurements) - closeness of agreement between the results of measurements of the same measure and carried out under changed conditions of measurement. A valid statement of reproducibility requires specification of the conditions changed. The changed conditions may include principle of measurement, method of measurement, observer, measuring instrument, reference standard, location, conditions of use, (and) time.

Reproducibility may be expressed quantitatively in terms of the dispersion characteristics of the results.

Detection Limit(s)

The term "detection limit" is used to describe the lowest analyte level that can be confidently identified. There are many specific definitions for this term, and it is used to describe the detection capabilities of detectors, instruments, and analytical methods. The term "detection limit" must be defined, and a description of how it was evaluated during method validation must be provided. Limits derived from mathematical definitions or statistical models must be verified by testing materials containing analyte at the claimed detection level.

Limit of detection (LOD): is the smallest concentration of analyte in the test sample which can be reliably distinguished from Zero. It is the concentration of analyte which induce signal (S) that is 3 times higher than the background noise level (N). S/N=3

Limit of quantification (LOQ): is the smallest concentration of analyte in the test sample which can be reliably quantified. It is the concentration of analyte which induce signal (S) that is 10 times higher than the background noise level (N). S/N=10 (EC, 2002, Loh Saw *et al*., 2012 and Ranjit, 2013).

2.11. Acceptance level for Aflatoxin by different Organizatio*n*

National and international institutions and organizations such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have recognized the potential health risks to animals and humans posed by consuming aflatoxin-contaminated food and feed. To protect consumers and farm animals regulatory limits have been adopted. The current maximum residue levels (MRL) for aflatoxins set by the United States" Food and Drug Administration (USFDA) is 20 µg/kg (FDA, 2011) and safe limit of 20µg/kg is also established by WHO (Mwihia *et al.,* 2008;). Maximum level of 2 µg/kg for aflatoxin B1 and 4 µg/kg aflatoxin total has been established in all cereals (EU, 2010).

Table 1: some countries maximum tolerated levels of aflatoxin in human foodstuffs, dairy products and animal feedstuffs in 2002/2003 survey.

Country	Food Item	Aflatoxin	Limit (µg/kg)
Algeria	Peanut, nut, cereals	B1	10
		B1,B2/G2,G1	20
Egypt	peanut and cereal	B1	5
		B1,B2/G2,G1	10
	Corn	B1	10
		B1,B2/G2,G1	20
Israel	nuts, peanuts, maize flour, figs and their products and other foods	B1	5
		B1,B2/G2,G1	20
Kenya	peanut (product)s, vegetable oil	B1,B2/G2,G1	20
Ethiopia	?	?	?

Source: Khoshpe *et al.,* 2011

3. METHODOLOGY

3.1. Description of the study site

Mirab Gojjam (or "West Gojjam") is a zone in the Amhara Region of Ethiopia, with an area of 13,311.94 square kilometers. West Gojam of the Amhara region is the highest maize-producing zone not only in the region but also in Ethiopia. Based on the five years data the average annual production of maize this zone is 3,209,274 quintals. This amount represents 46% of the regions and 10% of the national average production. The study was conducted in three woredas: Burie (Latitude(N) 10°42′ , Longitude(E) 37°4′E,Altitude 2091m,annual average temp. 14 -24 C^0, annual rain fall 1200 mm. Population 23292); Finoteselam (Latitude(N) 10°42′, Longitude (E) 37°16′, Altitude 1917m, annual average temp. 9.8-23.5 C^0, annual rain fall 1250mm Population 25913); Jabitehnan (Latitude(N) , 10°41'53" Longitude (E) 37°10'35", Altitude 1500-2300 m, annual average temp. 14 ^{0} - 32 ^{0}C , annual rain fall 1250mm, Population 231,232) (EATA, 2012 and ECEA, 2003**).**

Fig 5: Location map of the study area

3.2. Study Design

A Nested study design was conducted to assess the level of *Aspergillus* species and Aflatoxin contamination in pre harvest and post-harvest maize in west Gojam Ethiopia.

3.3. Study Period

The study was conducted from September 1, 2014 to May 1 2015.

3.4. Materials

3.4.1. Apparatus, chemicals and glass wares

All chemicals used for analysis will have analytical grade and obtained from well-known company. Similarly, the glass wares will be cleaned free from any possible contamination prior to analysis.

The major equipments used during the analysis listed below:

For Aflatoxin determination

Hewlett-Packard HPLC 1050 or equivalent Liquid chromatograph ,Fluorescence Detector, Adjustable Pipettes, Micro filter ,Centrifuge, timer, Analytical balance, Variable Volumetric flask, Plastic test tube, Glass funnels (100 mm), Disposable pipet tips (50 and 200µl) and Variable beakers (100 and 250 ml)

For moisture content determination

Analytical balance, Desiccators, Oven, Moisture dish (metal dish), Mechanical mill

For Isolation and identification of fungi

Petri plates, Pipette, Autoclave, Variable Volumetric flask, test tube, Glass funnels (100 mm)

The main reagents used during the analysis are listed below:

For Aflatoxin determination

Extraction solvent (100% ACN), HPLC methanol grade, n-hexane, MgSO4 anhydrous salt, Nacl, Aflatoxin standards solutions

For Isolation and identification of fungi

Potato dextrose agar (PDA), Sterile distilled water, NaOCl

3.5. Sample size and Sampling Method

A total of 30 samples of 15 for pre harvest and 15 for post-harvest were collected from farmers in 3 woredas of west Gojam zone (Burie, Finoteselam and Jabitehnan). Five for pre harvest and five for post-harvest samples from each woredas were collected. For pre harvest samples we were collected samples through Systematic random sampling in which maize grown and storages within 10 Km radius of the respective woredas. For post-harvest, samples were collected from the same farmers.

3.6. Sample collection and transportation

For pre harvest 1kg of maize samples were collected from the standing maize in the field from each farmers were immediately visually assessed for insect damage, discoloration due to fungal agents and these Samples were taken immediately to the laboratory, hand-shelled and sun dried for 2-3 days and the samples were then divided into two; one third of the sample for mycological analysis and two-thirds for aflatoxin analysis packed and stored in bags at 4ºC in the laboratory until they were be analyzed . Pre harvest maize samples were collected in December 20 14.

For post-harvest 1 kg of sample were collected after 3 month of harvest , the collected maize samples were placed in clean polyethylene bag with hermetic sealing, labeled and shipped to the laboratory of Food Science and Nutrition Program, Addis Ababa University. The samples were then divided into two; one third of the sample for mycological analysis and two-thirds for aflatoxin analysis packed and stored at 4ºC in the laboratory until they were analyzed. Post-harvest samples were collected in March 2015.

Fig 6: pre and post- harvest sampling areas.

3.7. Pre and post-harvest practice survey

Valid and reliable standard questionnaire was designed and instituted to obtain relevant pre and post-harvest practice information for the study. The questionnaire was translated to the local language (Amharic) and Pre-tested before the main study on clients. The data was collected by two trained data collector

3.8. Laboratory studies

3.8.1. Determination of moisture content (AOAC 2000)

Moisture contents of the maize samples were determined according to AOAC (2000), using the official method 925.05. The dishes used for the moisture determination were dried at 105 ^{0}C for 1 hr. In Memmert drying oven of model 40050 and placed in desiccators for about 30 min. The mass of each dishes were measured (W1) and about 5 g of the sample was weighed in to each of the dishes (W2). The sample was then mixed thoroughly and dried at 105^{0}C for 3hr. After drying is completed, the mass was measured (W3). The moisture content was calculated from the equation:

Calculations

$$\text{Moisture content in \%} = \frac{W2-W3}{W2-W1} * 100$$

Where: W_1= Weight of a given sample in gram

W_2= weight of crucible plus weight of sample before dry

W_3= weight of crucible plus sample after drying

3.8.2. HPLC-FLD determination of aflatoxins in Maize sample

Validation for HPLC–FLD detection of aflatoxins in maize samples was made in accordance with method validated by Faculty of Pharmaceutical Science Laboratory of Food Analysis Ghent, Belgium, Europe for the determination of aflatoxin in maize sample.

This method has been widely used for the determination of aflatoxins in food; no post column derivitization needed Robust; easy and relatively fast procedure for sample preparation; Quick, Easy, Cheap, Effective, Rugged, and Safe.

Sample preparation

Samples were homogenized prior to milling and a representative sample were taken and milled to a desired particule size (0.5mm -1.0 mm)

Mycotoxin standards

Aflatoxin B1, B2, G1, G2: supplied as 1 mg solid by Sigma Aldrich, Belgium

Making calibration curve

Prepare a mixture of AFB2, AFG2 AFB1and AFG1 at concentration of 0.2 µg/mL, 0.2 µg/mL, 2 µg/mL and 1 µg/mL, respectively1.0± 0.002 g of ground maize was weighed accurately in 6 test tube, one of the 5 samples was served as a blank sample. The other five samples were spiked with the respective concentration as shown in the table 2.

Table 2: spike volume and concentration of aflatoxins for making calibration curve

	Concentration (µg/kg)		
Spike levels	**AFB1**	**AFG1**	**B2 and G2**
1	10	5	1
2	20	10	2
3	40	20	4
4	80	40	8
5	160	80	16

Extraction and clean-up

Sample (1.0 g) was weighed in a polypropylene centrifuge tube (15 mL) and 5 mL deionized water was added and Vortex briefly, and then the samples were allowed to stand for 30 min. But spiked samples were left for equilibration for 30mins before addition of water. After 30 minute 5 mL of extraction solvent (100% ACN) was added and briefly mixed using a vortex mixer and then it was shacked for 30 minute at position 7 using an end –over-end shaker, Subsequently,

MgSO4 anhydrous salt 2.0 ± 0.05 g and NaCl 0.5 ± 0.01 g were added and then shaken briefly to prevent agglomeration of the salts and was mixing using a vortex for 2 min. Afterwards, the tube was centrifuge at 4000 g for 15 min. Then 4 ml of the top organic layer was transferred to a new tube and evaporated under N2 at 40 ^{o}C. To the residue 200 µL of injection solvent (A: B 50/50) and 200 µL of n-hexane was added and dissolved using a vortex and then filtered with micro filter and Centrifuge for 10 min at 10,000 g. Finally 150 µL was collected from the lower phase into HPLC vials

Chromatographic conditions

The HPLC analyses were carried out with Agilent 1100 system, consisting of a degasser, binary pump, auto sampler, column oven and a fluorescence detector. The chromatographic separation was performed with the Zorbax SB RP C18, 150×4.6 mm, 5 µm chromatographic column. The mobile phase was of A: Milli Q water (100%) and B: MeOH/ACN (71.5/28.5, v/v). A gradient elution profile was used (see Table 3). The column temperature was 30 ^{o}C at the flow rate of 1.5 ml/min to achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 20 µl for both standard and sample solutions.

