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COMPARATIVE STUDY OF AFLATOXINS LEVEL BETWEEN TRADITIONAL AND INDUSTRIAL BARLEY MALT IN ETHIOPIA

BY

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Declaration

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

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List of Abbreviations and Acronyms

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AOAC	Association of Official Analytical chemists
CSA	Central statistics agency
EU	European Union
FB1	Fumonisin B1
FDA	Food Drug Administration
EFMHACA	Ethiopian Food, medicine and health care administration and control authority
GAP	Good agricultural practice
HACCP	Hazard Analysis and critical control point
HL	Hectoliters
HPLC-FLD	High-performance liquid chromatography with fluorescence Detector
HP-TLC	High performance thin-layer chromatography
IAC	Immunoaffinity Column
IARC	International Agency for Research on Cancer
KAP	Knowledge, attitude and practice
LAB	Lactic acid bacteria
LC	Liquid chromatographic
LOD	Limit of Detection
LOQ	Limit of Quantification
NGOs	Non-governmental organizations
OTA	Ochratoxin A
PBS	phosphate buffer saline
RSD	Relative standard deviation
RP-HPLC	Reversed phase high performance liquid chromatography
STDEV	Standard Deviation
TFA	Tri floro acetic acid
TLC	Thin-layer chromatography
WHO	World health organization

Abstract

Aflatoxin is the secondary metabolite produced by specific strains of Aspergillus species. It is a global health concern, which leads to contamination of cereal grains. This research was conducted to evaluate and compare the level of aflatoxins in both traditional and industrial barley malts. Aflatoxins B_1, B_2, G_1 and G_2 were determined by immunoaffinity column clean up and high performance liquid chromatography with fluoresce detection. Twenty four traditionally produced barley malts (eight samples from each site) were collected from Debremarkos, Finoteselam and Enjibarra. Six samples (three local and three imported) were collected from three different industries. Besides, a purposive survey for the evaluation of knowledge, attitude and practice (KAP) about aflatoxin were conducted in both traditional and industrial producers. The mean aflatoxin level of Debremarkos, Finoteselam and Enjibarra malts were 5.30 µg/kg, 1.90 µg/kg and 6.72 µg/kg, respectively, whereas the mean aflatoxin level of industrially produced local and imported barley malt samples were 1.22 µg/kg and 1.69 µg/kg, respectively. The finding of this study was compared to the European Legislation, the aflatoxin B_1 contamination of four samples for traditional and none of industrially produced barley malts were above 2 µg/kg of the tolerable limit. Similarly, the total aflatoxin content of eight for traditional and one for industrially produced barley malt were above 4µg/kg of the tolerable levels. Furthermore, comparing the aflatoxin content of traditionally and industrially produced barley malt, the aflatoxin content of traditional barley malt was higher than the aflatoxin content in industrially produced barley malt. On the other hand, the knowledge, attitude and practice towards mycotoxin specifically aflatoxin in traditional barley malt producers were lower than industrial barley malt producers. In conclusion, the aflatoxin contaminations of traditionally produced barley malt were more at risk than barley malt produced industrially. Therefore aflatoxin control and management needs attention from farm to fork in the value chain as malt is in input to industrial or traditional beverages where a very large number of peoples are used to consume.

Keywords: *Aflatoxin, traditional barley malt, industrial barley malt, KAP, HPLC*

1. Introduction

1.1 Background

Barley (*Hordeum vulgare L.)* is one of the most important cereal crops that are largely produced in the central and south east mid and high altitude areas of Ethiopia. It is the fifth important cereal crop after tef, maize, wheat and sorghum with annual production of 1.9 million tons in 2014 (CSA, 2014). Ethiopia is the second largest barley producer in Africa next to Morocco accounting for about 25 percent of the total barley production in the continent (FAO, 2014). Barley belongs to the oldest and economically most important crops. It is one of the staple food crops used to prepare (injera, porridge and bread) and local drinks (Tella and Besso). Its use for malting and animal feed in Ethiopia makes it an important crop in terms of the lives and source of revenue of small farmers. Barley farmers in Ethiopia have not fully adopted modern inputs like fertilizer and modern seeds that help boost production (CSA, 2014).

Cereal crops such as barley, sorghum, maize, wheat and corn are susceptible for mycotoxin contamination. Mycotoxins are naturally occurring secondary metabolites of fungi and toxic to humans, animals and plants. *Fusarium, Aspergillus* and *Penicillium* species are the most significant fungal pathogens attacking agricultural commodities. Grain contamination and damage may occur in the field during kernel maturation, in harvest, transportation and storage processes, if conditions of high moisture and temperature are present (Volkova, 2013). Barley germination in the malting process plays a significant role for brewing; it has high moisture and temperature conditions which are favorable for yeast and mould growth. During this time, the production and accumulation of extra amounts of mycotoxins mostly *Fusarium* toxins may occur.

Aflatoxins are a group of naturally-occurring mycotoxins and are produced by many species of Aspergillus fungi most notably *Aspergillus flavus* and *Aspergillus parasiticus*. It can be classified as B_1, B_2, G_1, G_2 and the M_1 and M_2 are after metabolizing of B_1 and B_2 respectively. Aflatoxin production is the consequence of a combination of species, substrate and environment while in the substrate production can happen in the field and in storage conditions between 20 and 40 ºC with a 10- 20% of moisture and 70-90% of relative humidity of the air. Aflatoxins are produced at the end of the exponential phase or at the beginning of the stationary phase of the mould growth. The formation of aflatoxins is influenced by physical, chemical and biological

factors. The physical factors include temperature and moisture. The chemical factors include the composition of the air and the nature of the substrate. Biological factors are those associated with the host species (Hesseltine, 1983).

Aflatoxins are naturally occurring carcinogenic substances and are extremely toxic to humans when consuming aflatoxin contaminated foods (Songsermsakul & Razzazi-Fazeli, 2008). They are recognized by the International Agency for Research on Cancer (IARC, 2002) as hepatotoxic and carcinogenic agents to humans and capable of inducing liver cancer and cirrhosis, as well as reducing individuals' immune resistance, causing outbreaks of type B viral hepatitis. One of the reasons which make aflatoxin one of the most challenging mycotoxin is the fact that it could be produced by the responsible fungi not only at pre-harvest time but also at post-harvest stages including storage. However, lack of regulations which makes the use of such contaminated commodities inevitable could lead to severe human and animal diseases too.

Malt is the main brewing grain material prepared from barley by germination and modification processes (Volkova, 2013), where as malting is the process of converting barley or other cereal grains into malt for use in brewing, distilling or in foods and takes place in a malting sometimes called a malt house or a malting floor. Malting process which, involves cleaning, soaking, germination and drying aims to change the grain into malt with high enzymes and vitamin contents. Barley malt in the brewing process has a major influence on the organoleptic quality of the final product. Sugars, amino acids and lipids contribute to colour and flavor compounds, while proteins and polysaccharides are accountable for foam formation and the body of beer (Ullrich, 2011).

Therefore, the present study was conducted to assess the level of aflatoxin contamination in traditional and industrial barley malt produced in Ethiopia, using High Performance Liquid Chromatography with Immunoaffinity column cleanup. In addition, a purposive survey was conducted to assess the existing knowledge, attitude and practice (KAP) on barley malt related to food safety issues.

1.2 Statement of the problems

Cereals are the main source of food in the diet of people around the world. However, it can easily be contaminated with fungi in various stages of production and storage. Mycotoxins are poisonous and are known to cause chronic health risks. Prolonged exposure to mycotoxins through diet has been linked to cancer and immune-system diseases in human beings (Bhat and Vasanthi, 2003). Aflatoxin contamination of food causes hepatotoxicity, carcinogenicity, mutagenic, teratogenic and also causes induction of tumors, impaired central nervous system, skin disorders, hormonal defects and decreased bone strength (Saleemullah *et al*., 2006). Aflatoxin exposure has also been associated with childhood stunting and it is a chronic form of malnutrition. It is potentially associated with many health problems including an increased rate of infectious illnesses, impaired learning capabilities and reduced work productivity (Black *et al.,* 2013).

The economic impact of aflatoxins leads directly to cereals and livestock losses as well indirectly to costs of health care and regulatory programmes designed to reduce risks to animal and human health. Aflatoxins are extremely persistent under most conditions of storage, handling and processing. It is impossible to eliminate them once the food stuffs are contaminated (Ubwa *et al*., 2014).

In Ethiopia, the problem of aflatoxin contamination in agricultural commodities is much more serious and considered to provide a favorable condition for aflatoxigenic mould production of agricultural products. For instance a recent study on *Aspergillus species* and aflatoxin levels in sorghum (*Sorghum bicolor L.*) stored at different period and storage system in Northern Shewa, indicated that about 56.7%, 16.7%, and 23.3% of the *sorghum* samples were found to be contaminated with *Aspergillus flavus, Aspergillus niger* and *Aspergillus parasiticus*, respectively and the level of aflatoxin B1, B2, G1, and G2 were in the range of 3.95 to 153.72µg/kg, 1.17 to 91.82µg/ kg, 9.87 to 139.64µg/kg, and 3.22 to 52.02µg/kg, respectively (Geremew *et al*., 2016)

In another research conducted in Ethiopia for natural occurrence of toxigenic fungi species and aflatoxin in freshly harvested groundnut kernels in Tigray, Northern Ethiopia, showed all samples were found 100 percent positive for *Aspergillus* species, with the detected aflatoxin concentrations ranging from 0.1 to 397.8 ppb (Dereje et al., 2012).

In developing countries such as Ethiopia the risk of human and animal exposure to consumption of mycotoxin contaminated food or feed is very high. This is mainly due to lack of awareness, regulations, management recommendations and grain-test check points and at times due to serious food shortage when people tend to consume moldy grains.

However, there were little related researches conducted on the aflatoxin level of traditionally and industrially prepared malt on this area. Therefore, the study of the status of aflatoxin level in traditional and industrial barley malt is significant at this time to understand the current situation in that particular study area.

1.3. **Significance of the study**

The finding of this study is helpful to indicate the level of aflatoxin contamination in traditional and industrially barley malt grains and the findings also helpful in safeguarding the public health of the nation by increasing knowledge and awareness of the aflatoxin contamination on agricultural food products. Besides, to reduce the healthcare costs as well as increase the well-being and healthy life style of the consumers.

Finally, this study provides a baseline data for barley malt manufacturers and traditional producers on aflatoxin contamination in barley malt grains and also to provide data to the concerned regulatory authorities on the level of aflatoxin contamination in barley malt grains.

1.4.Research Questions

- Is there a significant aflatoxin contamination in Ethiopian traditionally and industrially produced barley malt?
- What is the existing knowledge, attitude and practice (KAP) on traditionally and industrially producer barley malts in relation to mycotoxin particularly aflatoxin contamination?

1.5.Objectives

1.5.1. General objectives

To compare the level of aflatoxin between traditional and industrial barley malts in Debremarkos, Finoteselam, Enjibarra, Gonder, Assela and Addis Ababa.

1.5.2. Specific objectives

- To determine the level of aflatoxins (B_1, B_2, G_1 and G_2) in traditional and industrial barley malt in Ethiopia.
- To compare the level of aflatoxins in traditionally and industrially produced barley malts in Ethiopia with the International standards.
- To assess the knowledge, attitude and practice (KAP) of aflatoxin in traditionally and industrially produced barley malts in Ethiopia.
- To avail the information to the regulatory bodies for their preventive and controlling.

2. Literature Review

2.1.Overview of barley malt production

Barley malt is essential to the beverage industry as it is the predominant raw material in beer production world-wide (Ross *et al*., 2002). The main purpose of malt is to supply fermentable sugars, amino acids and vitamins required for growth and alcoholic fermentation during beer brewing (Hattingh, 2013). A basic rule is that for malt to be made the barley must be capable of germination, with a minimum germination of 98 % and maltsters often struggle to deliver a constant malt product as they are faced with inconsistent malt batches and poor germination capacities of dormant barley (FAO, 2009).

Worldwide, there are few malt processing companies. For instance, Malteurop (France), the largest malting company in the world with a current annual production capacity of more than 2.2 million tons. Belgium has taken over the biggest US brewering Anheuser-Busch and this newly created company in Belgium is the leading global brewer and one of the world's top five consumer product companies. The top ten malting companies produce approximately 9.4 million tons or 44% of global malt production (FAO, 2009).

Table 1. Main malt producing countries (thousand tons).

Country	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**Trend 2005/2004**
World total	**17820**	**18487**	**18296**	**18640**	**19140**	**19704**	**+3%**
World leaders:							
1. China	2870	2954	2870	2380	2730	3220	+18%
2. United States	2404	2060	1952	1923	1990	2086	+5%
3. Germany	1635	2000	2000	2072	1797	1436	-20%
4. United Kingdom	1452	1490	1477	1501	1425	1332	-7%
5. France	1155	1162	1183	1211	1211	1225	+1%

Source: FAOSTAT internal follow-up, 2008

Worldwide, major malt producers are integrated with the grain trading business, which reduces the risks, related to the origination of malting barley from producers where as some maltsters offer specific contracts to farmers (contract farming) to secure their supplies. In 2006–2007, the

global trade of malting barley decreased to 5.5 million tons in grain equivalent 0.2 million tons less than the previous year, this decrease was largely due to a reduced supply on the global market, high prices and increasing local production (FAO, 2009).

2.2.Production of barley malt in Ethiopia

Malt is the major raw material for both commercial and traditional beer production. The product has an increasing demand with beer production. The practice of traditional barley malt in Ethiopia is different from one place to another but the main processing steps are removing of impurities like sand and stones from the cereals, soaking for 3-4 days depending on the production year of the cereals and also the environment, removing the water by using sieve then cover the cereals by leaf and put inside of the basket and put mud or stone over the leaf in order to produce heat then stay for 3-5 days then remove the leaf from the malt by using hand manually finally, put the malt on the floor or on the roof of the house in order to dry.

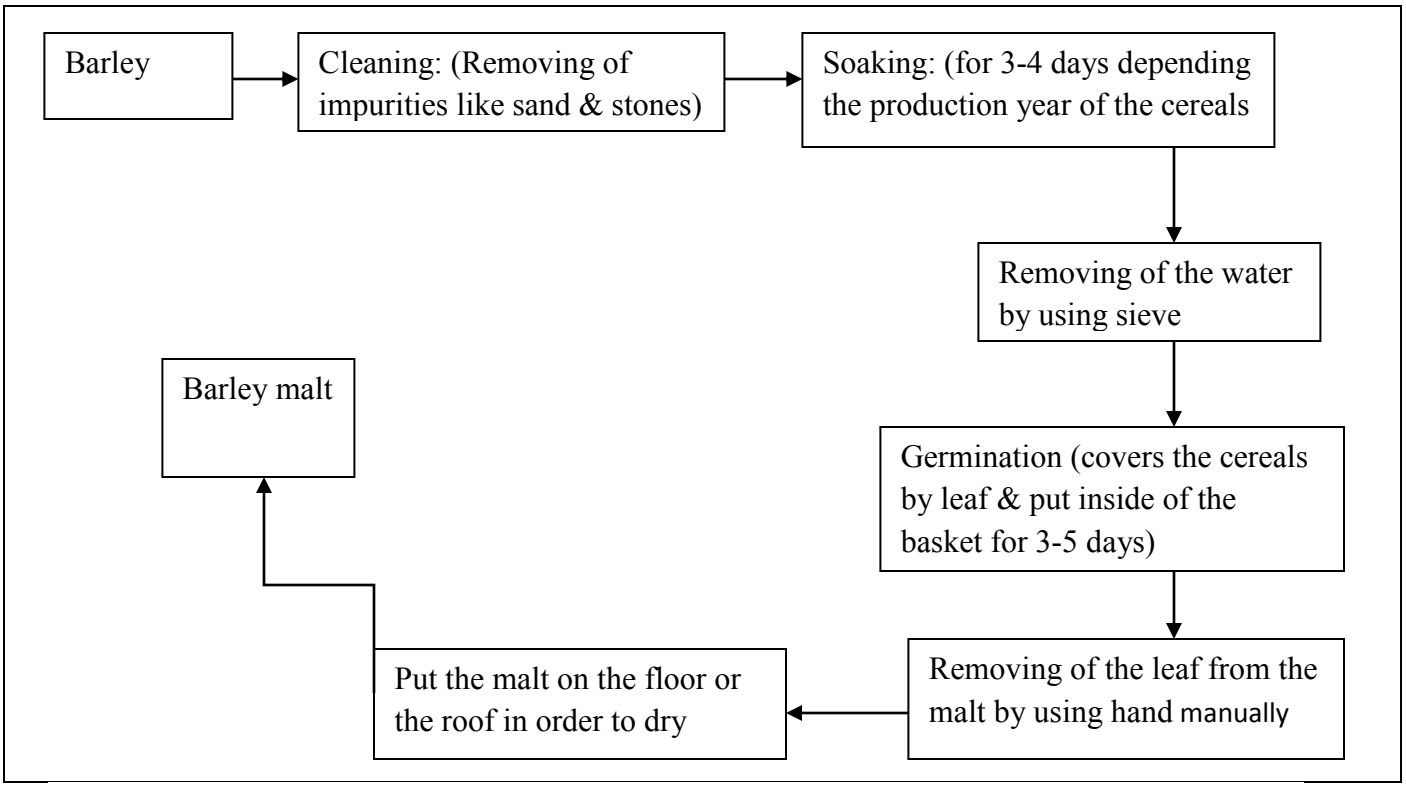


Figure 1. Traditional barley malt processing flow chart.

The above figure.1 shows the overall process for traditional barley malt but the whole process and practice is not in a controlled manner where as in industrial process everything is controlled. The main potential area for supplying of barley malts are Arsi, Bale, East and West Gojam, North and South Gonder and Awi zones for both traditional and industrial process (Mulatu and Grando, 2011).

In Ethiopia, there are two malt plants i.e. Assela malt factory -located in Assela town of Oromiya regional state which was established in 1984 with the aim of supplying malt to the local breweries and the plant is located in area where barley can be grown potentially where as Gonder malt factory located in Gonder town of Amhara regional state which was recently established factory and it had almost the same potential to Assela malt factory by producing malt. Since the existing plants in the country do not satisfy the demand for malt, breweries are forced to import from abroad as shown in Table 2 (Malt profile in Ethiopia).

Table 2. Import of malt in Ton

Year	**Quantity**	**Value(Birr)**
2000	3042	13,117,368
2001	9624	36,749,085
2002	5509	21,557,400
2003	5428	24,143,453
2004	6184	30,032,464
2005	10913	52,942,054
2006	26967	126,449,016

Source: - Customs Authority

Table 2 depicts that import of malt has been increasing since year 2004. The imported quantity which was 5,428 tons in the year 2003 has increased by about 14% and reached at 6,184 tons by the year 2004. A substantial increase of import is registered during the recent two years. The imported quantity in year 2005 was 10,913 tons. Compared to year 2004 it has shown an increase of about 76%. Similarly a huge amount of malt has been imported during 2006 which amounts 26,967 tons. The imported of quantity in 2006 is 2.5 times higher compared to the previous year.

The main reason for such huge increase of import is due to the in ability of the existing factory to satisfy the demand of breweries which have experienced production increase in the last few years.

The production of modern barley malt has a very short history associated with beer making in Ethiopia, which started in the early twenties with the establishment of the St. George Brewery. Even though Ethiopia has favorable environment and potential market opportunity, the share of malting barley production is quite low (about 15%) as compared with food barley. At present there are six breweries in Ethiopia and the existing total capacity of these breweries is 2.7 million hectoliters (HL) which needs 45,679 tones of malt every year. However, the capacities of Asela and Gonder malt factories are supplying 15,000 tones and 16200 tones of malt respectively and the remaining are imported (Getachew *et al*., 2007).

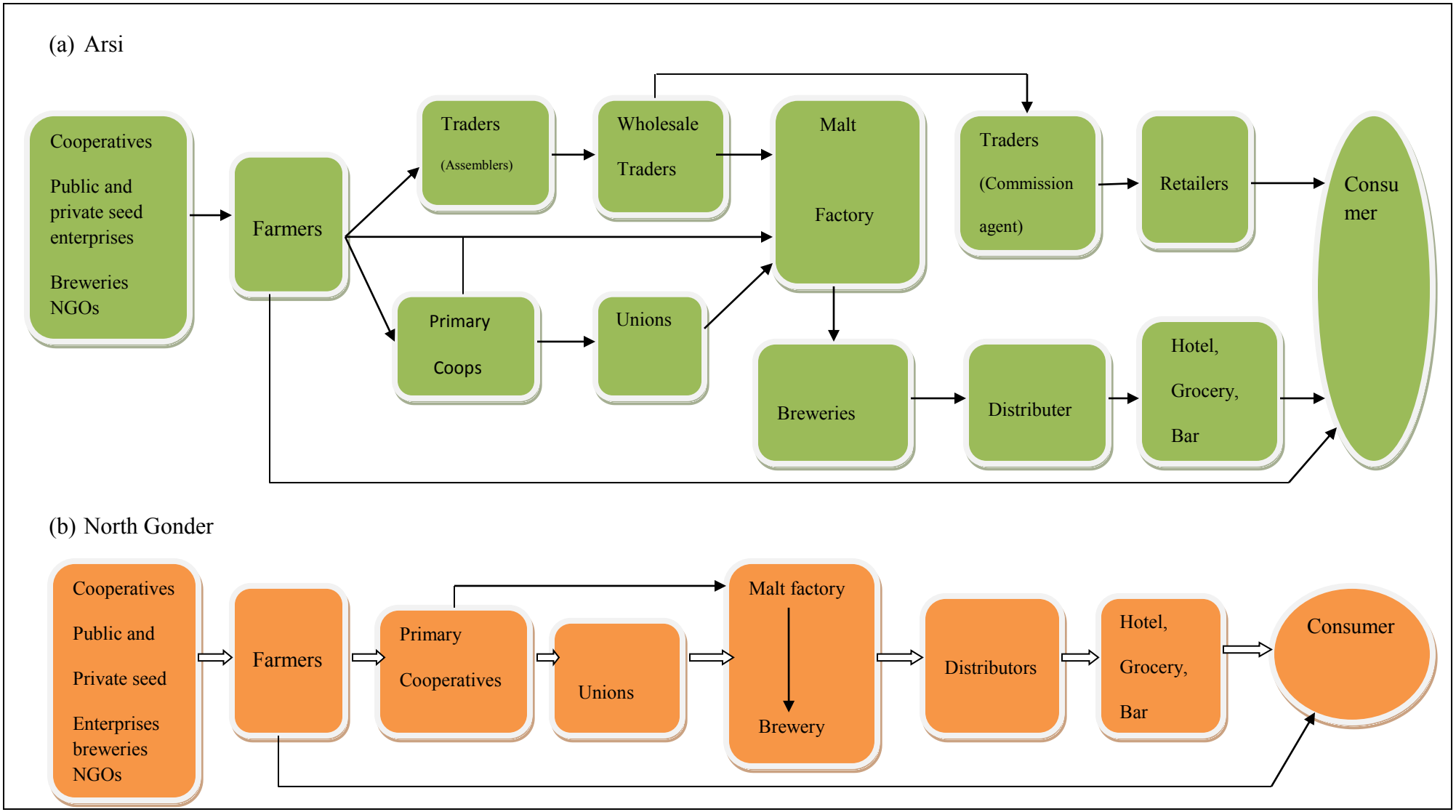


Figure 2. Value chain map for barley malt in Arsi and North Gondar, Ethiopia.

Marketing chains vary depending on the infrastructure and other market fundamentals at the location of production. Two zones Arsi and Gondar selected for the rapid appraisal appear to vary significantly in terms of barley marketing. While farmers in Arsi produce both food and malt barley and market through cooperatives and traders, farmers in Gondar exclusively market their malt barley through cooperative. Figure 2 depicted that a large number of actors are involved at the beginning of the marketing chain, that is, in providing input supplies. In both Arsi and Gondar, there are several government agencies (national research system, seed enterprises), cooperatives, holding companies, NGOs and private seed companies that are involved in seed marketing. In the Arsi marketing chain, both traders and cooperatives appear and compete in marketing both food and malt barley. This is a bit counter-intuitive given our earlier results that cooperative's share in total marketed volume is small, while the north Gondar is unique in that cooperatives market all of the malt barley and the cooperative share in total marketed volume is large (Shahidur *et al*, 2015).

2.3. Industrial malt processing

2.3.1. Malting process

Malting begins with the getting of quality grain and this incoming grain is received with moisture content not exceed 12% (UK Malt, 2011). This prevents the growth of mold and decreases the likelihood of mycotoxin build up within the stored grain. Every load is sampled, inspected and tested at the intake point. Once tipped the grain is cleaned through screeners to remove stones, foreign bodies, dust and straw whereas once cleaning and drying processes are complete then the grain is stored in silos. The malting process usually consists of four stages: grain cleaning and grading, steeping, germination and kilning (Hattingh, 2013). Each stage in the process is carefully monitored to ensure good malt quality.

a) Cleaning and grading

Barley kernels undergo a series of pre-cleaning and grading processes before malting can begin. Initial cleaning involves the passing of grains through several sieves to remove dust and foreign materials such as stones and straw. This is followed by grading, which includes the separation of kernels based on size and grading into different sizes is principally performed to germinate similar sizes of grain in the same batch to have an even germination during the process.

Broken or damaged grains are rich in polysaccharide-producing microbes such as Pseudomonas spp. and Flavobacterium spp. that may cause filtering problems during brewing (Follstad and Christensen, 1962). Grading usually separates kernels on sieves of 2.5 mm in diameter. The plumper fractions are malted, while the thin grains are sold as animal feed (Ullrich, 2011).

b) Steeping

During the steeping process the cleaned barley ready for malting is submerged in water to raise the moisture content in the barley. In modern industrialized malt houses the grain is mixed with water into slurry at temperatures between 14 -16 °C (Schwarz and Li, 2011). Over the course of 24 - 40 hours, the steep will undergo a minimum of two or three water changes that are interspersed with ventilated air rests between tank refills. Above the line of the steep tank immersions and air rests, the grain reaches 35% moisture content and this is enough to stimulate germination, steeping continues until the grain reaches a final moisture content of 42-48% (Schwarz and Li, 2011).

c) Germination

The objective of germination in malting is to synthesize enzymes that initiate the modification of endosperm components to make them available for brewing. The quiescent barley grain is composed of stabilized raw materials, including carbohydrates, proteins and lipids. Through germination, these raw materials are modified by enzymatic activity into smaller components, such as sugars, amino and fatty acids (Shewry and Ullrich, 2014).