Table 3: HPLC Gradient Elution Profile

	Water (Milli-Q)	MeOH/ACN (71.5/28.5, v/v)
Time(minutes)	75	25
1	75	25
12	60	40
25	50	50
28	0	100
30	0	100
32	75	100
35	75	25

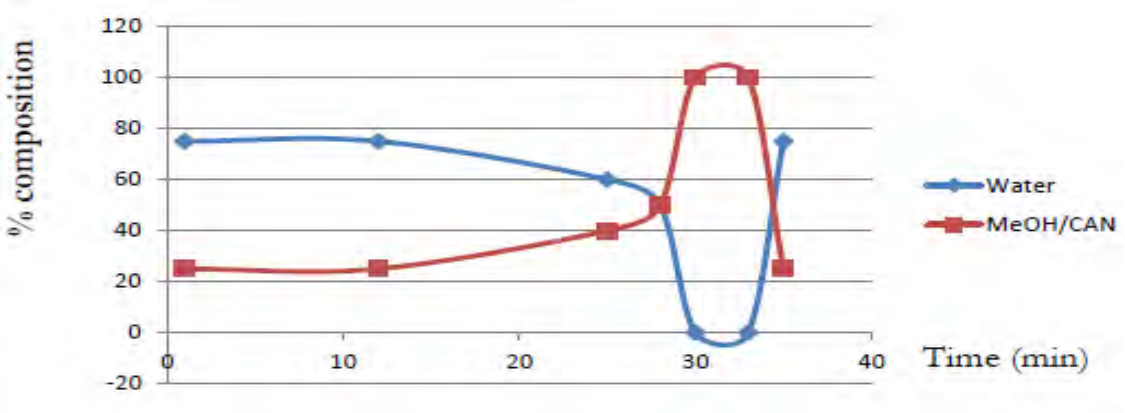

Fig 7: HPLC Gradient Elution Profile

3.8.3. Isolation and identification of fungi

Fungal isolation

Fifty seeds per sample were surface sterilized with 10% NaOCl C solution for 1 min, followed by immersion in sterile distilled water for 1 min. Surface sterilized seeds were then

placed on freshly prepared potato dextrose agar (PDA) plates (five seeds per plate) with in which 0.05mg of streptomycin sulphate has been added to suppress the growth of bacteria in petri plates and incubated for three days at 25°C. Pure cultures of different out growing fungi were obtained by transferring fungal colonies to new PDA plates using sterile toothpicks, and incubating the plates for 5-7 days at 25°C. Pure cultures of each isolate were then stored at 4°C in vials containing 2.5 ml of sterile distilled water for further use.

Species identification

Isolates were identified to a species level based on morphological (phenotypic) features as described by Abdi *et al*., (2014); Negero *et al*., (2014); Mohammed *et al.*, (2013); and Cotty *et al.,* (1994). For this purpose: Isolates representing each pure culture were grown on PDA at 25°C for 5-7 days. Fungal colonies that grew rapidly and produced colors of white, yellow, yellow-brown, brown to black or shades of green, mostly consisting of a dense felt of erect conidiophores were broadly classified as *Aspergillus* spp. The major distinction of *Aspergillus* species are described below in table 4.

Table 4: The color of the colony in various *Aspergillus* species

SPECIES	SURFACE	REVERSE
A. clavatus	Blue-green	White, brownish with age
A. flavus	Yellow-green	Goldish to red brown
A. fumigatus	Blue-green to gray	White to tan
A. glaucus group	Green with yellow areas	Yellowish to brown
A. nidulans	Green, buff to yellow	Purplish red to olive
A. niger	Black	White to yellow
A. terreus	Cinnamon to brown	White to brown
A. versicolor	White at the beginning, turns to yellow, tan, pale green or pink	White to yellow or purplish red

Source: Rahul *et al.,* 2014

Variations in growth rate and thermo-tolerance were also used in identification of Aspergillus species. Aspergillus colonies are downy to powdery in texture. Aspergillus

fumigatus is a thermo-tolerant fungus and grows well at temperatures over 40°C. This property is unique to A. fumigatus among the Aspergillus species. A.flavus can be readily distinguished from other Aspergillus species by lack of growth at 5 °C, by rapid growth at both 25 and 37°C, and by the production of a bright yellow-green conidial color (Negero *et al.,* 2014)

3.9. Data processing and analysis

Data obtained from questionnaire and laboratory were entered into Microsoft Excel sheets and exported the software STATA Version 11 and SPSS version 20.0 for analysis. The data were summarized and organized using graphs, tables and texts. Chi-squared or Fisher Exact tests, when appropriate, were used to verify the statistical significance. Student‟s t-test for paired samples was used to see if there was significance difference in aflatoxin level between pre and post-harvest samples. The data were also analyzed by using one way analysis of variance (ANOVA) and least significant difference (LSD). P-value of <0.05 was considered to be significant.

4. RESULT AND DISCUSSION

4.1. Pre and post-harvest practice among participants

A questionnaire was supplied to farmers in order to obtained information on the pre and post-harvest practice of the participant. As shown in table 7, A total of 15 farmers with aged 28-61 years (mean age: 38 years; std. dev.: 9.2 years) that were involved in the study (*Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize in west Gojam Ethiopia) were enrolled in this study. The 20-40 years age group recorded the highest participation in this study, 10 (66.7%) and the > 60 year-olds had the least of 1 (6.7 %). Majority (77.3 %) of the maize farmers has been heard about mould. 86.7 % of the participants practiced crop rotation and all off them ploughed their fields manually or used animal ploughs. None of them were decreasing the moisture content to the required level or had information about the required level of moisture content after harvesting. With regard to drying method 86.7 % of farmers were drying their maize by sun drying and 13.3 % of drying by using fire smoke. 80 % of the participants cleaned their storehouse before storage. Most of them 80 % stored maize in the house and 20% Courtyard. Most of them (70%) did not store maize together with other product. Most of the participants (80%) stored maize product with Gota" made from teff straw and mud followed by in plastic bags overlaying in house 20%.

Table 5: Scio demographic and pre and post-harvest practices among maize growers in the study area

Sociodemographic and harvesting practices		N %
Age	20-40	10 (66.7)
	40-60	4 (26.7)
	>60	1 (6.6)
Education	Illiterate	12 (80)
	Primary school	2 (13.3)
	Secondary school	1 (6.7)
Sex	Female	**2** (13.3)
	Male	13 (86.**7)**
Heard about Mould	Yes	11 (77.3)
	No	4 (26.7)
Crop rotation	Yes	13 (86.7)
	No	2 (13.3)
Harvest the crop as soon as maturity	Yes	4 (26.7)
	No	11 (77.3)
After harvesting decrease the moisture content to the required level	Yes	0 (0)
	No	15 (100)
Dry after harvesting	Solar drying	13 (86.7)
	Professional dryer	0 (0)
	Drying using fire smoke	2 (13.3)
Clean the storehouse before storage	Yes	12 (80)
	No	3 (20)
Storage location	Field	0 (0)
	In the house	12 (80)
	Courtyard	3 (20)
Store other products in the store, together with maize	No	11 (77.3)
	Yes	4 (23.3)
Storage method	"Gota" made from teff straw and mud	12 (80)
	underground pits	0 (0)
	In plastic bags overlaying in house	3 (20)

4.2. Moisture content of pre and post-harvest maize

Moisture level of pre-harvest maize varied between 12.99 - 15.71 %, while the post-harvest sample recorded moisture levels of 10.23-13.81%. The average moisture content of pre and post-harvest maize was 14.38 ± 0.95 and 12.40 ± 0.94, respectively.

Crop is usually physiologically mature 7–8 weeks after flowering, at which time the grain contains 35–40% moisture and has maximum dry weight. This is the time at which the crop should be harvested to avoid unnecessary losses in the field. However, in Ethiopia, the time and method of harvesting maize depend on weather conditions, the size of the crop and how quickly the farmer wants to utilize the crop. In this study samples were collected 2-3 weeks before harvest but the maize aged more than 4 month after flowering and average moisture content of 14.38 ± 0.95 which was unacceptable. Timing of harvest greatly affects the extent of aflatoxin contamination. Extended field drying of maize increased insect infestation and fungal contamination. Aflatoxin levels increased by about 4 times by the third week and more than 7 times when maize harvest was delayed for 4 weeks (Kaaya *et aI.,* 2005) .

4.3. Validation of HPLC–FLD method for aflatoxins analysis in maize sample

Validation for HPLC–FLD detection of aflatoxins in maize samples was made in accordance with method validated by Faculty of Pharmaceutical Science Laboratory of Food Analysis Ghent, Belgium, Europe for the determination of aflatoxin in maize sample. The analytical methodology was validated in terms of linearity, reproducibility, repeatability; percent recovery limits of detection (LOD) and quantification (LOQ). Retention times in naturally contaminated samples and standards containing aflatoxins AFG2, AFG1, AFB2 and AFB1 were: 19.228, 21.428, 23.343 and 25.81 min, respectively (Figure 8).

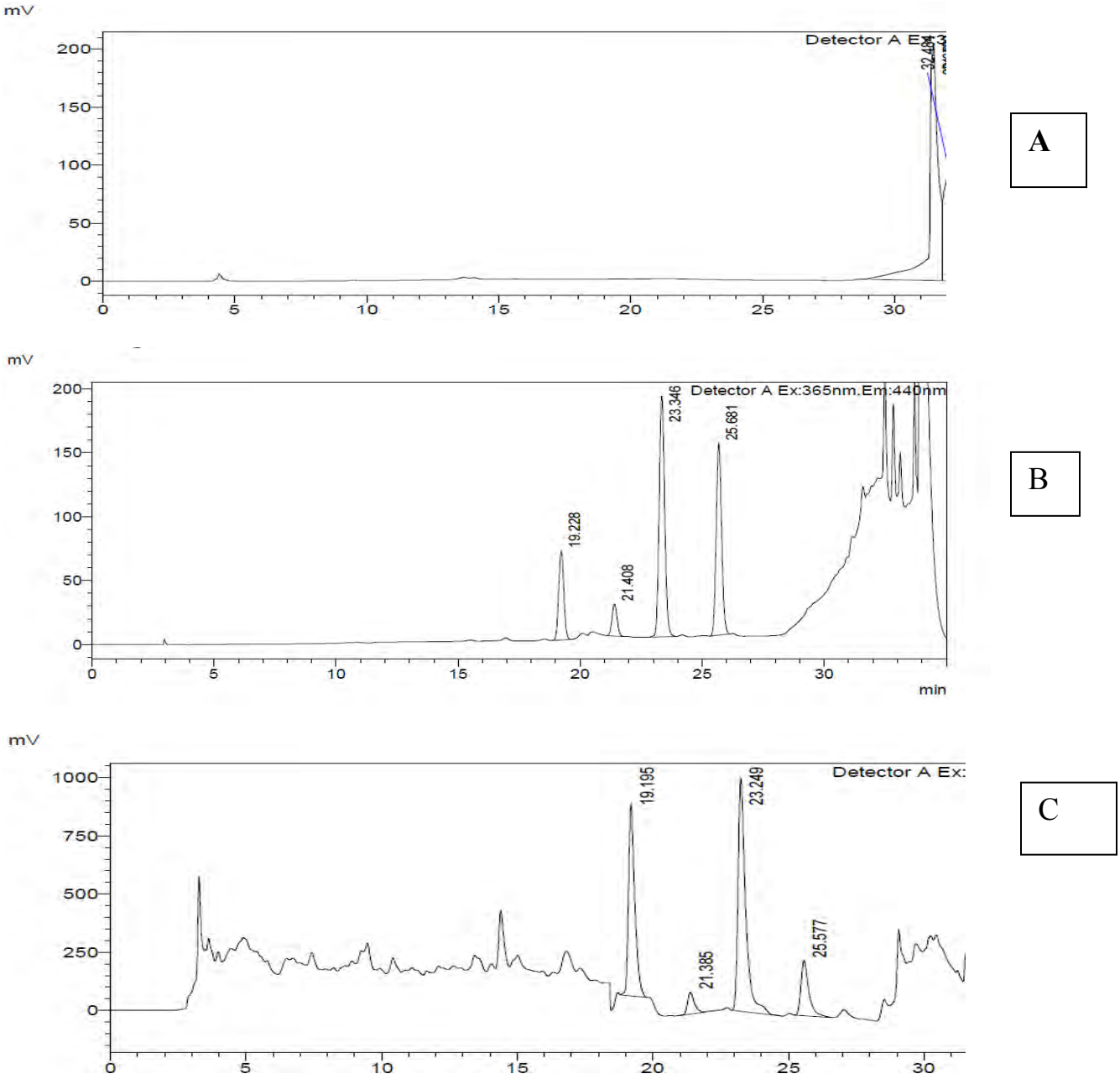

Fig 8 : Chromatogram of (A) blank sample, (B) standard calebration curve and (C) naturally contaminated fungal sample (elution order AFG2,AFG1,AFB2 and AFB1.

Five-point calibration curve graphs were obtained with concentration of 1-16 ppb of AFG2, 5-80 of AFG1, 1- 16 ppb of AFB2 and 10 - 160 ppb of AFB1. Calibration graphs were drawn by linear regression of the least-squares method using the peak area of standard as response versus concentration as shown in Figure 9. The correlation coefficients were >0.998, which was considered as evidence of an acceptable fit of the data to the regression line (Ranjit, 2013).

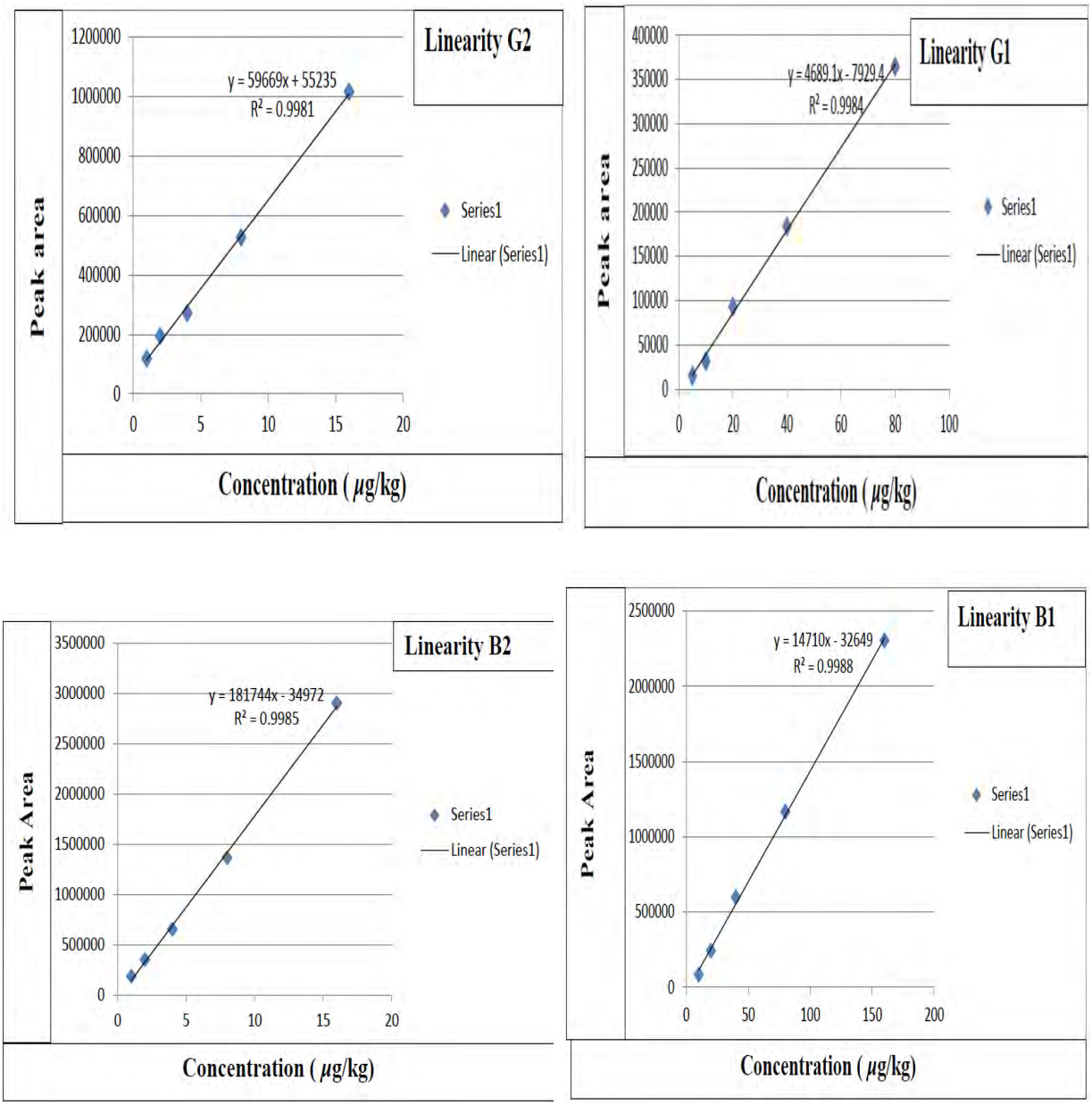

Fig 9: Linear response of peak area against Aflatoxin concentration (AFG2, AFG1, AFB2 and AFB1.

The test for the precision of the method was checked and verified by repeatability, inter-day and intra-day precision as shown in Table 6. It was checked by injecting Aflatoxin (G2=1, G1=5, B2=1 and B2= 10) at the low level concentration and Aflatoxin (G2=8, G1=40, B2=8 and B2= 80) at the high level concentration for 5 times on the same day for inter day precision. Similarly, for the inter-day precision similar concentration was analyzed on different days.

Table 6: Validation of Method for Analysis of Aflatoxins

	spike level (μg/kg)	Area and retention time	AFG2	AFG1	AFB2	AFB1
limit of detection (μg/kg)			0.03	0.3	0.014	0.15
Limit of quantification(μg/kg)			0.11	1.28	0.05	0.50
Repeatability, RSDr (%)	G2=1,G1=5 B2=1and B1=10	Area	3.26	0.75	0.82	3.39
		Retention time	0.05	0.06	0.05	0.05
	G2=8,G1=40 B2=16 and B1=80	Area	0.50	5.13	1.12	5.30
		Retention time	0.23	0.24	0.21	0.19
Reproducibility, RSDr (%)	G2=1,G1=5 B2=1 and B1=10	Area	1.37	2.18	1.39	5.5
		Retention time	0.15	0.15	0.16	0.15
	G2=8,G1=40 B2=16 and B1=80	Area	0.85	5.04	1.48	5.25
		Retention time	0.04	0.03	0.03	0.03

As shown in table 6, relative standard deviations (RSDr) for within-day samples for peak area at the low level assayed concentration were G2=3.26,G1=0.75, B2=0.82 and B1=3.39 and G2=3.26,G1=0.75, B2=0.82 and B1=3.39 at the high level. Similarly, for retention time relative standard deviations (RSDr) for within-day samples at the low level assayed concentration were G2=0.05, G1=0.06, B2=0.05 and B1=0.05 and G2=0.23, G1=0.24, B2=0.21 and B1=0.19 at the

high level. Similarly, relative standard deviations (RSDr) for inter-day samples for peak area at the low level assayed concentration were G2=1.37, G1=2.18, B2=0.1.39 and B1=5.5 and G2=0.85, G1=5.04, B2=1.48 and B1=5.25 at the high level. Similarly, for retention time relative standard deviations (RSDr) for inter-day samples at the low level assayed concentration were G2=0.15, G1=0.15, B2=0.16 and B1=0.15 and G2=0.04, G1=0.03, B2=0.03 and B1=0.03 at the high level (table 6). Relative standard deviations for retention time and peak area of five replicates were <0.2 and < 6% respectively, which showed high precision. (Loh Saw *et al*., 2012; Ranjit, 2013).