Small size grains usually germinate faster than large size grains. During the process the temperature and the moisture are controlled to the desired conditions then the maltery has been observed not to have any difficulty in such processes. In the malting process, germination generally takes between four and five days, at which point the green malt is moved to the kiln and modification is discontinued ((Bamforth and Barclay, 1993); (Papazian, 2003)).

d) Kilning

During kilning the green malt is evenly spread across the floor and hot air is blown through the grain bed. This process kills the embryo and terminates germination while preserving the malt extracts and retaining its enzyme activity. Over the course of approximately 24 hours the green

malt undergoes two major phases that is withering and curing, while withering takes place over the first 10 hours of kilning at temperatures ranging from 50-75^{o}C (Schwarz and Li, 2011). The purpose of withering is to slowly remove the moisture from the grain so that modification is arrested and the enzymes stop working but are not denatured, however curing entails gradually increasing the temperature to between 80-110˚C (depending on malt style) over the remainder of the kilning time (Bamforth and Barclay, 1993). This enables the maltsters to develop the desired malt color and aroma for the particular malt they are creating.

After the ageing process the finished malt has reached a moisture content of approximately 3-5% (Bamforth and Barclay, 1993). This moisture content keeps enzymatic activity dormant and the malting process (steeping, germination and kilning) has decreased the possibility of spoilage. The malt is then stored in climate controlled storage bins prior to being transferred to the brew house. These storage bins maintain a climate that is low in humidity (less than 10.5%) and temperatures of 10 - 20˚C (Queensland, 2015).

2.4.Mycotoxin

The term mycotoxin was first used in the 1960s to describe the toxin associated with infected peanuts in animal feed and the loss of turkeys in England or Turkey-X-disease. This mycotoxin was later identified as the *Aspergillus flavus* toxin, aflatoxin B_1. Mycotoxins are toxic secondary metabolites produced by many species of microscopic filamentous fungi occurring on field cereals, including barley and these fungi evoke a toxic response when introduced in low concentrations to higher vertebrates and other animals by a natural route (Bennett, 1987).

The most abundant fungal genera affecting the malting barley are *Alternaria*, *Aspergillus*, *Penicillium* and *Fusarium,* which simultaneously showed relatively high producing potential for a wide range of mycotoxins almost 30% of alternaria, 20% of Aspergillus and 88% of Fusarium fungi isolated from barley grains was able to produce alternaria toxins, aflatoxins, ochratoxin A, deoxynivalenol and zearalenone (Medina *et al*., 2006). Additionally, to the relatively common micro mycetes also the *Claviceps purpurea* causing the ergot disease belongs to frequent barley pathogens (Schwarz *et al*., 2007). A research was done in Czech Republic on the determination of seventeen mycotoxins in barley and malt by using ultra-performance liquid chromatography coupled to mass spectrometry. A total of 52 barley and malt samples were determined and

among those, none of samples contained any of neither four aflatoxins nor ochratoxin A, but Fumonisin B_1 occurred only in one sample, Fumonisin B_2 and zearalenone were found in two barley samples finally Enniatins were detected in all samples. The values did not exceed the maximum allowable limit for the selected mycotoxins in unprocessed or processed cereals set by the European Union (Bolechova *et al*., 2014)

Traditionally, toxigenic fungi contaminating agricultural grains or products have been conventionally divided into two groups; those attack seed crops have been described as field fungi such as *Cladosporium*, *Fusarium, Alternaria spp*. which reputedly gain access to seeds during plant development and storage fungi such as *Aspergillus*, *Penicillium spp*. which proliferate during storage (Legan, 2000). Currently, this division is not so strict because according to (Miller, 1995) four types of toxigenic fungi can be distinguished: (1) Plant pathogens as *Fusarium graminearum* and *Alternaria alternata;* (2) Fungi that grow and produce mycotoxins on senescent or stressed plants, such as *Fusarium. moniliforme* and *Aspergillus flavus*; (3) Fungi that initially inhabit the plant and increase the feedstock's susceptibility to contamination after harvesting, such as *Aspergillus flavus;* (4) Fungi that are found on the soil or decaying plant material that occur on the developing kernels in the field and later proliferate in storage if conditions permit, such as *P. verrucosum* and *A. ochraceus.*

Mycotoxins are usual contaminants in raw materials, food and feeds such as mould species that can grow on a wide range of substrates under a wide range of environmental conditions and they occur in agricultural products all around the world (Bennet and Klich, 2003). It has been estimated that 25% of the world's crops are affected by fungal growth and commodities may be both pre- and post-harvest contaminated with mycotoxins. The mycotoxins that can be expected in food differ from country to country in relation to the different crops, agronomic practices and climatic conditions (Bryden, 2007). For instance, a research conducted in Russia on mycotoxin in brewing grain raw material that was barley malt of 40 samples analyzed during 2007-2011, seven were contaminated with mycotoxin and in two incidents the mycotoxin levels were higher than maximum allowable levels. It was T-2 (150 μg/kg) and OTA (10 μg/kg). At the same time 120 malt samples were examined, 67 were contaminated by mycotoxin and the mycotoxin concentration were found to be higher than the maximum allowable levels, that were Aflatoxin in four cases with concentrations ranging from 5 μg/kg to 15 μg/kg; OTA in three incidents,

samples consisted respectively 25, 30 and 40 µg/kg; T-2 in three incidents, ranging from 200 µg/kg to 400 µg/kg; ZEA in one incident, 150 µg/kg (Volkova, 2013).

Exposure to mycotoxins may occur through ingestion, inhalation and dermal contact and it is almost always accidental. Most cases of mycotoxicoses result from eating contaminated food and human exposure can be direct via cereals or indirect via animal products such as meat, milk and eggs (CAST, 2003). Most mycotoxins are relatively heat-stable within the conventional food processing temperature range (80–121°C), therefore so little or no destruction occurs under normal cooking conditions such as boiling and frying, or even following pasteurization (Milicevic *et al*., 2010). In general, the application of a food process includes physical treatments (cleaning and milling) and thermal processing like cooking, baking, frying, roasting and extrusion reduces mycotoxin concentrations significantly, but does not eliminate them completely. The different treatments have various effects on mycotoxins and those that utilize the highest temperatures have the greatest effects; roasting or cooking at high temperatures (above 150 °C) appears to reduce mycotoxin concentrations significantly (Bullerman and Bianchini, 2007).

2.4.1. Toxicity of mycotoxin

Mycotoxins are potent toxic to humans and animals at low concentrations when they are absorbed through consumption, inhalation or dermal absorption. It has been shown that ingestion of contaminated food or feed is the main source of mycotoxin exposure to both human and animals (Ajoy *et al*., 2010; Ilunga, 2012). Although mycotoxicoses caused by direct consumption of contaminated food products and feed stuffs produce undesirable effects and poses the greatest risk in human or animals and it have been characterized as food or feed related, non-contagious, non-transferable and non-infectious (Zain, 2011). Mycotoxins have various acute and chronic effects on humans and animals depending on the species. Within a given species, the impact of mycotoxins on health is influenced by age, sex, weight, diet, exposure to infectious agents and the presence of other mycotoxins and pharmacologically active substances (Milicevic *et al*., 2010); Zain, 2011). The majority of mycotoxins currently known are grouped according to their toxic activity, under chronic conditions as mutagenic, carcinogenic or teratogenic where as grouping according to their site of action results in hemo-, hepato-, nephron-, dermato-, neuro- or immunotoxins (Niessen, 2007).

2.4.2. Influence of environmental factors on mycototoxin production

The production of mycotoxins is highly susceptible to temperature, moisture, water activity, pH and oxygen concentration, the same environmental factors that affect the growth of toxigenic fungi. Moisture and temperature are two factors that have a crucial effect on fungal proliferation and toxin biosynthesis (Bryden, 2007; Palumbo, 2010). The incidence and level of mycotoxin contamination are closely related to the geographic position and to seasonal factors as well as to the cultivation, harvesting, stocking and transport conditions (Milicevic *et al*., 2010). Mycotoxin contaminations can be categorized into the one that occurs in the developing crop or pre-harvest and the one that develops after maturation or post-harvest. In the pre-harvest period, preventive measures are incorporated in good agronomic practices, such as the careful use of insecticides and fungicides, irrigation to avoid moisture stress, harvesting at maturity and improvement by genetic resistance to fungal attack. Throughout the post-harvest period, the control of the moisture and temperature of the stored commodity will largely determine the degree of fungal activity and consequently the mycotoxin synthesis (Bryden, 2007). Treatments with chemicals including sodium bisulfite, ozone and ammonia, acids and bases, represent an opportunity to control fungal growth and mycotoxin biosynthesis in stored grains (Bozoglu, 2009; Magan, 2006; Magan and Aldred, 2007). In recent years, a good control of mycotoxigenic fungi has been achieved using plant products such as extracts and essential oils as environmental friendly fungicides (Nguefacka *et al*., 2004; Reddy *et al.,* 2010; (Thembo *et al.,* 2010). Likewise, biological control represents a new opportunity in control strategies. There is evidence that Bacillus sp., propionic acid bacteria and lactic acid bacteria (LAB) are able to inhibit fungal growth and mycotoxin production (Bianchini and Bullerman, 2010).

2.5.Aflatoxin

Aflatoxin belongs to a group of fungal toxins known as mycotoxins and produced by fungi Aspergillus species, such as *Aspergillus flavus* and *Aspergillus parasiticus*; in particular, *Aspergillus flavus* is common in agriculture. *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius* and *Aspergillus pseudotamari* are also aflatoxin-producing species but they come across much less frequently (Bennett and Klich, 2003).

Aflatoxins in agricultural commodities are primarily produced by *A. flavus* and *A. parasiticus* and this *A. flavus* produces only aflatoxins B while *A. nomius* and *A. parasiticus* produce both B

and G toxins ((Rustom, 1997; Yu *et al*., 2002). Depending upon colour of the fluorescence, aflatoxins are divided into aflatoxin B_1 and B_2 for blue and G_1 and G_2 for green (Dalvi, 1986) where as Aflatoxin M_1 and M_2 known as milk-aflatoxins, are the metabolites of AFB_1 and AFB_2, respectively. Among the known aflatoxins, AFB_1 is the most commonly produced mycotoxin and the most potent and it has been reported to be the most powerful natural carcinogen in mammals (Creppy, 2002).

Also aflatoxin is the secondary metabolite produced by specific strains of *Aspergillus*; these species contaminate various agricultural commodities either before harvest, at post-harvest stages or storage under favorable conditions of temperature and humidity. They have been found in mouldy human food and animal feeds and have been implicated in numerous health disorders (Russell, 2007).

Aspergillus is a large genus of mould which grows at an optimal range of temperature of 28-33°C and at water activity of about 0.83-0.97. The aflatoxigenic moulds namely *A. flavus; A. parasiticus* and *A. nomius* are principally found in soils, decaying vegetation and in warmer parts of the world such as tropical regions where temperature and moisture are high. They have a higher affinity of growth in nuts and oilseeds but *Aspergillus flavus* may be the predominant producer of the B group (Russell, 2007). Moreover, aflatoxin M_1 is usually considered to be a detoxication by-product of aflatoxin B_1 and it is also the hydroxylated metabolite present in animal products that eat foods containing the aflatoxin B_1 toxin.

A research related to aflatoxin was conducted in Botswana on fungi, aflatoxins, fumonisin B_l and zearalenone contaminating sorghum-based traditional malt, wort and beer. Out of the 46 malt samples, 72% contained *Rhizopus stolonifer*, 63% *Fusarium verticillioides* (syn. *Fusarium moniliforme)* and 37% *Aspergillus flavus*. Although *Aspergillus flavus* was isolated from malt samples, aflatoxins B_1, B_2, G_1 and G_2 were not detected in any of the samples analyzed. When the malt, wort and beer samples were analyzed for fumonisin B_l and zearalenone, fumonisin B_1 was detected in 3 malt samples, with concentrations ranging from 47 to 1316 μg/kg, while zearalenone was detected in 56%, 48% and 48% of the malt, wort and beer samples respectively. Zearalenone concentration in samples ranged from 102 to 2213 μg/kg in malt, 26 to 285 μg/l in wort and 20 to 201 μg/l in beer. Zearalenone carry-over from wort to beer ranged from 23 to

403%. Therefore, although aflatoxins and fumonisin B_1 do not appear to be major contaminants, zearalenone is common and could pose a potential problem in traditional beer in Botswana (Nkwe *et al*., 2005).

2.5.1. Causes of aflatoxins

Cereal crops grown under warm and moist weather in tropical or subtropical countries are especially more prone to aflatoxin contamination than those in temperate zones. Groundnuts and groundnut meal are by far the two agricultural commodities that seem to have the highest risk of aflatoxin contamination. Although these commodities are important as substrates, fungal growth and aflatoxin contamination are the consequence of interactions among the fungus, the host and the environment. The appropriate combination of these factors determines the infestation and colonization of the substrate and the type and amount of aflatoxin produced. Water stress, high-temperature stress and insect damage of the host plant are major determining factors in mould infestation and toxin production.