Limit of detection (LOD) is the concentration of analyte which induce signal (S) that is 3 times higher than the background noise level (N). S/N=3 The detection limits in this study were 0.03, 0.3, 0.014 and 0.15 μg/kg for aflatoxins B1, B2, G1 and G2, respectively, calculated based on three times the standard deviation of the noise (Table 6).

The limit of quantitation (LOQ) of the aflatoxins in sample is the concentration of analyte which induce signal (S) that is 10 times higher than the background noise level (N). S/N=10.The limit of quantitation (LOQ) were 0.11, 1.28, 0.05 and 0.5 μg/kg for aflatoxins B1, B2, G1 and G2, respectively, calculated based on 10 times the background noise level(Table 6). .

The accuracy of the method was measured from analytical recovery in duplicate sample at spike levels of Aflatoxin (G2=4, G1= 5, B2= 4 and B1= 40 μg/kg)). Recovery percentage of each spiked sample was calculated as recovery= (amount found/amount added)*100. The average recovery were AFG2= 92.1%, AFG1= 82.8%, AFB2= 97.5% and AFB1=98.3%. The average recoveries for spiked samples ranged from 82.8-98.3 %. The recoveries obtained were within the range of 70 to 125%, which were acceptable according to AOAC International guidelines for method validation (Loh Saw *et al*., 2012) (Table 7)

Table 7: Recovery test of Aflatoxin from maize sample

AFLATOXIN	Aflatoxin concentration in sample (µg/kg)	Aflatoxin level added (µg/kg)	Result found(µg/kg)		Replicate % recovery		Average recovery %	RSD %
			1	2	1	2		
AFG2	0.000	4	3.81	3.560	95.25	89.03	92.14	4.8
AFG1	12.280	5	14.05	14.56	81.31	84.26	82.79	2.5
AFB2	0.000	4	4.051	3.750	101.3	93.75	97.53	5.4
AFB1	13.878	40	53.357	52.53	99.03	97.50	98.3	1.1

4.4. Levels of Aflatoxin in maize samples from west Gojam

In the present study 30 samples consisting of 15 pre- and 15 post-harvest maize samples were collected from farmers‟ fields of West Gojam. Samples were analyzed in duplicate for AFG2, AFG1, AFB2 and AFB1 contamination by HPLC-FLD. Aflatoxin contamination in maize was observed in 77.7 % of pre harvest by level range 3.13-63.66 and 80 % by level range 9.02-139.8 of post-harvest sample. The samples were showed that the mean total aflatoxin level of 18.38 and 43.43 µg/kg for pre and post -harvest maize respectively (Table 8).

About 77.7% total aflatoxin contamination in pre harvest maize obtained in this study is consistence to the result of Ayyathurai *et al*., (2009) who reported that the total aflatoxin contamination about 79.7 % of pre harvest maize in India but However the results of this study is higher than that of reported in Benin with percentage of pre harvest maize aflatoxin

contaminated 42.5% in 1994 and 30% in 1995 (Sétamou *et al.,* 1997). This study was also higher than reported in Kenya which was 22.97% of total aflatoxin contamination in pre-harvest maize (Muthusamy *et al.,* 2012). The high total aflatoxin contamination in pre harvest may be due to delayed harvesting (high likelihood of kernel damage by pests, insect injury), differences in temperature, humidity, using crop resistance variety, crop rotation system and maintaining adequate irrigation schedule (Kaaya *et aI.,* 2005). Genotype, soil types, drought and insect activity are also important in determining the likelihood of pre-harvest Contamination (Muthomi *et al.,* 2012)

Aflatoxin contamination of post-harvest maize (80%) obtained in this study is consistence to result previously reported in Ethiopia, where Aflatoxin contamination of post-harvest maize was 88 % (Ayalew *et al.,* 2010). However the result of this study is remarkably higher than that of reported in Malaysia which was 40% (Loh Saw *et al*., 2012) and 58% in Nigeria (Onilude *et al*., 2012). This variation of percentage total aflatoxin contamination in post-harvest may be due to difference in handling process from the time of harvest to the time of consumption. The other reason may be due to once the crop becomes infected under field conditions, the fungal growth continues with increasing at post-harvest and storage conditions (Wagacha *et al*., 2008). It is also well known that growth of *Aspergillus* spp. and subsequent production of aflatoxins in maize is dependent on a number of factors such as temperature, humidity, insect injury, handling during pre-harvest, harvest and storage (Ayyathurai *et al*., 2009; Muthusamy *et al.,* 2012).

According to the present study, in pre harvest maize sample the average aflatoxin G2, G1, B2 and B1 concentration were 2.10 µg/kg, 10.10 µg/kg, 1.17µg/kg and 5.00 µg/kg respectively. In post-harvest maize sample the average aflatoxin G2, G1, B2 and B1 concentration in the maize sample were 8.14 µg, 18.11 µg/kg, 7.2 µg/kg and 9.86 µg/kg, respectively (Figure 10).

Among the naturally occurring aflatoxins (AFG2, AFG1, AFB2 and AFB1), AFB1 is usually predominant and is the most toxic aflatoxin. In this study the most frequently identified was aflatoxin B1 which was 66.7% in pre-harvest and 87.7% in post-harvest. The highest level of AFB1 (29.5 µg/kg) was recorded in post-harvest maize kernel sample. The levels of AFB1 ranged from 3.13 to 27.31µg/kg and 2.83 to 29.5 µg/kg in pre and post-harvest sample

respectively, AFB1 was detected in 60 % of pre harvest samples and 80% in post-harvest sample. The samples showed the mean aflatoxin B1 level of 5.00 µg/kg and 9.86 µg/kg for pre and post -harvest maize respectively (Table 9)

The current prevalence of AFB1 (66.7%) in pre-harvest and (87.7%) in post-harvest was consistent to results previously reported in India which was 79.7 % in pre harvest maize (Ayyathurai *et al*., 2009) and in Iran which was 77% of post- harvest samples (Khosrow *et al.,* 2015) but higher than reported by Muthusamy *et al.,* (2012) which was 22.97% of pre-harvest maize and 53.93% post-harvest maize by Ayyathurai *et al*., (2009) which was 22.97% of pre-harvest and 53.93% post-harvest maize. This variation may be due to number of factors such as temperature, humidity, insect injury, handling during pre-harvest, harvest and storage (Ayyathurai *et al*., 2009; Muthusamy *et al.,* 2012)

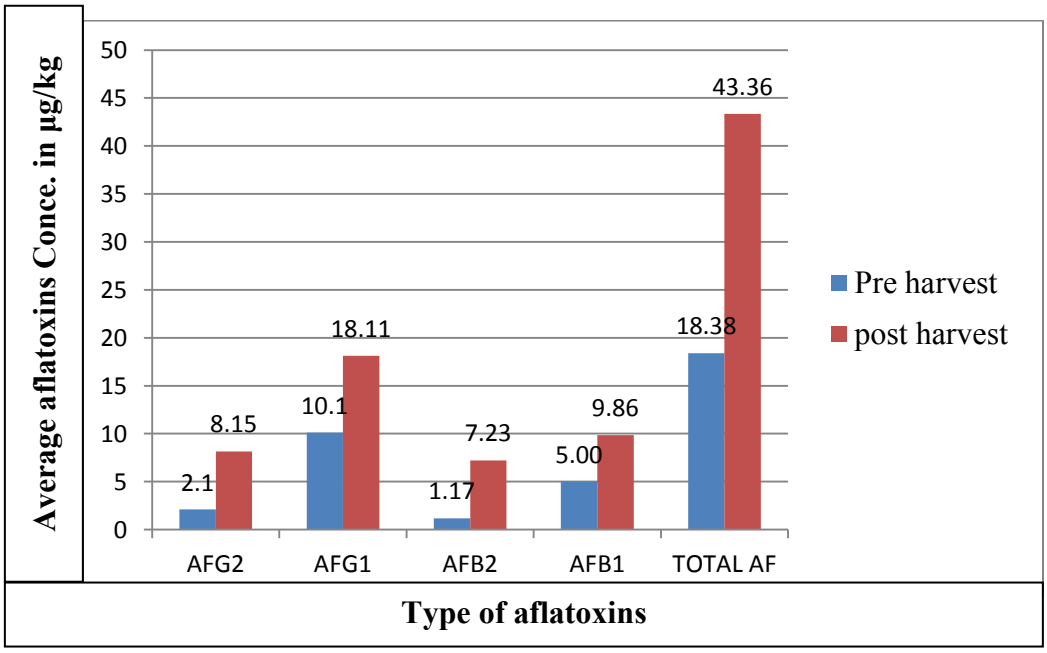

Fig 10: Average concentration of aflatoxin in pre and post- harvest maize

4.4.1. Comparison of the means of total aflatoxin level in pre and post-harvest maize.

Table 8 showed that the mean total aflatoxin level in post- harvest is much higher (43.43 µg/kg) than in pre harvest maize (18.38 µg/kg). Paired t-test statistical analysis for mean of total

aflatoxin level in pre and post- harvest maize sample is also showed that there was a significant difference in total aflatoxin level between pre and post- harvest maize (t=3.6, p=0.003) which was less than 0.05. Which means total aflatoxin level was significantly increase from pre harvest to post-harvest pre harvest maize. The reasons for high increment of aflatoxin level in post-harvest may be poor harvest and post-harvest practices of the maize (Kaaya *et al.,* 2005; Muthusamy *et al.,* 2012) and Once the crop becomes infected under field conditions, the fungal growth continues with increasing at post-harvest and storage conditions (Wagacha *et al*., 2008)

Table 8: Paired comparison of the mean difference of total aflatoxin level in pre and post-harvest maize

TYPE OF SAMPLE	Sample size	Mean (µg/kg)	Mean 0f difference	SD	Std. error mean	95% CL for mean	t-test	P-value
Pre harvest	15	18.38	25.0	26.7	6.9	10.2-39.8	3.6	0.003
Postharvest	15	43.4						

AF=aflatoxin, SD= standard deviation of the mean difference, cl=confidence interval

4.4.2. Comparison of the means of Aflatoxin B1 level in pre and post-harvest maize.

Paired t-test statistical analysis for mean of B1 aflatoxin in pre and post –harvest maize showed that the mean total aflatoxin level in post- harvest is higher (9.86 µg/kg) than in pre harvest maize (5.00 µg/kg).

Paired t-test statistical analysis for mean of Aflatoxin B1 level in pre and post- harvest maize sample is also showed that there was a significant difference in total aflatoxin level between pre and post- harvest maize (t=2.28 p=0.039) which is less than 0.05. Which means Aflatoxin B1 level was significantly increase from pre harvest to post-harvest pre harvest maize (Table 9).

The reasons for high increment of Aflatoxin B1 level in post-harvest may be poor harvest and post-harvest practices of the maize (Kaaya *et al.,* 2005 and Muthusamy *et al.,* 2012) and Once

the crop becomes infected under field conditions, the fungal growth continues with increasing at post-harvest and storage conditions (Wagacha *et al*., 2008)

Table 9: Paired comparison of the mean difference of Aflatoxin B1 level in pre and post-harvest maize

Type of sample	Sample size	Mean (µg/kg)	Mean 0f difference	SD	Std. error mean	95% CL for mean	t-test	P-value
Pre harvest	15	5.00	4.86	8.24	2.13	0.29-9.42	2.28	0.039
Postharvest	15	9.86						

AF=aflatoxin, SD= standard deviation of the mean difference, cl=confidence interval

4.4.3. Total aflatoxin level in pre harvest maize in relation to locations.

In the present study 15 pre- harvest maize samples were collected from farmers" fields of West Gojam (Brie=5, Finoteselam=5 and Jabitehnan=5). Samples were analyzed in duplicate for AFG2, AFG1, AFB2 and AFB1 contamination by HPLC-FLD. The mean total aflatoxin level was 8.08, 14.21 and 32.80 µg/kg in Burie, Finoteselam and Jabitehnan respectively although the difference was not statistically significant ($p>0.05$) (Table 10).

4.4.4. Total aflatoxin level in post-harvest maize of three locations.

Total aflatoxin contamination in post -harvest maize was observed in 80%, 60% and 100% of Burie, Finoteselam and Jabitehnan respectively. The mean total aflatoxin level was 25.10, 29.21 and 75.53 µg/kg in Burie, Finoteselam and Jabitehnan respectively although there was no statistically significant difference with these locations. This result is consistent with previous report although there was no statistically significant difference with these predisposing risk ($P>0.05$) (Table 10).

4.4.5. Aflatoxin B1 level in pre harvest maize in relation to locations

In the present study 15 pre- harvest maize samples were collected from farmers‟ fields of West Gojam (Brie=5, Finoteselam=5 and Jabitehnan=5). Samples were analyzed in duplicate for AFB1 contamination by HPLC-FLD. As shown in table 10, the mean Aflatoxin B1 level was 2.3, 6.09 and 6.62 in Burie, Finoteselam and Jabitehnan respectively although the difference was not statistically significant (P>0.05) (Table 10) .

4.4.6. Aflatoxin B1 level in post-harvest maize of three locations

Table 10 showed that mean Aflatoxin B1 level in post-harvest maize was 8.59, 11.92 and 9.08 µg/kg in Burie, Finoteselam and Jabitehnan respectively although there was no statistically significant difference with these locations (p>0.05) (Table 10).

The non-significant relation of location on B1 and total aflatoxin in the study may suggest that the difference in aflatoxin contamination was irrespective of the location and the difference might be due to other factors like poor harvest and post-harvest practices of the farmers on maize. The other possible explanations this might be due to the three locations have no significant difference in temperature, humidity, annual rainfall (Sheila et al., 2012; Atehnkeng *et al*., 2008).

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Table 10: Average aflatoxin level in relation to location

Parameter	Burie	Finoteselam	Jabitehnan
Pre harvest total aflatoxin (µg/kg)	8.08[a]	14.21[a]	32.8[a]
Post -harvest total aflatoxin (µg/kg)	25.12[a]	29.50[a]	75.47[a]
Pre harvest Aflatoxin B1 (µg/kg)	2.31[a]	6.09[a]	6.62[a]
Post -harvest Aflatoxin B1 (µg/kg)	8.59[a]	11.91[a]	9.08[a]

Means in the same raw followed by different letters are significantly different, $P < 0.05$ and using LSD

4.4.7. Total Aflatoxin level difference between pre and post- harvest maize In relation to Location

The mean total aflatoxin level difference between pre and post -harvest collected from Burie, Finoteselam and Jabitehnan was 11.79, 15.28 and 42.61 µg/kg respectively .The effect of location on total Aflatoxin level difference between pre and post- harvest maize was analyzed by one way ANOVA, first by taking the difference of each sample of pre and post-harvest as dependent variable and location as a factor. A very high mean aflatoxin level difference was observed in each locations although there is no significant difference in the increment of total aflatoxin level between the locations (P>0.05) (Table 11).

4.4.8. Aflatoxin B1 level difference between pre and post- harvest maize In relation to Location

Among the naturally occurring aflatoxins (AFG2, AFG1, AFB2 and AFB1), AFB1 is usually predominant and is the most toxic aflatoxin. The mean Aflatoxin B1 level difference between pre and post -harvest collected from Burie, Finoteselam and Jabitehnan was 6.28, 7.96 and 2.46 µg/kg respectively .The effect of location on Aflatoxin B1 level difference between pre and post-harvest maize was analyzed by one way ANOVA. The result showed that there was no significant difference between on the increment or decrement of Aflatoxin B1 on pre and post -harvest maize (p>0.05) (Table 11).

Table 11: Average aflatoxin level difference in pre and post-harvest in relation to locations

Parameter	Burie	Finoteselam	Jabitehnan
Total aflatoxin mean difference between pre and post-harvest (µg/kg)	11.79[a]	15.28[a]	42.61[a]
B1aflatoxin mean difference between pre and post-harvest (µg/kg)	6.28[a]	7.96a	2.46[a]

Means in the same raw with different letters are significantly different, $P < 0.05$ and using LSD

Table 11 showed that location had no significant effect on the increment of aflatoxin from pre harvest to post harvest. This study was showed that increment of aflatoxin contamination was irrespective of the location difference. This increment might be due to other factors like poor harvest and post-harvest practices of the maize, aflatoxin contamination of maize has further been shown to increase with storage period. Excessive heat, high humidity, lack of aeration in the stores, and insect and rodent damage resulting in the proliferation and spread of fungal spores (Kaaya *et aI.,* 2005; Muthusamy *et al.,* 2012) and the other reason may be due to once the crop becomes infected under field conditions, the fungal growth continues with increasing at post-harvest and storage conditions (Wagacha *et al*., 2008)

4.5. Comparison of aflatoxin results with different international standards

National and international institutions and organizations such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have recognized the potential health risks to animals and humans posed by consuming aflatoxin-contaminated food and feed. To protect consumers and farm animals regulatory limits have been adopted. The current maximum residue levels (MRL) for aflatoxins set by the United States‟ Food and Drug Administration (USFDA) is 20 µg/kg (FDA, 2011) and safe limit of 20µg/kg is also established by WHO (Mwihia *et al.,* 2008;). Maximum level of 2 µg/kg for aflatoxin B1 and 4 µg/kg aflatoxin total has been established in all cereals (EU, 2010).

30 samples consisting of 15 pre- and 15 post-harvest maize samples were collected from farmers‟ fields of west Gojam. Samples were analyzed for AFG2, AFG1, AFB2 and AFB1 contamination by HPLC-FLD. Table 12 showed that 33.3 % of pre harvest and 73.3 % of post-harvest maize samples were exceeded the US Food and Drug Administration (FDA), the World Health Organization (tolerance limit of 20 µg/kg). The current percentage is lower than reported in Ethiopia 90% in in cereal and legume samples (Habtamu et *al.,* 2001) higher than reported in Kenya 35.5% stored maize (Mwihia *et al.,* 2008), 12.05% reported In India. (Muthusamy *et al.,* 2012)

About 66.7% of pre harvest sample and 86.7% of post- harvest maize samples were exceeded of total aflatoxin levels of European Union (EU) recommended maximum limit 4 µg/kg.

European Union (EU) also established acceptance limit of Aflatoxin B1 (<2 µg/kg) but most of samples (pre harvest=66.7 % and post-harvest = 86.7%) of this study were exceeded the acceptance limit (Table 12).

Table 12: percentage of maize sample exceeded the limit of aflatoxin level of different international standards

	FDA/WHO	**EU**	
Sample	**Total AF <20 µg/kg N (%)**	**Total AF <4 µg/kg N (%)**	**AFB1 <2 µg/kg N (%)**
Pre-harvest maize sample	5 (33.3)	10 (66.7)	10 (66.7)
Post-harvest maize sample	11(73.3)	13(86.7)	13(86.7)

4.6. Isolation and identification of Aspergillus species.

Aspergillus species were isolated on potato dextrose agar (PDA) and species level were isolated by sub culturing Pure cultures of different out growing fungi that were obtained on PDA and by transferring fungal colonies to new PDA plates using sterile toothpicks, and incubating the plates for 5-7 days at 25°C.

The common *Aspergillus* symptoms (yellowing or chlorotic leaves, wilting, drying and brown or black mass covered by yellow or greenish spores) were observed in pre and post-harvest maize on PDA. Identification of *Aspergillus* at species level was identified by sub culturing on PDA and their Variations in growth rate and color of the colony.