Similarly, specific crop growth stages, poor fertility, high crop densities and weed competition have been associated with increased mould growth and toxin production where as the moisture content of the substrate and temperature are the main factors regulating the fungal growth and toxin formation. A moisture content of 18% for starchy cereal grains and 9-10% for oil-rich nuts and seeds has been established for maximum production of the toxin (WHO, 1979). On the other hand the minimum, optimum and maximum temperatures for aflatoxin production have been reported to be 12^0_c - 27^0_C and 40^0_c -42^0_C respectively. Frequent contamination of corn and other commodities with high levels of aflatoxins has been a serious problem all over the world resulting in significant economic losses to farmers and a health hazard to farm animals and humans as well.

2.5.2. Structure and physical properties of aflatoxin

Aflatoxins were isolated and characterized after the Turkey X disease that caused the death of more than 100.000 turkey poultries due to the intake of a contaminated peanut meal produced in South America originating from contaminated raw material (Blout, 1961; Goldblatt, 1969). The most important aflatoxins among the 13 compounds so far identified are the aflatoxin B_1, B_2, G_1 and G_2 and the aflatoxin metabolic by products M_1 and M_2. Aflatoxin B_1 is considered the most

toxic and is produced together with aflatoxin B_2 by both *Aspergillus flavus* and *Aspergillus parasiticus* where as aflatoxin G_1 and G_2 are produced exclusively by *Aspergillus parasiticus*. Aflatoxins M_1 and M_2 were originally discovered in the milk of cows which fed on moldy grain. Aflatoxin M_1 has been observed also in the fermentation broth of *Aspergillus parasiticus*. These compounds are products of a conversion process in the animal's liver that try to make these molecules more hydrophilic to be easily excreted from body via the kidney. Aflatoxin M_1 is a metabolite of aflatoxin B_1 in humans and animals where exposure at ng levels can come from mother's milk. Likewise, aflatoxin M_2 is a metabolite of aflatoxin B_2 in milk of cattle fed on contaminated food (Santini and Ritieni, 2013).

Chemically they are stable in foods and resistant to degradation under normal cooking procedures and their chemical structure incorporates dihydrofuran and tetrahydrofuran moieties coupled to a substituted coumarin. Accumulation of aflatoxin is dependent upon weather conditions and it is difficult to eliminate once it is produced. Before harvest, the risk for the development of aflatoxin is greatest during major droughts even though when soil moisture is below normal and temperatures are high the number of Aspergillus spores in the air increases. These spores infect crops through areas of damage caused by insects and bad weather.

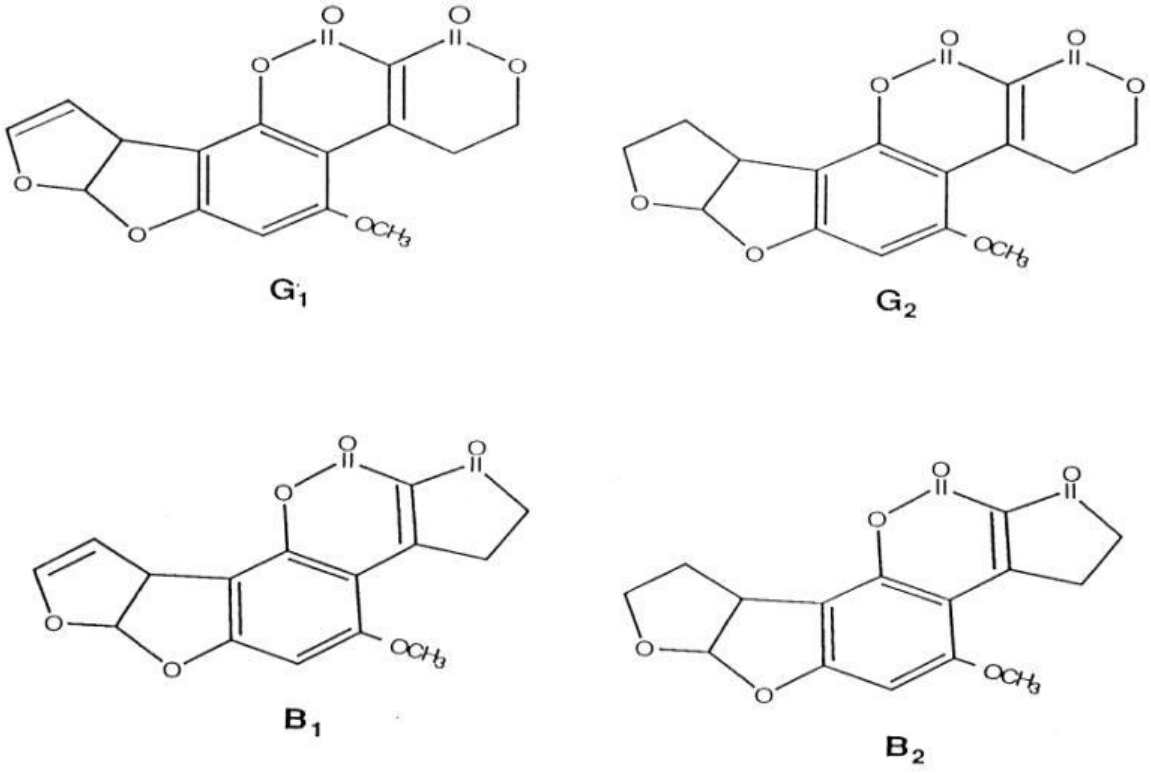


Figure 3. Chemical structure of aflatoxin. Source: **(**Cole, 1981).

Table 3. . Summary of physical properties and spectral characteristics of aflatoxins.

Property Aflatoxins	B1	B2	G1	G2	M1
Chemical Formula	$C_{17}H_{12}O_8$	$C_{17}H_{14}O_8$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$	$C_{17}H_{12}O_7$
Molecular weight	312	314	328	330	328
Melting point (0_C)	268-269 (D)1	287-289 (D)	244-249 (D)	230	299 (D)
Sorbent Pentane, +	Chloroform	Chloroform	Chloroform	Ethyl acetate	Methanol
Fluorescence emission	425 nm	425 nm	450 nm	425 nm	425 nm

D = Decomposition Source: (Cole, 1981)

2.5.3. Occurrence of aflatoxin in foods

Aflatoxin producing fungi particularly *Aspergillus flavus* are common and widespread in nature and most often found when certain grains are grown under stressful conditions such as drought. It often occurs in crops in the field of pre-harvest as well as most commonly in post- harvest. Post-harvest contamination can occur when the food commodities are stored under high moisture content and high temperature conditions which facilitate the rapid growth of moulds; the level of contamination depends on the plant stress, temperature, water activity and storage condition. The mould occurs in soil, decaying vegetation, hay and grains undergoing microbiological deterioration and invades all types of organic substrates when the conditions are favorable for growth particularly in hot and humid situations (Ominski *et al*., 1994). Under such suitable environment aflatoxigenic fungi contaminate foods directly or indirectly.

In direct contamination, the product is infected with aflatoxigenic fungi with subsequent toxin production. Indirect contamination occurs when food or feed was previously contaminated with aflatoxin producing fungi and although the fungi has been removed or killed during processing, aflatoxins still remains in the final product. Such contamination of cereals and oilseeds is the main point of entry of many mycotoxins in the human and animal dietary systems particularly in Africa (Smith and Moss, 1985).

Aflatoxins have been also detected in milk, cheese, corn, peanuts, cottonseed, nuts, almonds, figs, grape berries, spices and a variety of other foods and feeds but among this corn, peanuts and cottonseed are the highest risk of aflatoxin contamination. Milk, eggs and meat products are contaminated sometimes due to the consumption by the animal of aflatoxin contaminated feed. In general, all foods and feedstuffs are vulnerable to fungal contamination but the nature and degree of aflatoxigenic fungal contamination will depend on the presence or absence of aflatoxin in the product (Santini and Ritieni, 2013).

2.5.4. Aflatoxin and human health

Human exposure to aflatoxin is principally through ingestion of contaminated foods where as inhalation of the toxins may also occur occasionally due to the occupational exposure (HKSAR, 2001). Consumption of aflatoxin has a negative impact on health, great economic loss and causes diseases called aflatoxicosis and this can lead to acute or chronic aflatoxicosis based on the duration and amount of exposure and can compound existing health issues or the risk of disease

transmission such as aflatoxin-related immune suppression, liver cancer, liver cirrhosis, as well as nutrition-related problems in children such as stunted growth.

Acute toxicity of aflatoxins in humans however represents a serious threat. For example, in 1974 it has been reported in India as an outbreak of hepatitis and 100 cases of death attributed to the consumption is heavily aflatoxin contaminated maize causing an aflatoxins intake of two to six mg per day (Krishnamachari *et al*., 1975) Based on these data, it has been estimated that the acute lethal dose for adults is approximately ten to twenty mg of aflatoxins (Pitt, 2000). Aflatoxins have been in years associated to various health conditions such as kwashiorkor, a severe malnutrition disease, to a form of pediatric aflatoxicosis (Hendrickse, 1997).

Developed countries which have sufficient amounts of food combined with regulations that monitor aflatoxin levels protect human populations from significant aflatoxins ingestion. However, in countries where populations are facing starvation or where regulations are either not enforced or non-existent, routine ingestion of aflatoxin may occur (Cotty *et al*., 1994).

Worldwide, liver cancer incidence rates are two to ten times higher in developing countries than in developed countries (Henry *et al*., 1999). A joint Food and Agriculture Organization/World Health Organization/United Nations Environment Programme Conference Report stated that in developing countries where food supplies are already limited, extreme legal measure may lead to lack of food and to excessive prices.

2.5.5. **Toxicology of aflatoxin B_1**

Aflatoxin B_1 created great interest amongst the medical profession once it was established as a powerful carcinogen (Wogan, 1973). In fact it is claimed to be the most powerful naturally occurring carcinogen known. The toxin is also mutagenic and teratogenic (Raisuddin *et al*., 1993). Because of these toxic properties, several investigations have been carried out over the years since its discovery both *in vitro* and *in vivo* to elucidate its mode of action as a carcinogen, perhaps to the detriment of studying other toxic properties it exhibits as well as those of its congeners AFB_2, AFG_1 and AFG_2. To justify this obsession with AFB_1, it is fair to point out that it is the most commonly occurring and at the highest levels as well as being the most potent when compared with the other aflatoxin. However, it might be worth while investigating the

toxic properties of AFB_2 more closely, as this has all the molecular attributes of AFB_1 apart from the bishydro-furano double bond that confers the carcinogenic property.

AFB_1 has a tendency to specifically attack guanine, one of the four DNA bases (Taylor, 1992). Because AFB_1 is somewhat non-polar, it passes though membranes and other lipid barriers easily and also has slight water solubility so it passes from the aqueous phase at low concentrations and accumulates in fat soluble phases such as adipose tissues. Furthermore, the molecule itself, because it is primarily aromatic in nature, the main core is a coumarin structure, which is rather flat and therefore, can intercalate into DNA (Jones *et al*, 1998).

Because of the interaction of AFB_1 with DNA, it is reasonable to ascribe it to be its main toxicological action, certainly at low chronic level of exposure. AFB_1 can form AFB_1-DNA adducts, DNA strand breaks, DNA base damage and oxidative damage that can lead to cancer (Wang and Groopman, 1999). This damage can be repaired by various mechanisms, e.g. base excision repair (Wood, 1999). However, certain mutations that occur due to AFB_1's action may interfere in these repair mechanisms, in particular the xeroderma pigmentosum complementation gene group D which is one of the groups encoding for groups in the nucleotide excision repair pathways. Recent findings suggest that two loci in this group are of particular importance in modulating the AFB_1 related development of liver cancer (Long *et al*., 2009).

2.5.6. Management of aflatoxin

Aflatoxin production increases with high temperature, high humidity and insect damage, so control of temperature and humidity are therefore, important in aflatoxin management. *Aspergillus flavus* and *Aspergillus parasiticus* are unable to produce aflatoxin at water activity of less than 0.7 or relative humidity below 70% however, under stress condition such as drought; aflatoxin contamination can be higher (CAC, 2005).

The physical and chemical treatment of contaminated commodities includes detoxification of aflatoxins using physical means such as removal of contaminated commodities or inactivation of the toxin in the commodity. These methods include mechanical sorting and separation, washing, density segregation, solvent extraction, irradiation and oxidation (Awad *et al*., 2010). However, efficiency of these techniques will depend on level of contamination. Furthermore, results obtained are often uncertain and relatively expensive and could remove or destroy essential

nutrients in feed. Chemical methods of deactivating mycotoxins in feeds and also clay products that could be used in deactivating mycotoxins have been extensively been reviewed by Kolosova and Stroka (2011). However, aflatoxin management strategies can be divided into Pre-harvest and Post-harvest strategies.

❖ **Pre- harvest strategies, this strategy includes:-**

- Good agricultural practices (GAP) this involves adequate fertilizer application and crop rotation with non-host.
- Management of insect pests that affect crops for fungal infection through availability of infection channels such as wounds and other entry points.
- Optimal harvest time so that crops are not left in the field exposed to environmental factors that predispose crops to pathogen infection. Harvesting immediately after physiological maturity is recommended since aflatoxin level can increase with delayed harvest interval (Kaaya, 2006).
- Suitable management of crop residues as they harbor pathogens that are able to survive saprophytically (Awad *et al*., 2010).
- Management with fungicides has challenges due to environmental pollution and also emergence of resistant pathogen populations and also chemical residue in food products. Of fundamental value are environmentally friendly strategies. Polysaccharides and glycol proteins particularly β-glucans from *Basidiomycetes lentinulaedodes* (edible mushroom) is known to promote health effects in animals and human and have ability to inhibit aflatoxin biosynthesis by stimulating the antioxidant defence of the toxigenic fungus. Oxidative stress induced using paraquat enhanced the expression of β-glucan synthase gene and stimulated effect of β-glucans production that leads to a higher aflatoxin inhibiting capacity. Efficient inhibition could be due to higher content of β-glucans (Reverberi *et al*., 2011). Utilization of microorganisms or their enzymatic metabolites to detoxify mycotoxins in food and feed has advantages such as mild reaction conditions, target specificity, efficiency and environmental friendly.
- Biological control is use of one microorganism to control another microorganism such as Pseudomonas strains (Palumbo *et al*., 2010). It has been noted that *Aspergillus flavus* strains differ in aflatoxin production and this influences crop contamination. There are strains that produce a lot of aflatoxins and also produce numerous small sclerotia (<400μm). These are

the ‘S’ strains (toxigenic strain). Another strain the ‘L’ strain produces low aflatoxin levels and a few large sclerotia that are about >400µm and are atoxigenic (Cotty and Cardwell, 1999). There is competitive exclusion when one strain competes to exclude another in the environment. This implies that a shift of strain profile from toxigenic to atoxigenic is a viable biological control strategy.

- **Post-harvest management**

Some of the post-harvest strategies that could be used in Africa include rapid and proper drying of cereals or maize to moisture level of 13% or below. This will halt growth of fungi in the product however, products stored with high moisture increase growth of fungi in the stored product and this leads to increase of aflatoxin in the product (Hell *et al*., 2008). Post-harvest insect control can prevent damage to cereals and quality management systems for Hazard Analysis Critical Control Point (HACCP) should be employed for management of mycotoxins (Schamale and Munkvold, 2011).

2.5.7. Over view of aflatoxin studies in Ethiopia

As previously discussed before aflatoxin is a group of secondary metabolites produced by fungi *Aspergillus* species, such as *A. flavus* and *A. parasiticus;* in particular, *A. flavus* is common in agriculture and affect at all levels of value chain systems. Aflatoxin B1 is a contaminant of grain and fruit and has one of the highest levels of carcinogenicity of any natural toxin. AFB_1 and the fungi that produce it can also contaminate the raw materials used for beer and wine manufacture, such as corn and grapes. Prevalence of the status of aflatoxin in Ethiopia is common due to; (a) predisposing pre and post harvest factors, (b) Low or limited knowledge of aflatoxin by value chain actors, (c) Lack of regulation framework and monitoring facilities both at national and regional level and (d) limited research/lab facilities and trained personnel. Recent activities on aflatoxin in Ethiopia include survey, laboratory analysis of samples (mycological and toxin analysis) and field experiments were done (Alemayehu and Usha, 2014).

For instance, in a research conducted on natural occurrence of mycotoxins in staple cereals like barley, sorghum, teff (*Eragrostis tef*) and wheat by using high performance liquid chromatography (HPLC), AFB_1 and OTA were detected in all four crops samples at concentrations ranging from trace to 26 µg/kg and a mean concentration of 54.1 µg/kg respectively (Amare *et al*., 2006). A research conducted on fungal infection and aflatoxin

contamination in maize collected from Gedeo zone by using thin layer chromatography method and the result showed that all the samples tested were beyond the safety level of aflatoxins as determined by Food and Drug Administration and European Union (Chauhan *et al*., 2016).

Another research conducted in East Hararghe by Wondimeneh et al, 2016 showed that the aflatoxin B_1 and total fumonisin contamination and their producing fungi in fresh and stored sorghum grain were detected. Aflatoxin B_1 was detected at levels ranging from <LOD to 33.10 µg/kg grain. In addition a research done on screening of aflatoxins in Shiro and ground red pepper in Addis Ababa, the result showed that only aflatoxin B_1 was detected in both types of foodstuff and the levels of aflatoxin in Shiro and ground red pepper positive samples ranged from 100 to 500 ppb and 250 to 525 ppb, respectively (Fufa and Urga, 1996).

Moreover, a research on multimycotoxin analysis of sorghum (*Sorghum bicolor L. Moench*) and finger millet (*Eleusine coracana L. Garten*) were done and all the tested sorghum and finger millet samples were found to be contaminated by *Fusarium* and *Aspergillus* species (Alemayehu *et al*., 2014). These are a few of the research done and many investigators have detected aflatoxins, mycotoxins and incidence of Aspergillus in many agricultural foods in Ethiopia such as in ground nuts (Abdi and Alemayehu, 2014, Ephrem, 2015, Mawcha and Kahsay, 2014, Eshetu and Dawit, 2010), in maize, sorghum, barley, wheat, millet, tef (Binyam, 2016, Amare, 2010, Habtamu and Kelbesa, 2001) and in milk and milk product (Melkamu and Birhane, 2014, Dawit *et al*., 2016).

2.5.8. Determination of aflatoxin by HPLC and Immuno affinity Column

2.5.8.1.High performance liquid chromatography(HPLC)

Chromatography is one of the most popular methods to analyze aflatoxin which includes Thin-layer chromatography (TLC), high performance thin-layer chromatography (HP-TLC), and high-performance liquid chromatography (HPLC). Thin layer chromatography is also known as flat-bed chromatography or planar chromatography which is used for the separation, purity assessment and identification of aflatoxins. In this method there is lack of precision where as the high performance thin-layer chromatography method improves the precision by automating the sample application and plate interpretation steps. High performance liquid chromatography is by far the most reported chromatographic method using a variety of detection strategies. It was

developed rapidly in recent years, about 80% of the world organic compounds (health food efficacy composition, nutritional fortifiers, vitamins, protein etc.) use HPLC for separation and determination. The assessment of the quality of foods using this method provides an acceptable, accurate and alternative method to establish guidelines and to evaluate the status of aflatoxins in contaminated foods. Now-a-days, HPLC methods are widely used because of their superior performance and reliability as compared to TLC and this method has been developed for all major mycotoxins in cereals and other agricultural commodities. In the field of analysis of aflatoxins, HPLC is mainly used for final separation and detection of the analyte of interest and extraction and clean-up techniques have to be applied prior to detection with HPLC.

HPLC have high efficiency, high sensitivity (HPLC-FLD with as low as 0.1 ng /kg detecting limit (Herzallah, 2009) and high resolution. The most commonly used detectors for HPLC are fluorescence detectors (FLD), which rely on the presence of a chromophore in the molecules. A number of toxins already have natural fluorescence such as aflatoxins and can be detected directly by HPLC–FLD. In addition HPLC in determination of aflatoxins and their metabolites showed higher levels of accuracy and lower detection limits when using immuno affinity column (IAC) combined with application of FLD (Brera *et al*., 2007; Jaimez and Fente, 2000).

Chromatography columns are the most important part of the HPLC and there are two types of HPLC methods are commonly used i.e., normal and reversed phase columns are used for separation and purification of toxins depending on their polarity. In normal phase chromatography, a polar stationary phase such as silica gel and a non-polar solvent such as hexane are used whereas reversed-phase chromatography (RP-HPLC) utilizes non-polar stationary phase such as C-8 or C-18 hydrocarbons and polar mobile phase such as water, methanol or acetonitrile. Aflatoxins are naturally strongly florescent compounds, so the HPLC identification of these molecules is most often achieved by florescent detection (Akiyama *et al*., 2001). Therefore, it is more preferable to analyze aflatoxin from barley malt sample by using immuno affinity column, high-performance liquid chromatography with fluorescence detector.

2.5.8.2. Immunoaffinity column(IAC)

The immuno affinity column (IAC) occupies a special place among the immune analytical approaches being used many years as a method of sample purification and concentration in the aflatoxin analysis (Scott and Trucksess, 1997). Aflatoxins are low weight molecules and they are only immunogenic if they are bound to a protein carrier. The principle of the IAC is that an antibody (polyclonal or monoclonal) recognized by the analyte is immobilized onto a solid support such as agarose or silica in phosphate buffer, all of which are contained in a small column.

The column is initially conditioned with phosphate buffered saline (PBS) and reaches room temperature. After that the crude sample extract is applied to the IAC containing specific antibodies to aflatoxin at slow steady flow rate of 2-3 mL/min and this flow rate can be controlled by gravity or vacuum system. The aflatoxin binds to the antibody and is retained in the IAC, incidentally the crude sample extract must be in aqueous solution because organic solvents can damage the antibody and can interfere with the antibody-aflatoxin interaction. The binding strength of the antibody-aflatoxin will influence recovery of the IAC. The specificity of antibody is important to remove the structurally closely compounds which can cause interferences in the quantitation of aflatoxin. The capacity of the IAC (the total number of antibody sites available for binding aflatoxin) is also important as overloading the column will lead to poor recovery (Senyuva and Gilbert, 2010).

The column is washed with washing solution like water or phosphate buffered saline to remove impurities. After washing completely the IAC is blown to dryness by nitrogen stream. Then by passing a solvent such as methanol, water or acetonitrile through the IAC, breaking the antibody aflatoxin bond, the captured aflatoxin is removed from the antibody and thus eluted from the column. The big volume of sample loading and the small volume of solvent eluting make the analyte concentrate. The eluate containing aflatoxin is then further developed by addition of fluorescence enhancer or directly measured by HPLC method.

Figure 4. 1 mL Aflaclean™ Immunoaffinity columns. Source: LC Tech Application Note).

2.5.9. Validation of the chromatographic method

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. It is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that a target analyte will be analyzed.

2.5.9.1.Identification

Identification of aflatoxins determined by retention time of individual and mixed aflatoxins (AFG_2, AFG_1, AGB_2 and AFB_1) injecting at the same condition.

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

Limit of detection (LOD) and limit of quantification (LOQ) are terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level S/N > 3 where as LOQ is defined as the lowest amount of analyte which can be reproducibly quantitated above the baseline noise, that gives S/N >10 (FDA, 2000).

2.5.9.3. Precision

Precision is the closeness of agreement between independent test results obtained from homogenous test material under stipulated conditions of use and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. A precision criterion the instrument precision (repeatability) will be ≤ 5% RSD.

2.5.9.4.Linearity and working range

A calibration curve should be generated in which the linear relationship is evaluated across the range of the expected matrix concentrations. Linearity should be described by a linear, polynomial or other (as appropriate) regression plot of known concentration vs. response using a minimum of 5 different concentrations. Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. The regression coefficient (r^2) is > 0.998 is generally considered as evidence of acceptable fit of the data to the regression line (Ghulam, 2004).

2.5.9.5. Accuracy and recovery

Accuracy refers to the closeness of agreement between the true value of the analyte concentration and the mean result that is obtained by applying the experimental procedure. Accuracy is closely related to systematic error (analytical method bias) and analyte recovery (measured as percent recovery). The International Conference on Harmonization (ICH) document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations with three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals. Accuracy criteria for an assay method (FDA) is that the mean recovery will be 100 ± 2% at each concentration over the range of 80-120% of the target concentration (Bioanalytical Method Validation, 2013).

3. Materials and Methods

3.1. Description of the study site

The study was conducted in Debremarkos, Finoteselam, Enjibarra, Gonder, and Addis Ababa. Debremarkos is located in north western Ethiopia, in Amhara National Regional State, its distance is 300 km from Addis Ababa. Its astronomical location is 10º 21" North Latitude and 37º 43' East Longitude, it is the East Gojam Zonal- administrative town. Finoteselam, is located in north western Ethiopia, in Amhara National Regional State, west Gojam zone, Jabatehnane woreda, at a distance of 387 km from Addis Ababa, Its astronomical location is 10º 41' North Latitude and 37º 16' East Longitude. It is the west Gojam Zonal- administrative town. Enjibarra is located in northern Ethiopia, in Amhara National Regional State, Awi zone, Babja Woreda, at a distance of 420 Km from Addis Ababa. Its astronomical location is 10º 53' North Latitude and 36º 56' East Longitude. It is also the Awi- zonal- administrative town. Gonder is located in north western Ethiopia, in Amhara National Regional State, its distance is 725 km from Addis Ababa. Its astronomical location is 12º 36" North Latitude and 37º 28' East Longitude, it is the North Gonder Zonal- administrative town. Addis Ababa is located almost in the center of Ethiopia and it is in an altitude of about 2,400 meters above sea level. It had a built up area of 290 square km in 2004 and has an estimated population of 4 million (UN-Habitat, 2007).