Three different *Aspergillus* spp. were found to be associated with the pre- and post-harvest maize samples collected from west Gojam (Figure 11). The first species isolated from the collected samples was *A. flavus*. Colonies of this fungus were characterized by a velvety, yellow to green or the old colony was brown mould with a goldish to red-brown on the reverse (figure 11). This species also distinguished from other *Aspergillus* species by lack of growth at 5 °C, by rapid growth at both 25 and 37°C. The second species were *A. niger*, the major distinction from the other species of *Aspergillus* is the production of carbon black or dark brown spores of biseriate phialides .The current study also confirmed the production of black or brown-black or black conidia by this species (Figure 11). *A. parasiticus* was the third species isolated from maize samples tested in the current study. Colonies representing this species produced dark green

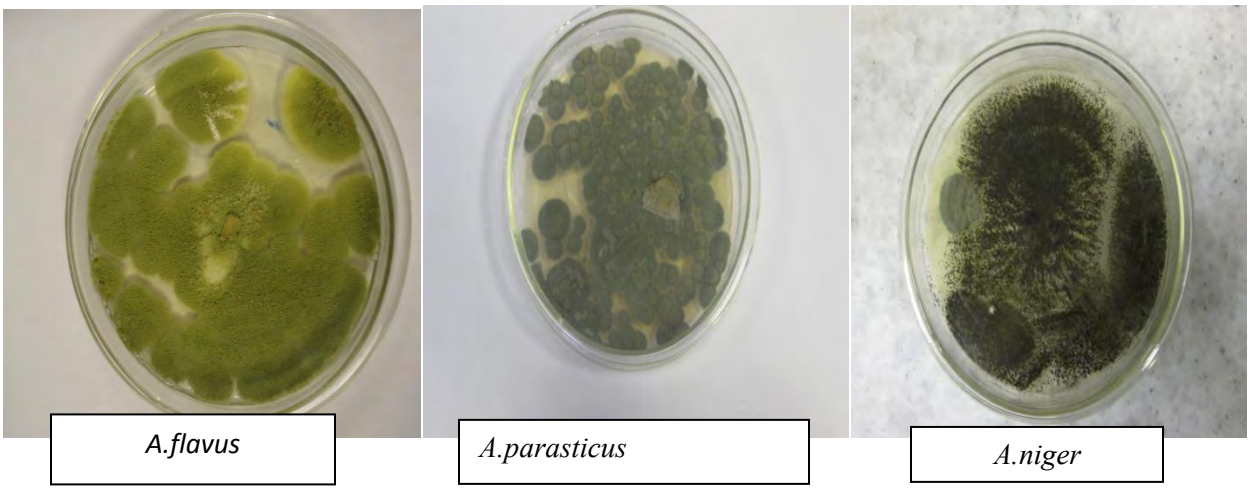

Fig: 11 *Aspergillus* spp. isolated from maize sample

These observations all the above species were consistent with the findings of (Abdi *et al*., 2014; Negero *et al*., 2014; Mohammed *et al.*, 2013; and Cotty *et al.,* 1994).

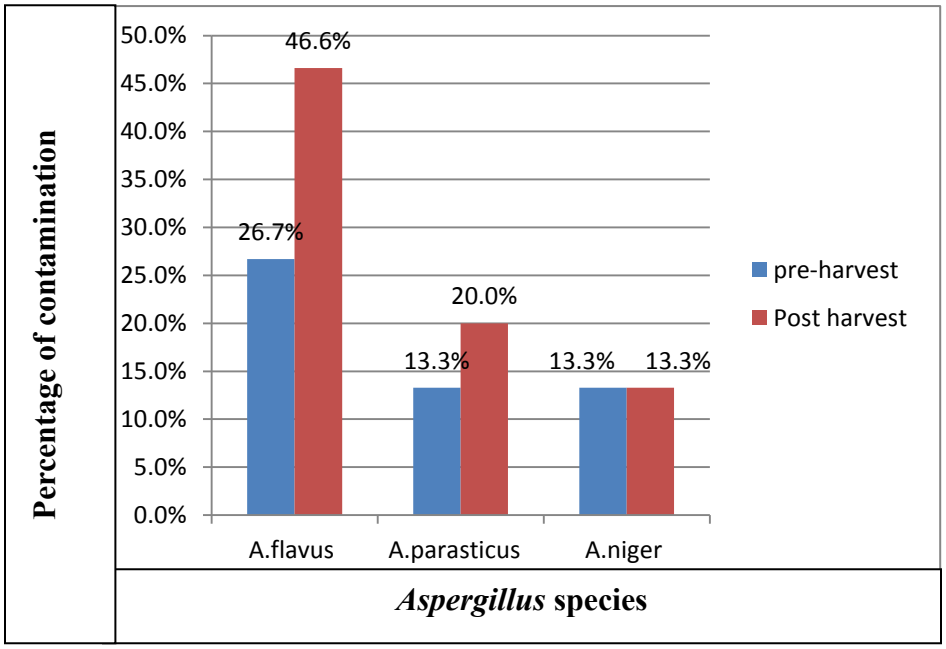

Fig 12: percentage of *Aspergillus species* pre and post-harvest maize

From 15 pre- harvest and 15 post-harvest maize samples collected from West Gojam *A. flavus*, *A. parasiticus* and *A. niger. A. flavus* were isolated on PDA. In pre harvest sample 53.3 % of samples were contaminated by *Aspergillus* species (26.7% = A.flavus, 13.3%= *A. parasiticus and 13.3%= A. niger)* and in post-harvest maize sample were contaminated *Aspergillus* species 80 % *(*46.6 % = *A.flavus, 20.0 %= A. parasiticus and 13.4 %= A. niger)* (Figure 9).

Aspergillus flavus is the main fungal species infecting maize grains. In the current study also the most prevalent *Aspergillus* species in pre-harvest was *A.flavus* (26.7%). This was lower than 80% reported in 1994 and 60% in 1995 in Benin (Sétamou *et al.,* 1997) and 65% in Nigeria (Bankole *et al.,* 2004).

Contamination *Aspergillus flavus* in post-harvest maize was 46.6%. This was lower than reported in Ethiopia 88% from cereal sample (Dawit *et al*., 1985) and in Kenya 78.5% (Benard *et al*., 2013) and higher than reported in Iran 27% (Khosrow *et al.,* 2015).

Aspergillus species contamination variation in different countries may be due pre and post - harvest practice of the farmers and also different in temperature, humidity, not (using crop resistance variety, crop rotation system, maintaining adequate irrigation schedule) also the cause of this variation (Kaaya *et aI.,* 2005). Genotype, soil types, drought and insect activity are also important in determining the likelihood of Contamination (Muthomi *et al.,* 2012. Moreover, poor harvesting practices, improper storage, and less than optimal conditions during transport and marketing can also contribute to fungal growth and increase the risk of mycotoxin production (Negero *et al*., 2014)

4.6.1. *Aspergillus* species contamination in relation to harvesting time

Table 11 showed that contamination of maize sample in post-harvest (80%) higher than pre harvest (53.3%) although there was no significant difference in contamination by *Aspergillus* species in pre and post-harvest(p=0.245).This may be due to short time (3 month difference between pre and post-harvest sample collection) for new A*spergillus* contamination.

Table 13: *Aspergillus* species contamination in relation to harvesting time

Harvesting time	No	Positive for No. (%) *Aspergillus* species	P-value
Pre harvest	15	8 (53.3)	0.245
Post-harvest	15	12 (80)	
Total	30	20 (66.7)	

4.6.2. *Aspergillus flavus* contamination in relation to harvesting time

A summary of Aspergillus flavus contamination associated to harvesting time is presented in Table 14. The result showed that the contamination of pre- harvest maize by *A.flavus* was 26.7% and post-harvest maize was *46.6 %* and statistical analysis showed that there was no significant relationship between *Aspergillus flavus* contamination and harvesting time (P=0.450).

Table 14: *Aspergillus flavus* contamination in relation to harvesting time.

Harvesting time	No	Positive for A. flavus No. (%)	P-value
Pre harvest	15	4(26.7)	0.450
Post-harvest	15	7(46.6)	
Total	30	11(36.6)	

4.6.3. Pre harvest *Aspergillus flavus* contamination in relation to locations

The result showed that the pre harvest *Aspergillus flavus* contamination was 0, 20 and 60% in Burie, finoteselam and Jabitehnan, respectively although there was no significant difference in Pre harvest *Aspergillus flavus* contamination between locations (P=0.231) which is <0.05(table 15).

Table 15: *A. flavus* contamination in pre harvest maize sample in relation to location

Location	Number of sample	*A.flavus*	p-value
		Positive N (%)	
Burie	5	0(0)	0.231
Finoteselam	5	1(20)	
Jabitenhan	5	3 (60)	
Total	15	4 (26.7)	

4.6.4. Post- harvest Aspergillus flavus contamination in relation to locations

The result showed that the post-harvest *Aspergillus flavus* contamination was 60, 20 and 60% in Burie, finoteselam and Jabitehnan, respectively although there was no significant difference in Pre harvest *Aspergillus flavus* contamination between locations (P=0.534) which is <0.05(table 16).

Table 16: *A. flavus* contamination in post-harvest maize sample in relation to location

Location	Number of sample	*A.flavus*	
		Positive N (%)	p-value
Burie	5	3(60)	0.534
Finoteselam	5	1(20)	
Jabitenhan	5	3 (60)	
Total	15	7 (26.7)	

The non-significant effect of location on *Asprgillus* species in our study may suggest that the difference was irrespective of the location and the difference might be due to other factors like poor harvest and post-harvest practices of the farmers on maize. The other possible explanations this might be due to the three locations have no significant difference in temperature, humidity and pre and post- harvest practice. Similar study was reported in Kenya (Sheila et al., 2012) and Nigeria (Atehnkeng *et al*., 2008)

5. CONCLUSIONS

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This research has shown that the level of total aflatoxin contamination is very high in pre and post-harvest maize. In addition, the pre and post maize sample also showed that high Aflatoxin B1 level in pre and post- harvest sample.

Paired t-test statistical analysis for mean of total and Aflatoxin B1 in pre and post- harvest maize samples were showed that both total and B 1 aflatoxin increase significantly from pre harvest to post - harvest maize.

Most of pre and post -harvest samples were exceeded the US Food and Drug Administration (FDA), the World Health Organization (WHO) maximum limit of total aflatoxin 20µg/kg. In this case also the contaminated maize sample in post- harvest was more exceeded the standards limit than the pre harvest.

Most of pre and post -harvest samples were also exceeded the maximum level of 2 µg/kg for aflatoxin B1 and 4 µg/kg aflatoxin total has been established by European Union (EU). Post-harvest- maize was more exceeded the European standard limit than the pre harvest.

This research has also shown that high *Aspergillu*s species contamination in pre and post-harvest maize samples. Post-harvest maize samples were more contaminated than the pre-harvest maize.

6. RECOMMONDATIONS

Aflatoxin contamination of agriculture commodities is gaining public prominence in Africa. This toxin is now perceived to have many more health effects than previously thought. Aflatoxins appear to be much more pervasive than previously thought, with a large percentage of foods and a high percentage of the population in Africa affected. The current study also showed that high percentage of *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize. The current study with its own limitation has investigated *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize but the following issues should also be considered in the future based on the outcomes of the current study.

- Prevention through pre- harvest, harvest and postharvest control management practice should be done to ensuring a safe final product.
  - Farmers should harvest maize not later than 3 weeks after the crop has attained physiological maturity
  - Sorting out of damaged cobs or grains at harvest and choice of storage structures that ensure good drying of maize are recommended.
  - Maize storage structures should be designed to meet standards taking into consideration the psychometric characteristics of the wind in order to prevent moisture drift into grains in order to maintain optimum storage temperatures.
- Investigation should focus on the effect of different pre- and post-harvest crop management systems on aflatoxin contamination in different agro-ecologies in Ethiopia and technologies that result in a significant reduction in aflatoxin levels should be promoted.
  - Further study with larger sample size will be helpful for confirmation of the difference factors associated with respect to the level of *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize
  - Further investigation on risk factor for *Aspergillus* species and Aflatoxin contamination in pre and post-harvest maize at different areas should be done, to determine the common sources of aflatoxin contamination in pre and post-harvest maize.
  - Further study should be done on the effect of delay harvesting time on aflatoxin level.

- Studies should be carried out on the aflatoxin contamination of standing maize in the different storage locations used for the study.
- Studies should be carried out on local alcohol drinks (like Tela and Areke) in relation to aflatoxin level.
- Studies should also be carried out on aflatoxin in relation to effect on human health.

- Educating stakeholders on the danger of commercializing and consuming mouldy foods.
  - The Agricultural Extension worker should educate farmers on the need to adopt Good Agricultural practices (GAP) in order to produce food free of hazards such as aflatoxins.
  - The media should also educate the public on the health hazards of aflatoxins
  - More awareness creation to improve the perception towards toxigenic fungi and associated mycotoxins should be done
  - Humans should learn to consume good quality grains as well as feed their livestock and poultry with uncontaminated feeds.
- Regular monitoring/ surveillance is needed
  - Maize grown for human consumption in areas where conditions are frequently favourable to fungal invasion and mycotoxin production in the field should be tested for mycotoxin contamination before use.

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ANNEX-1: Consent Form and Questionnaire, English Version

Addis Ababa University college of Natural science

Center for food science and Nutrition

Questionnaire for assessment of the *Aspergillus* species and aflatoxin contamination of pre harvest and post harvest maize in west gojam ethiopia

001. Date of interview ___/___/___

002. Questionnaire identification number _____

003. Interviewer name_____signature_____

Checked by supervisor. Signature _____date_____

Introduction

"My name is…….I am working as a data collector for a research being conducted by AAU, college of Natural science Center for food science and Nutrition. We are interviewing and testing *Aspergillus* species and aflatoxin contamination of pre harvest and post-harvest maize in west Gojam Ethiopia. Aflatoxin contamination is a potential source of health hazards and the spoilage of agricultural commodities**.** Your farm has been selected for this study. This finding of the study will be used as a basis for better planning of prevents possible aflatoxin contamination.

Verbal consent and confidentiality

Your answers are completely confidential. Your name will not be written on this form, and will never be used in connection with any of the information you tell me. You don't have to answer any questions that you don't want to answer, and you may end this interview at any time you want to. However, your honest answers to these questions and giving blood sample will help us better understand the *Aspergillus* species and aflatoxin contamination of pre harvest and post-harvest maize in west Gojam Ethiopia **.**We would greatly appreciate your help in responding to this survey. The study will be taken two times before and after harvest only 2 kg of sample will be taken .Would you be willing to participate?"……

(Signature of interviewer certifying that informed consent has been given verbally by respondent).

Age _____ sex _____ Education level_____

No	Questions	Coding categories	Skip to
101	Have you ever heard about Mould?	Yes…….....1 No……..2 →	103
102	How do you describe the type of color	_____	
103	Do you plow the land after growing maize before growing the next crop?	Yes……1 No……..2	
104	Do you use a crop rotation schedule to avoid planting the same commodity in a field?	Yes……1 No……..2	
105	Did you harvest the crop as soon as the crop is mature?	Yes……1 No……..2	
106	After harvesting did you dry to decrease the moisture content to the required level?	Yes……1 No……..2	
107	Do you have storage problems?	Yes……1 No……2 →	110
108	Which storage problem is the most important?	Insects……1 Rodents……..2 Birds……...…….3 Mould…….4	

		Others_____		
109	What did you do to solve this problem?	No treatment……..1 Storage insecticides…….……..…….2 Smoke……..3 Other (specify)_____		
110	How do you dry after harvesting	Solar drying……..…..……..1 Professional dryer……2 Drying using fire smoke……..3 Other specify_____		
111	Do you clean the storehouse before storage?	Yes…….……1 No……...2		
112	Where is your storage structure located?	Field……..…….,……1 In the house…….…..…….2 Courtyard…….…….3		
113	Do you store other products in the store, together with maize?	Yes…….,,,……..1 no……..……2 list?_____		
114	What storage method do you use?	_____		
115	For how long do you store the maize?	_____		

ANNEX 2: Consent Form and Questionnaire, Amharic Version

<u>አዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና የኒዉትሪሽን ማዕከል</u>

በአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና የኒዉትሪሽን ማዕከል የአፍላ ቶክሲን ብከላ በበቆሎ ላይ ከምርት በፊቲና በሁዋላ በተመለከተ ለሚደረግ ጥናት የተዘጋጀ መጠይቅ

001.ቃለ መጠይቅ የተካሄደበት ቀን _____/_____/_____

002.መለያቁጥር _____

003.የጠያቂውስም_____ፊርማ_____

የሀላፊው ማረጋገጫ. ፊርማ _____ቀን_____/____/___

<u>መግቢያ</u>

ሰላምታ። ስሜ _____ይባላል። እኔ ለአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና የኒዉትሪሽን ማዕከል ለሚደረግ ጥናት መረጃ ሰብሳቢ ነኝ፡፡ እኛ እዚህ አካባቢ በሚገኙ የበቆሎ ምርት ላይ የአፍላ ቶክሲን ብከላ በበቆሎ ላይ ከምርት በፊቲና በሁዋላ ለሚደረግ ጥናት ቃለ መጠይቅና በቆሎ እየሰበሰብን እንገኛለን፡፡ አፍላ ቶክሲን ማልት ፈንግስ በተባለ በተለይም ዐስፓጅን በተባለ የሚፈጠር መርዛማ ነገር ሲሆን በሰወች ጤና ላይ ጉዳት የሚያመጣ ሲሆን በተጨማሪም በምርታማነት ላይ ጉዳት ያመጣል፡፡

የሚሰጡት መረጃ ሚስጥርነቱ ሙሉ ለሙሉ የተጠበቀ ነዉ። መጠይቁላይ የርሰዎን ስም የሚገልፅ ማንኛዉም አይነት ነገር አይጠቀስም ወይም አይያያዝም፣ በመጠይቁ ወቅት የማይፈልጉትን ማንኛዉንም አይነት ጥያቄ መተዉ ወይም በማንኛዉም ሰዓት መጠይቁን ማቋረጥይችላሉ። ሆኖም ግን እርሰዎ የሚሰጡን እዉነተኛ መረጃ ወደ ፊት አፍላ ቶክሲን በበቆሎ ላይ ከምርት በፊቲና በሁዋላ ለተመለከተ ለሚያደርጉ ጥናቶች ጠቃሚ ይሆናል። ለዚህ ጥናት ለሚያደርጉልን ትብብር ምስጋናችን ከልብ የመነጨ ነዉ። ስለዚህ በመጠይቁ ለመሳተፍ ፈቃደኛነዎት?