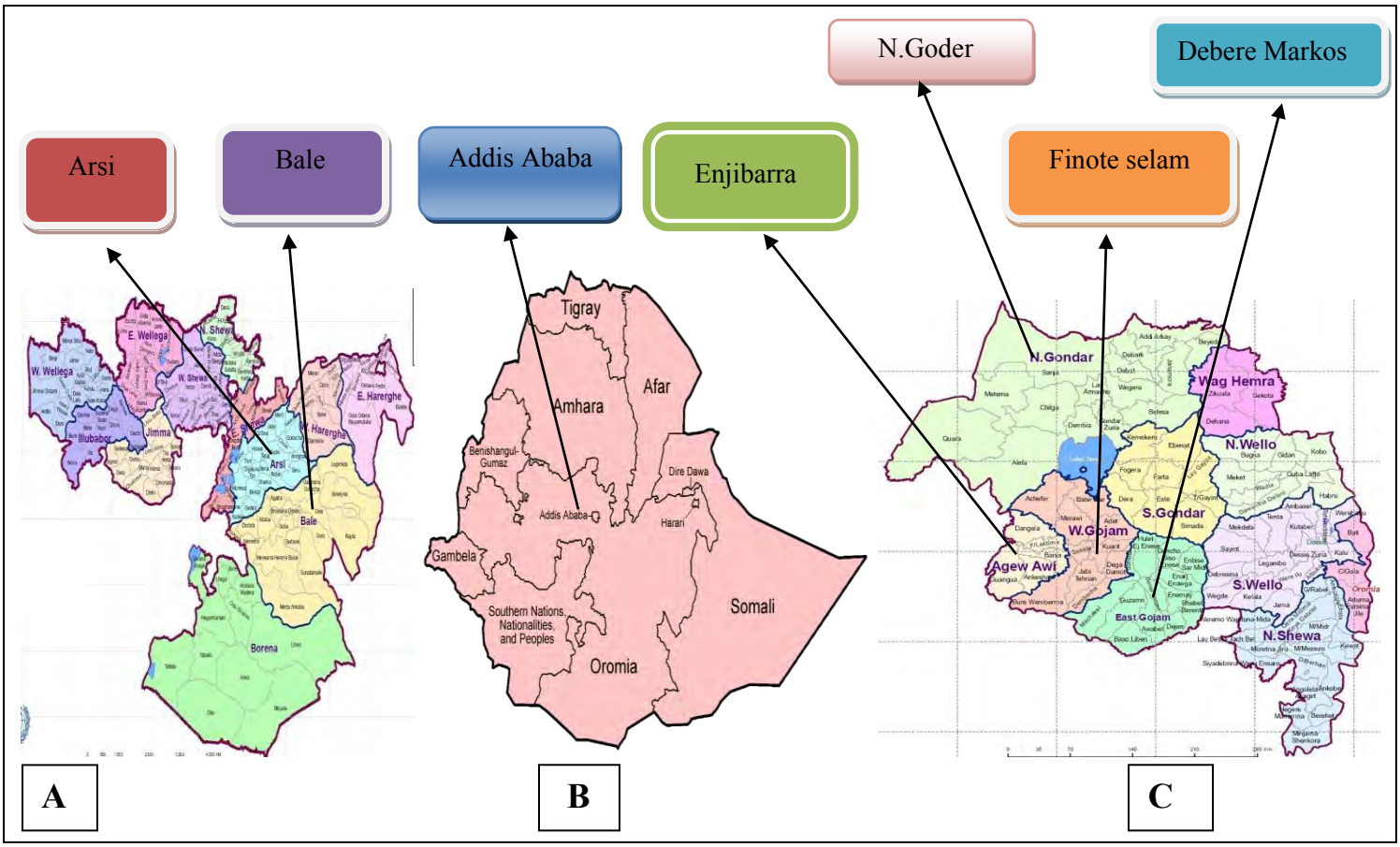


Figure 5.(A)Map of Oromiya regional state. (B) Map of Ethiopia. (C) Map of Amhara regional state.

3.2.Sample and sampling

Traditional (homemade) barley malts were collected from three different zonal towns which were Debere Markos; Finoteselam and Enjibarra. A total of 24 samples (eight samples from each town) the reason why this area was selected is the potential area for the production of tella and this traditional beer is made up of barley which is susceptible to aflatoxin. Even though there is a huge production of barely in these areas the way of handling after the postharvest and storage by the farmers may contribute for the occurrence of aflatoxin. The remaining six samples were collected from three different industries: from each industry one sample was taken from locally produced malt factory and one was taken from imported malts which were found in beer processing factories. As shown below in figure 6 the flow chart of sampling

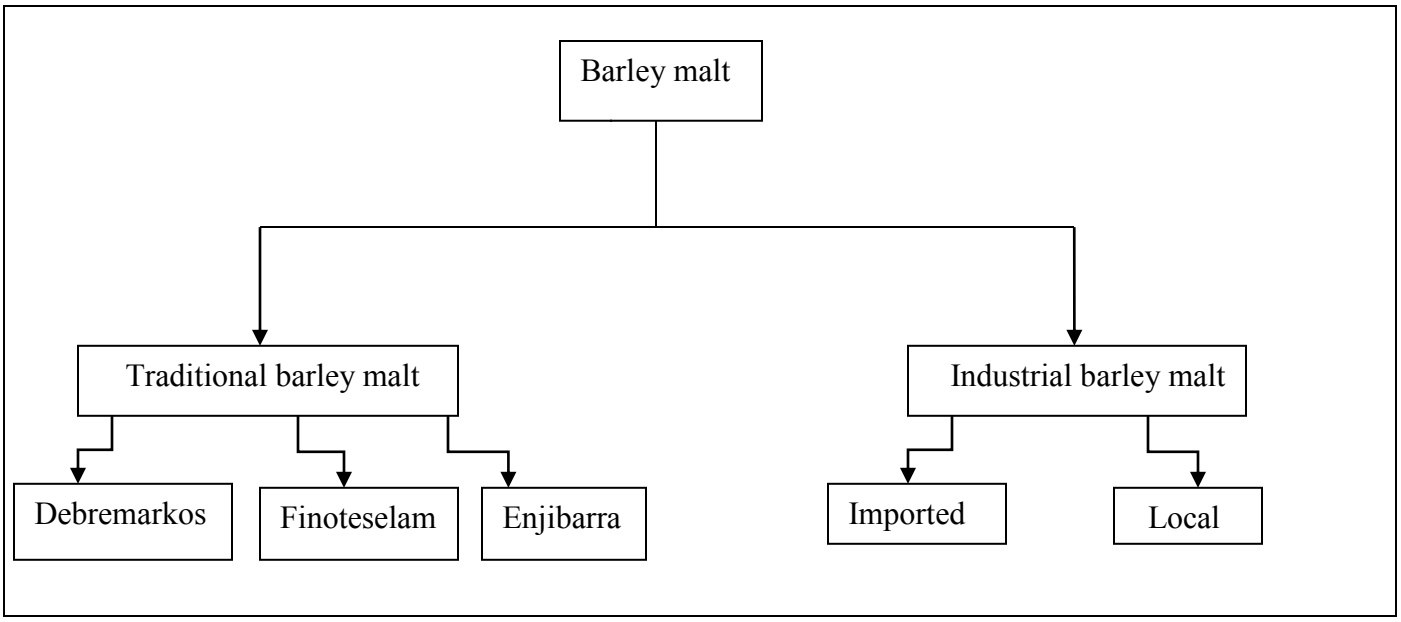
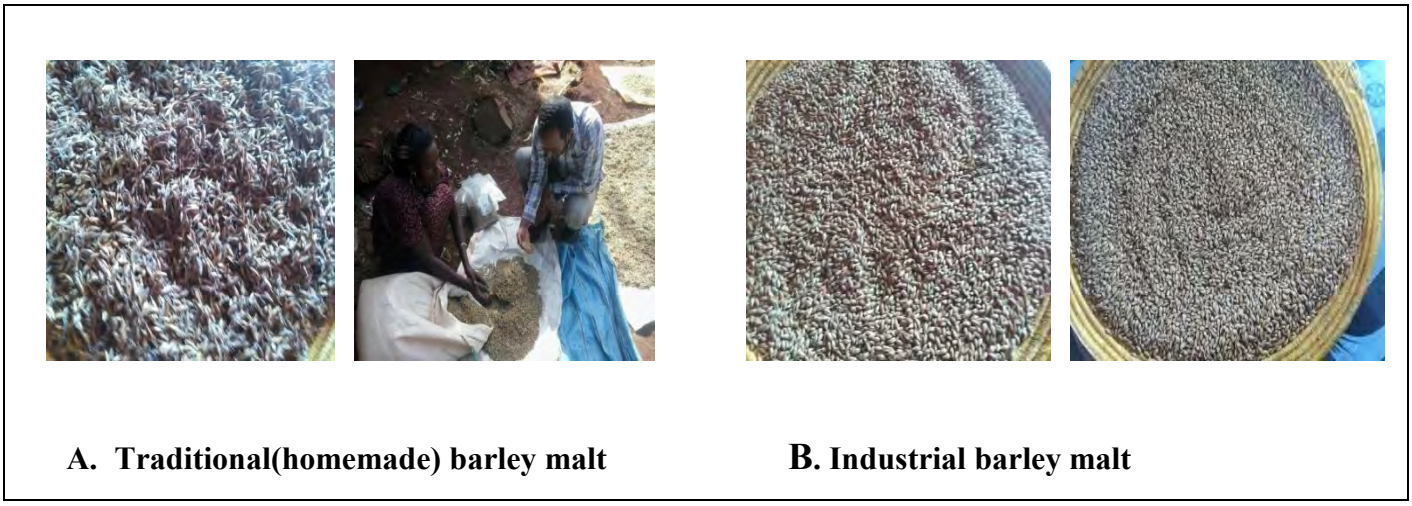


Figure 6.Flow chart of sampling

The reason why collection of samples from both traditional and industrial barley malt were a research were done in both traditional and commercial beer and a result showed contamination of aflatoxins.



A. Traditional(homemade) barley malt **B. Industrial barley malt**

Figure 7. Traditional and industrially barley malt

3.3.Study design of KAP

The study design employed for the KAP survey was a purposive sampling technique and semi-structured questionnaire was used to assess the KAP by randomly selecting traditional barley malt producers and industrial barley malt employees. The assessment was carried out in parallel with the sample collection. A total of 30 interviews were done, for traditional barley malt producer twenty four peoples (eight from each site) from Debremarkos, Finoteselam and Enjibarra and the remaining 6 for industrial barley malt employees were from local malt and beer factories. For those traditional barley malt producer the questionnaire used was translated in to local language whereas for industrial barley malt employees English language was used (Annex 1&2).

3.4.Equipment

The equipment used in the experiment were Whatman glass microfiber filter paper ,Immunoaffinity column, plastic bags, lab stand with clump, Volumetric and Graduated pipettes (1ml, 5ml, 10ml, 25ml and 50ml), volumetric flask (10ml, 25ml, 50ml, 100ml, 500ml and 1000ml), Measuring cylinder (50ml and 100ml), Beaker (50ml, 100ml and 500ml), conical flask(250, 500 and 1000ml), Mixer, Stirrer, Ultra bath Sonicator, Micropipettes, Micropipette tips, 0.45 μm Millipore Filter, Electronic balance, syringes(5ml and 10ml), Sample label, Vials with screw cap. SHIMADZU HPLC system setup contains auto sampler, injector, oven, column, Link, Degasser, fluorescence detector and desktop computer with chromatography software.

3.5.Chemicals and reagents:

Chemicals and reagents used in the sample preparation and analysis were HPLC grade acetonitrile and methanol, aflatoxin standards (B_1, B_2, G_1, G_2 and mixed), distilled water and phosphate buffered saline (PBS: NaCl 8g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l; and adjust pH to 7.2 using 0.1M HCl or 0.1M NaOH) were purchased from local market.

Mobile phase:

The mobile phase was a mixture of de-ionized water-methanol-acetonitrile (65:25:15, v/v/v) isocratic method applied for better resolutions of the chromatographic peak. The mobile phases were filtered by applying vacuum in a filter unit and degassed.

Chromatographic system:

Shimadzu HPLC instrument with, Auto sampling system, fluorescence detector and software were used for analysis. A Shim-pack FC-ODS column (5µm, 150 x 4.6mm diameter) at 25^0c temperature and 1.2ml/min flow rate was used. The run time was 25 minutes, injection volume 20µl, diluent methanol and Needle wash (Water: Methanol 90:10 v/v). Aflatoxins were detected at 365 nm excitation and 440 nm emission wavelengths.

3.6.Procedure for aflatoxin analysis

3.7.Method adaptation

To evaluate the analytical performance of the instrument and validity of the method, first standard preparation then identification, LOD and LOQ, precession, linearity and working range, recoveries and asserting the working range were arrived out.

3.7.1. Standard preparation

Aflatoxin standards were obtained from Sigma Aldrich (St. Louis, MO, USA). From the stock solution, aflatoxin mixed standard which has a concentration of (2, 5, 10, 20, 30, 50 and 100) ppb were prepared for method validation. Standard solutions were prepared in 10ml volumetric flasks using HPLC grade methanol as a diluent. The prepared standards were transferred to vials and stored at 4 ^{O}C and protected from light to avoid deterioration of the aflatoxins in the solution.

3.7.2. Identification

Identification of aflatoxin standards were determined by retention time of individual and mixed aflatoxins (AFG_2, AFG_1, AGB_2 and AFB_1) injecting at the same condition to know the elution order of the specific aflatoxin standard peak to be revealed thereby to determine which of the aflatoxin peak is detected first and sequentially to the desired last and its precision determined by percent relative standard deviation (%RSD).

3.7.3. LOD and LOQ

LOD was determined by injecting (0.01, 0.2, 0.01, and 0.2) ppb of individual aflatoxins G_2, G_1, B_2 and B_1 respectively to obtain the lowest amount of analyte greater than three times of noise level S/N > 3 . In the same way LOQ was determined by injecting (0.05, 0.8, 0.05, and 0.8) ppb

of individual aflatoxins G_2, G_1, B_2 and B_1 respectively to obtain the lowest amount of analyte which can be reproducibly quantitated above the baseline noise, that gives S/N >10.

3.7.4. Precision

Precision of the method was evaluated through the repeatability of the method by assaying ten replicate injections of aflatoxin mixed standard at the same concentration (30 ppb) during the same day under the same experimental conditions to obtain an acceptable %RSD.

3.7.5. Linearity and working range

Linearity was determined by injecting a serious of (2, 5, 10, 20, 30, 50 and100) ppb aflatoxins standard. The concentration range (2-100) ppb and regression equation was found by plotting the peak area (Y) versus the aflatoxins concentration (X) expressed in ppb.

3.7.6. Recovery

Recoveries were determined by spiking known concentration of samples with aflatoxin standards after extraction the final solution were diluted by PBS (14 ml) and purified through Immunoaffinity column. The column washed with PBS (20 ml) and aflatoxins was slowly eluted with methanol and water by 1 ml respectively into a graduated glass vial. The extract was injected in HPLC system for recovery and accuracy check.

3.8.Determination of moisture content

Moisture content was determined according to AOAC (2000) using the official method 925.09. A crucible was dried in an oven at 105^{o}C for 1 hour and placed in desiccators to cool. The weight of the crucible (W1) was determined. 5gm samples was weighed in the dry crucible (W2) and dried at 105^{o}C for 3 hours and after cooling to room temperature in desiccators it was again weighed (W3). The moisture content was determined by using Eq. (1).

$$\text{Moisture content in \%} = \frac{W2-W3}{W2-W1} * 100 \quad \ldots\ldots\ldots\ldots (1)$$

3.9.Sample preparation and clean up

The barley malt grains were taken and ground in miller. By taken 50 gram of ground barley malt sample, 5 gram sodium chloride, 80% methanol and 20% water were mixed into a 1 liter capacity, solvent resistant blender jar at a higher speed for 2 minutes. Then the solvent were filtered through whatman no.113 or 4 filter paper. 2 ml was collected from filter sample and diluted with 14 ml phosphate buffer saline (PBS) then purified through AflaCLEAN Immunoaffinity column at a flow rate of 2 ml per minute or the sample could be allowed to pass through the column by gravity if preferred. A slow, steady flow rate was essential for the capture of the toxin by the antibody. The column washed with 20 ml of PBS through at a flow rate of approximately 5 minutes and passed air through the column to removed residual liquid. Aflatoxin was slowly eluted from the immuno affinity column at a flow rate 1 dropped per second using 1 ml of 100% methanol and de-ionized water respectively and collected in an amber glass vial. The extract filtered with Millipore 0.45 nm and injected 20 µl on to the HPLC.

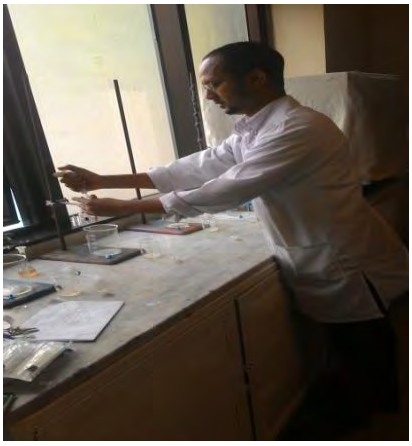


Figure 8. Sample preparation, clean up and reading.

3.10. HPLC determination and calculations

The eluate aflatoxins (B1, B2, G1 and G2) methanol solution were determined at parts per billion (μg/kg) levels in barley malt by Immunoaffinity column cleanup and high performance liquid chromatography with fluorescence detection and calculated according to the following equation.

Aflatoxin, μg/kg = A (T/I) (1/W) ----------eq (2)

Where;-

A= μg/kg of aflatoxin as eluate injected

T= final test solution eluate volume (ml)

I= volume eluate injected into LC (ml)

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

3.11. Experimental Design

Completely randomized experimental design was followed to determine the level of aflatoxin in traditionally and industrially produced barley malt in Ethiopia.

3.12. Statistical analysis

For statistical analysis, Excel and IBM SPSS statistics version 20 software were used. In the SPSS method, one - way analysis of variance (ANOVA) and paired samples T-test was performed to evaluate the level of total aflatoxin mean comparison between the study sites, between the industries and between study sites and industries respectively. After checking of ANOVA if p- value less than 0.05 ($P<0.05$) this shows there was a statistical significance where as in the reverse if p- value greater than 0.05 ($P>0.05$) there was no significance between them. Level of aflatoxin was the dependent variables and study sites were independent variables if locality affected the aflatoxin level.

4. Results and Discussion

4.1. Socio- demographic characteristics and KAP survey result of traditional barley malt producers

According to the socio demographic survey, there were a total of 24 females interviewed, among of them 54% of the female age were above 25 years and most of them were married. Majority of the malt producers had private workers and their educational statuses were illiterate. Their averagely family income was less than one thousand birr per month this shows that the economic class was low.

Based on the knowledge, attitude and practice (KAP) questionnaire, all the traditional malt producers barley was their main raw materials and majority of them i.e. 75% of the ingredient were found from farmers where as the remaining 25% were found from retailers. With regards to the knowledge on cereals storage system of suppliers, 83.3% of the producers were known their storage system were under silo, however the remaining 16.7% of them did not know where they store the cereals. About seven producers (29.2%) of the respondents reported that they were aware of mould contamination and the remaining seventeen producers (70.8%) were not. In other way 62.5% of the producers answered that the colour of mould is white and 37.5% answered as black. The majority (83.3%) of the producer didn't have knowledge know the problems related to mould growth where as the remaining 16.7% have knowledge.

However, their attitudes towards the production of barley malt majority of the respondents (79.2%) prefer mouldy cereals for the purpose of malting where as the other respondents (20.8%) were chosen that mould cereals for the purpose of kolo. In addition to this, 75% of the producers had lack of knowledge on the health impact of moldy cereals while 25% of them had some information about the health impact of mouldy cereals.

As shown in Table 3 below most (70.8%) of the producers bought mouldy cereals from the market due to their lack of knowledge where as the others did not bought, on the other hand majority of them did not store their ingredient for a long time before preparing the product but some of them stored. With regards to storage condition of the final products fourteen of the producer stored their products on the floor whereas the remaining ten producers put it on the roof.

Table 4.Summary of Socio- demographic characteristics and KAP survey on traditional barley malt producer in study site

Statement	Response		
Socio demographic characteristics		Frequency	Percentage
Age	25-30	13	54
	30-45	8	33.33
	>45	3	12.5
Sex	Female	24	100
Marital status	Single	5	20.80
	Married	12	50
	Divorced	4	16.7
	Widowed	3	12.5
Educational status	Illiterate	16	66.7
	Able to write and read	6	25.0
	Elementary school	2	8.33
Family income	<1000	18	75.0
	<2000	6	25.0
Occupation	Private workers	24	100.0
Knowledge			
What ingredient used to prepare malt?	Barley	24	100.0
	Wheat		
From where the ingredient is found?	Farmers	18	75.0
	Retailers	6	25.0
	Unions	-	-
Knowledge on cereals storage by suppliers	Silo	20	83.3
	Do not know	4	16.7
Do you hear about moulds?	Yes	7	29.2
	No	17	70.8
The colour of the moulds	Black	9	37.5
	White	15	62.5
	Yes	4	16.7

Problems related to moulds			
	No	20	83.3
Attitude			
Do you prefer mould cereals for malt making than to use kolo?	Yes	19	79.2
	No	5	20.8
Do you think that mould cereals used for malt preparation has a health impact?	Yes	6	25.0
	No	18	75.0
Practice			
Do you purchase if mould cereals are happen in the market?	Yes	17	70.8
	No	7	29.2
Have you put your ingredients for a long time before preparing the malt?	Yes	4	16.7
	No	20	83.3
Storage condition of the final product	At roof	10	41.7
	On the floor	14	58.3

4.2.Socio- demographic characteristics and KAP survey result of industrial barley malt producers

As shown in Table 4 below there was a total of six employees were interviewed, from the majority (50%) of the respondent age range lies between 25-30 ages whereas (33.3%) and (16.7%) of the respondents age range is between 30-45 and above 45 years of age respectively. Concerning their gender the majority (66.7%) of the respondents were males where as the left over were female. In relation to marital status 50% of employees were married whereas, 33.3% of the participants were single and 16.7% were divorced and almost all of them had a better educational status that was a bachelor and Msc degrees. Regarding the income status of the respondents the majority (75%) of the respondent income were medium levels. The table also portrays the occupation of the respondents. It is revealed that most (83.3%) of the study participants were the employee of the company whereas only (16.7%) of participant was the owner of the company.

The questionnaire with regards to knowledge, attitude and practice (KAP) of the respondents on industrial barley malt producers, all of the respondent used barley rather than wheat as a raw material and 66.7% of the ingredients were found from farmers where as 33.3% found from unions. About 83.3% of the employees responded that the storage condition of the finished product was in silos, whereas the 16.7% respond that they store in ware houses.

All of the respondents had information about moulds from which 66.7% they identified the colour as black where as the remaining 33.3% could identify the color as it was white. All of them had knowledge with regards to the health problems of moulds; majority of them (83.3%) had knowledge about food toxin like mycotoxin but the remaining had not knowledge. Likewise their attitudes towards mouldy cereals for the production of malt 83.3% indicated that mouldy cereals had health impacts.

Majority of the interviewee stated that there were lack of training related to food safety system. On the other hand 83.3% of them did not store their ingredients used for malt production for a long period of time whereas 16.7% of them store for a long time.

Table 5. Socio- demographic characteristics and KAP survey on industrial barley malt producer in study site.

Statement	Response		
Socio demographic characteristics		Frequency	Percentage
Age	25-30	3	50.0
	30-45	2	33.3
	>45	1	16.7
Sex	Male	4	66.7
	Female	2	33.3
Marital status	Single	2	33.3
	Married	3	50
	Divorced	1	16.7
Educational status	High school	1	16.7
	BSC degree	4	66.7
	MSC degree	1	16.7
Family income	<2000	1	16.7
	<4000	3	50.0

	>5000	2	33.3
Occupation	Company employer	5	83.3
	Company owner	1	16.7
Knowledge			
What ingredient used to prepare malt?	Barley	6	100
	Wheat	-	-
From where the ingredient is found?	Unions	2	33.3
	Farmers	4	66.7
Where the finished malt product store?	Silo	5	83.3
	Warehouse	1	16.7
Do you hear about moulds?	Yes	6	100.0
	No	-	-
The colour of the moulds	Black	4	66.7
	White	2	33.3
Problems related to moulds	Yes	6	100.0
	No	-	-
Attitude			
Do you have knowledge about food toxin like mycotoxin?	Yes	5	83.3
	No	1	16.7
Do you think that mould cereals used for malt preparation has a health impact?	Yes	5	83.3
	No	1	16.7
Practice			
Have you taken training with related to food safety system?	Yes	2	33.3
	No	4	66.7
Have you put your ingredients for a long time before preparing the malt?	Yes	1	16.7
	No	5	83.3

4.3.Results of method validation

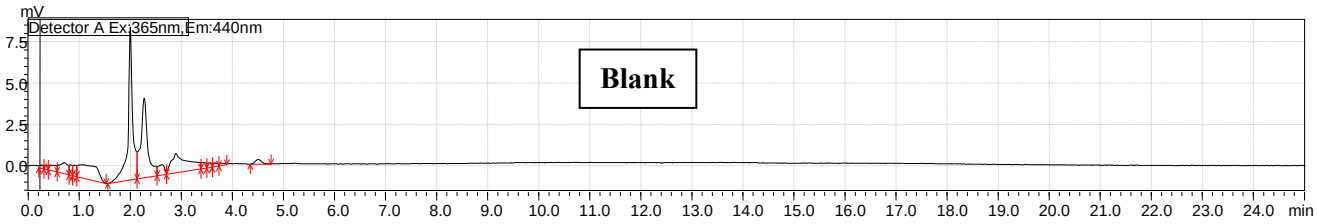
4.3.1. Identification of aflatoxin

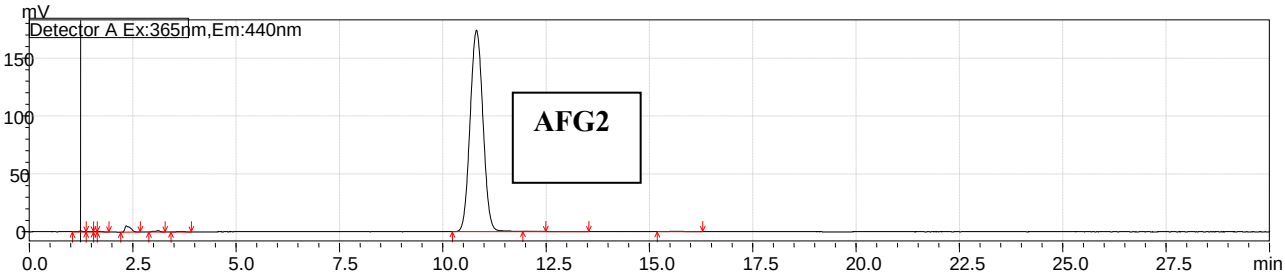
Peak identification results were as shown in Table 6 the retention time of individual and mixed aflatoxins gives a good precession having a range between (0.43-1.53) %RSD, that was acceptable according to FDA standard which is less than (<2%RSD). The elution order of individual aflatoxin was in the order of AFG_2, AFG_1, AFB_2 and AFB_1 with 10.818, 13.197, 15.109 and 19.082 retention times respectively.