_____

(የጥናቱንተሳታፊሙሉፈቃደኝነትያረጋገጠዉየጥናቱመረጃሰብሳቢፊርማ)

ጾታ _____ እድሜ_____ የትምህርት ደረጃ_____

ተቁ	ጥያቄ	መልሶች	ይለፍ
101	ስለ ሻጋታ ሰምተው ያውቃሉ?	አዎ……....1 አላውቅም……..2 →	103
102	እባከወ ቀለሙ ምን ነት ዐይነት እንደሆነ ይግፁልን ;	----------	
103	እህል ካመረታችሁ በኋላ ሌላ እህል ለመዝራት ማሳችሁን ታርሳላችሁ?	አዎ……..……..1 አናርስም……..2	
104	ማሳችሁ ላይ የተለያዩ እህሎች አፈራርቃችሁ ትዘራላችሁ	አዎ አፈራርቀን እንዘራለን ……1 አይ አፈራርቀን አንዘራም……..…….....2	
105	እህሉን የምትሰበስቡት ወዲያውኑ እንደደረሰ ነው?	አዎ ……..1 አይደለም……..2	
106	እህሉን ከወቃችሁ በኋላ የእህሉን የእርጥበት መጠን በተጨማሪነት ለመቀነስ ታደርቁታላችሁ?	አዎ ……..1 አናደርቀውም ……..2	
107	ምርተዎን ለርጅም ግዜ ስያስቀምጡ ችግር ገጥሞወት ያዉቃል	አዎ……..1 አልነበረም……2 →	110
108	አዎካሉ ምን አይነት ?	ነፍሳት……1 አይጦች ……..2 ወፎች……...……...…….3 ሻጋታ……..…….4 ሌላ _____	

109	እነዚህን ለማጥፋት ምን ተጠቀሙ?	ምንም……..……..1 ተባይ ማጥፍያ……..……..……2 ጭስ ……..……..3 ሌላ _____	
110	ምርተዎን ለማድረቅ ምን ይጠቀማሉ	ፀሀይ ……..……..……..….1 የማድረቅያ መሳርያ…….....….2 ሌላ _____	
111	ምርተዎን ለርጅም ግዜ ከማስቀመጠዎ በፊት ቦታዉን ያፀዳሉ?	አዎ……..1 አላፀዳም…….……2	
112	ምርተዎን ለርጅም ግዜ የት ያስቀምጣሉ?	ሜዳ ላይ……..……,……1 ቤት ዉስጥ…….……...…….2 ከቤት አጠገብ…….……3	
113	ከበቆሎ ጋር ሌላ ምርት ቀላቅለዉ ያስቀምጣሉ?	አዎ……1 አላስቀምጥም……..2	
114	የበቆሎ ምርተዎን በምን ያስቀምጣሉ?	_____	
115	የበቆሎ ምርተዎን ለምን ያህል ግዜ ለያስቀምጣሉ?	_____	

ANNEX 3: Location, Level of Aflatoxin and moisture content in Pre harvest maize sample from west Gojam

CODE	LOCATION	Moisture content (%)	AFBG2 µg/kg	AFG1 µg/kg	AFB2 µg/kg	AFB1 µg/kg	AFTOTAL µg/kg
PRE001	Burie	14.04	6.40	ND	ND	4.48	10.88
PRE002	Burie	14.30	10.39	7.94	ND	ND	18.32
PRE003	Burie	14.46	ND	3.27	0.54	3.87	7.68
PRE004	Burie	15.70	ND	ND	ND	ND	ND
PRE005	Burie	15.67	ND	ND	0.33	3.20	3.53
PRE006	Finoteselam	15.13	1.56	2.60	0.12	ND	4.28
PRE007	Finoteselam	15.71	ND	ND	ND	ND	ND
PRE008	Finoteselam	13.87	ND	ND	ND	3.13	3.13
PRE009	Finoteselam	13.60	5.21	20.72	10.42	27.31	63.66
PRE010	Finoteselam	13.57	ND	ND	ND	ND	ND
PRE011	Jabitehnan	15.17	1.26	2.37	0.20	3.28	7.11
PRE012	Jabitehnan	14.65	2.07	20.39	1.03	10.95	34.45
PRE013	Jabitehnan	13.29	1.81	19.72	ND	6.68	28.21
PRE014	Jabitehnan	13.13	1.64	36.28	3.96	5.65	47.54
PRE015	Jabitehnan	12.99	1.18	38.27	1.00	6.52	46.97
Mean		14.35	2.10	10.10	1.17	5.00	18.38

ANNEX 4: Location, Level of Aflatoxin and moisture content in Post- harvest maize sample from west Gojam

CODE	LOCATION	moisture content	AFBG2 µg/kg	AFG1 µg/kg	AFB2 µg/kg	AFB1 µg/kg	AFTOTAL µg/kg
POSOO1	Burie	12.25	24.62	2.84	0.88	23.99	52.32
POSOO2	Burie	13.60	9.76	6.56	0.66	4.95	21.93
POSOO3	Burie	12.70	ND	9.45	1.27	5.35	16.07
POSOO4	Burie	11.59	ND	ND	23.41	2.83	26.24
POSOO5	Burie	12.45	ND	ND	3.18	5.85	9.02
POSOO6	Finoteselam	13.81	0.80	11.96	10.85	4.47	28.08
POSOO7	Finoteselam	13.32	ND	ND	ND	ND	ND
POSOO8	Finoteselam	13.30	0.68	6.49	3.43	25.55	36.15
POSOO9	Finoteselam	12.78	16.23	27.86	9.58	29.58	83.26
POSOO10	Finoteselam	12.89	ND	ND	ND	ND	ND
POSOO11	Jabitehnan	12.58	1.35	3.74	0.95	19.64	25.68
POSOO12	Jabitehnan	12.18	50.07	82.85	3.92	2.54	139.38
POSOO13	Jabitehnan	11.93	13.26	42.58	ND	6.74	62.59
POSOO14	Jabitehnan	10.98	ND	35.50	48.98	9.69	94.17
POSOO15	Jabitehnan	10.23	5.47	41.85	1.41	6.77	55.51
	Mean	12.44	8.15	18.11	7.23	9.86	43.36

ANNEX 5: Statistical test of total aflatoxin level in pre harvest maize in relation to locations (One way ANOVA and descriptive)

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1664.794	2	832.397	2.285	0.144
Within Groups	4371.176	12	364.265		
Total	6035.97	14			

Location	N	Mean	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Burie	5	8.0820	3.15315	-.6725	16.8365
Finoteselam	5	14.2140	12.39056	-20.1877	48.6157
Jabitehnan	5	32.8560	7.42229	12.2484	53.4636
Total	15	18.3840	5.36122	6.8853	29.8827

ANNEX 6: Statistical test of total aflatoxin level in post- harvest maize in relation to locations (One way ANOVA and descriptive)

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	7778.969	2	3889.485	3.523	0.063
Within Groups	13246.78	12	1103.898		
Total	21025.75	14			

Location	N	Mean	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Burie	5	25.1160	7.39048	4.5967	45.6353
Finoteselam	5	29.4980	15.29195	-12.9593	71.9553
Jabitehnan	5	75.4660	19.33587	21.7810	129.1510
Total	15	43.3600	10.00613	21.8990	64.8210

ANNEX 7: Statistical test of Aflatoxin B1 level in pre- harvest maize in relation to locations (One way ANOVA and descriptive)

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	**55.17**	**2**	**27.585**	**0.534**	**0.6**
Within Groups	**620.01**	**12**	**51.668**		
Total	**675.181**	**14**			

Location	N	Mean	95% Confidence Interval for Mean	
			Lower Bound	Upper Bound
Burie	5	2.3100	-.3677	4.9878
Finoteselam	5	6.0899	-8.7386	20.9185
Jabitehnan	5	6.6156	3.1636	10.0676
Total	15	5.0052	1.1594	8.8510

ANNEX 8 : Statistical test of Aflatoxin B1 level in post- harvest maize in relation to locations (One way ANOVA and descriptive)

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	32.265	2	16.133	0.148	0.864
Within Groups	1304.299	12	108.692		
Total	1336.564	14			

Location	N	Mean	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Burie	5	8.5935	3.88306	-2.1876	19.3746
Finoteselam	5	11.9190	6.47043	-6.0458	29.8838
Jabitehnan	5	9.0793	2.87582	1.0948	17.0639
Total	15	9.8639	2.52281	4.4530	15.2748

ANNEX 9: Statistical test of total aflatoxin level difference between pre and post- harvest maize in relation to location (One way ANOVA and descriptive).

	Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Between Groups	2848.442	2	1424.221	2.215	0.152
Within Groups	7715.568	12	642.964		
Total	10564.01	14			

Location	N	Mean	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Burie	5	11.7860	7.53754	-9.1416	32.7136
Finoteselam	5	15.2840	6.60650	-3.0586	33.6266
Jabitehnan	5	42.6100	16.89136	-4.2879	89.5079
Total	15	23.2267	7.09259	8.0146	38.4388

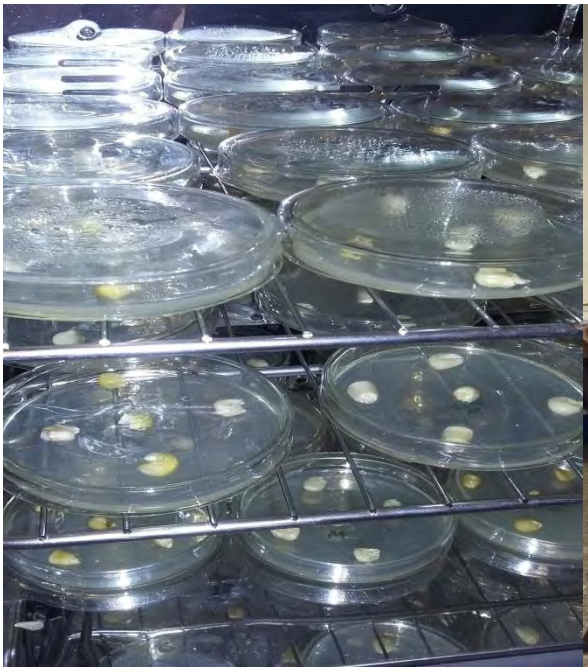
ANNEX 10: Statistical test of location and aflatoxin B1 level difference between pre and post- harvest maize (One way ANOVA and descriptive)

	Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Between Groups	79.376	2	39.688	0.491	0.624
Within Groups	969.039	12	80.753		
Total	1048.415	14			

	N	Mean	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Burie	5	6.2812	3.35419	-3.0315	15.5939
Finoteselam	5	7.9617	4.57957	-4.7532	20.6767
Jabitehnan	5	2.4637	4.02850	-8.7212	13.6486
Total	15	5.5689	2.23438	.7766	10.3611

ANNEX 11: Method validation and aflatoxin analysis on HPLC-FLD pictures

ANNEX 12: *Aspergillus* species isolation and identification pictures



DECLARATION

I, the undersigned, declare that this is my original work and has never been presented in this or any other University and that all the source materials used for this thesis have been suitably acknowledged;

Name **Signature**

Masresha Ahmed _____

Place: Addis Ababa University, Addis Ababa, Ethiopia

Date of Submission: June 15, 2015

The thesis has been submitted for examination with my approval as a University Advisor.

Name: Dr. Ashagrie zewdu

Signature: _____

Date: June 15, 2015