Table 6. Statistics for aflatoxin retention time identification.

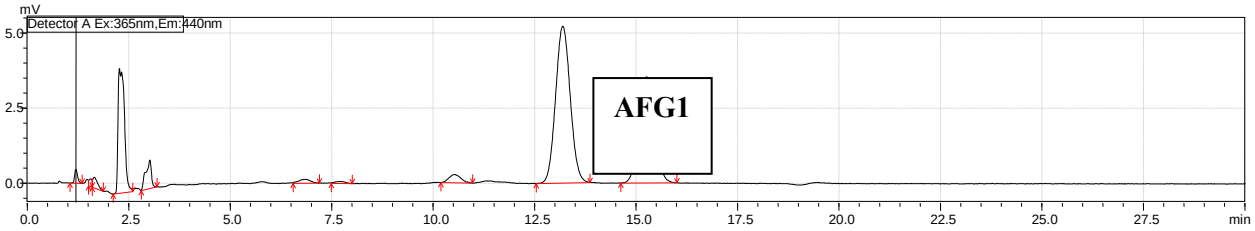
Aflatoxin	Aflatoxin 30 ppb injection retention time(min)		N	Mean	Std.Devation	%RSD
	For single run	For mixed run				
AFG_2	10.818	10.586	2	10.702	0.1640	1.53
AFG_1	13.197	13.277	2	13.237	0.0566	0.43
AFB_2	15.109	15.354	2	15.2315	0.1732	1.14
AFB_1	19.082	19.487	2	19.2845	0.2864	1.49

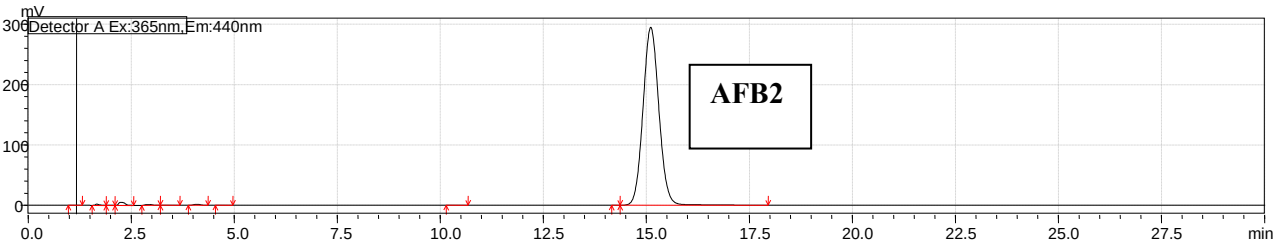
Figure 9 shows below chromatographic result for Blank, individual (AFG_2, AFG_1, AFB_2 and AFB_1) and mixed aflatoxins demonstrate the qualitative aspect of identification test is more defined and acceptable.




mV
Detector A Ex:365nm,Em:440nm
150
100
50
0
AFG2
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
min

AFG1


mV
Detector A Ex:365nm,Em:440nm
5.0
2.5
0.0
AFG1
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
min


mV
Detector A Ex:365nm,Em:440nm
300
200
100
0
AFB2
0.0
2.5
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7.5
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12.5
15.0
17.5
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22.5
25.0
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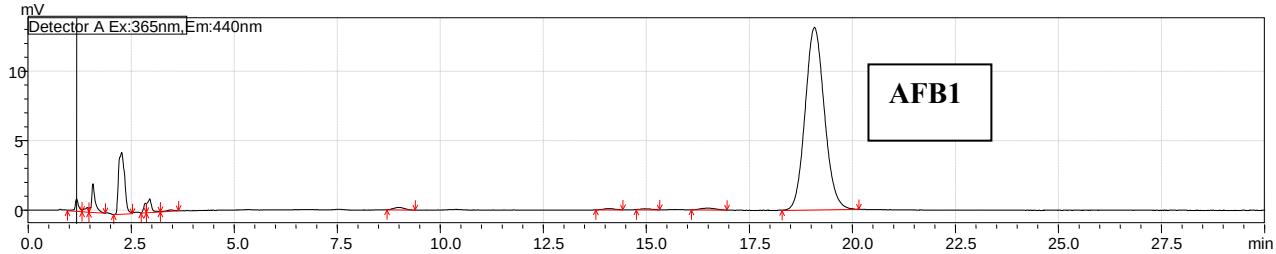
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Detector A Ex:365nm,Em:440nm
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AFB1
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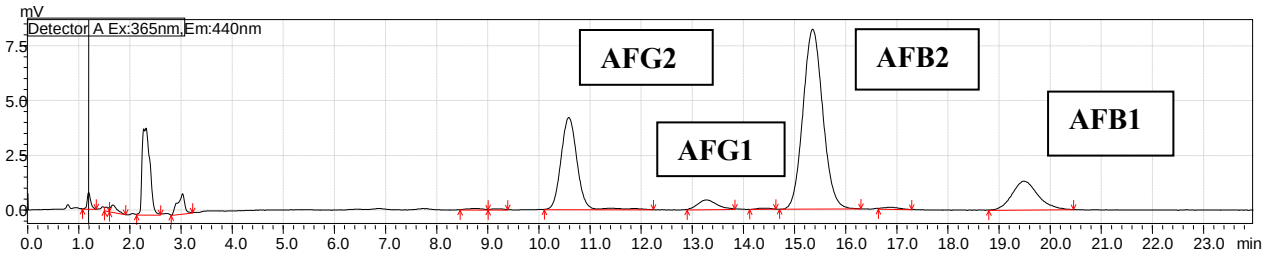

Figure 9. Chromatogram for aflatoxin identification

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

Detection performance of the HPLC was determined by the limit of detection and was found to be 0.01, 0.2, 0.01 and 0.2 ppb for aflatoxin AFG_2, AFG_1, AFB_2 and AFB_1 respectively. LOD was determined by the amount of analyte that can be detected above baseline noise; typically, three times the noise level S/N > 3, where as the limits of quantification for individual aflatoxin (AFG_2, AFG_1, AFB_2 and AFB_1) were 0.05, 0.8, 0.05 and 0.8 ppb respectively. In Table 7 LOQ was determined based on the amount of analyte which can be reproducibly quantitated above the baseline noise, that gives S/N >10. The limits of quantification were in the range of (0.05-0.8) µg/kg which shows satisfactory quantification of the instrument on the desired working range.

Table 7. Limit of detection (LOD) and limit of quantification (LOQ)

Aflatoxin	**LOD**		**LOQ**	
	Ppb	Signal to noise ratio (s/r>3)	ppb	Signal to noise ratio (s/r>10)
AFG2	0.01	4.67	0.05	11.92
AFG1	0.20	3.34	0.80	10.41
AFB2	0.01	5.41	0.05	12.86
AFB1	0.20	4.08	0.80	11.16

4.3.3. Precision

The precision was evaluated through the repeatability of the method by assaying ten replicate injections of aflatoxin mixed standard at the same concentration (30 ppb), during the same day, under the same experimental conditions. This result shows an acceptable result of %RSD and the precision criterion of the instrument precision and is normally expressed as the percent relative standard deviation for a statistically significant number of samples should be ≤ 5% RSD in FDA standard.

Table 8. Statistical analysis for precision

Aflatoxin type	**N**	**%RSD for peak area**	**%RSD for retention time**
AFG2	10	3.23	1.04
AFG1	10	2.38	1.85
AFB2	10	3.11	3.50
AFB1	10	2.83	1.09

4.3.4. Linearity and working range

Linearity was studied by selecting seven concentrations (2, 5, 10, 20, 30, 50 and 100) ppb in order to demonstrate a proportional relationship of peak area versus analyte concentration over the working range as shown in Table 9. Regression equation was found by plotting the peak area (y) versus the aflatoxins concentration (x) expressed in ppb as presented in **Figure 10** below.

Table 9. Linearity check

Aflatoxin	**N(point)**	**Calibration curve equation**	**R^2**
AFG2	6.0	Y = 42517x + 2308	0.998
AFG1	6.0	Y = 1361x + 214.6	0.998
AFB2	6.0	Y = 10459x + 4081	0.999
AFB1	6.0	Y = 5235x + 1316	0.998

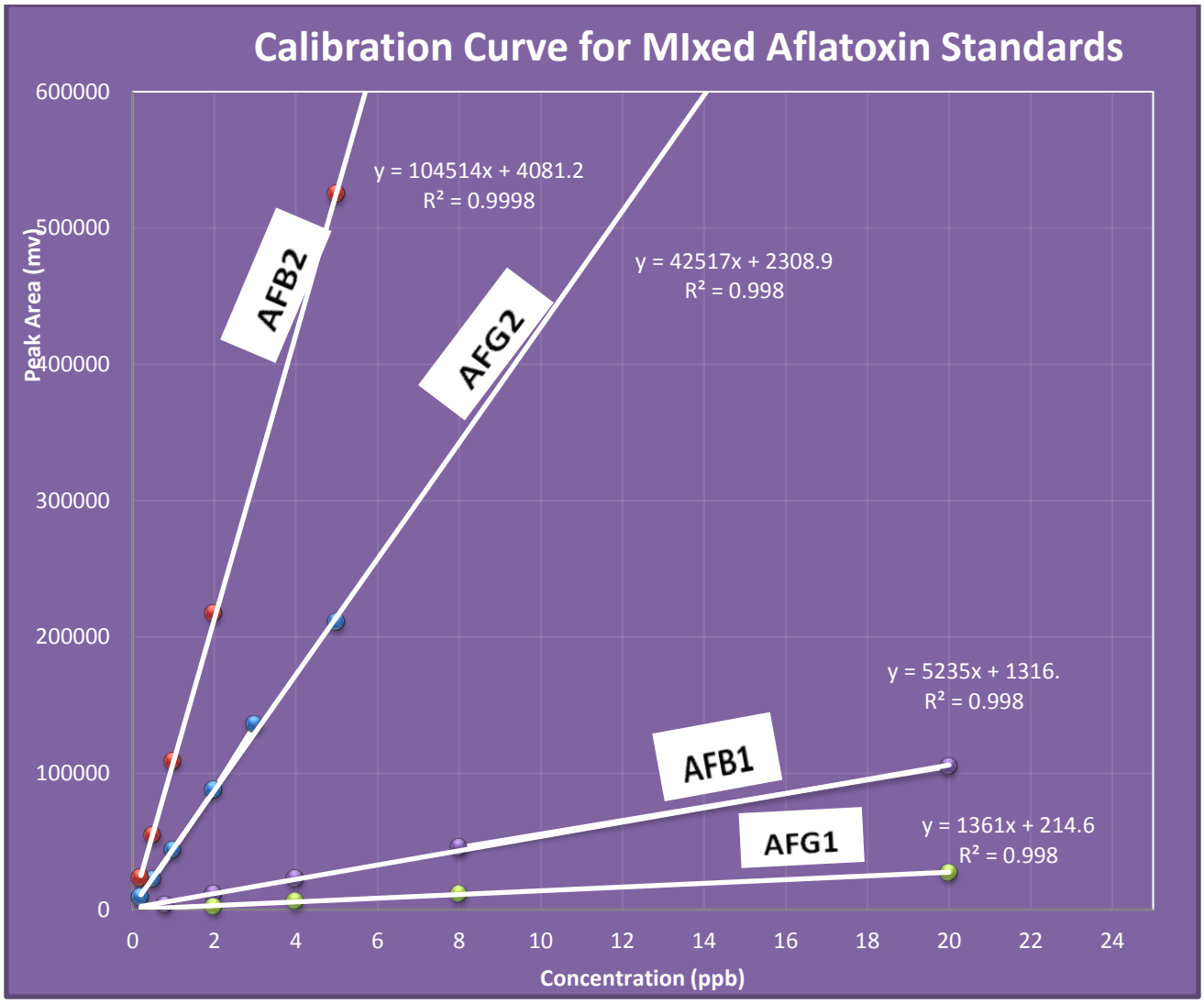

Figure 10. Calibration curve for aflatoxin B1, B2, G1 and G2

Working range

The range was resulting from linearity studies and depends on the intended application of the test method. The range is normally expressed in the same units as the test results obtained by the method. As shown below in Table 10 the data obtained during the linearity studies was used to assess the range of the assay method.

Table 10. Working range

Aflatoxin sum in ppb	Aflatoxin in ppb			
	AFG_2	**AFG_1**	**AFB_2**	**AFB_1**
2	0.20	0.80	0.20.	0.80
5	0.50	2.0	0.50	2.0
10	1.0	4.0	1.0	4.0
20	2.0	8.0	2.0	8.0
30	3.0	12.0	3.0	12.0
50	5.0	20.0	5.0	20.0
100	10.0	40.0	10.0	40.0

4.3.5. Accuracy and recovery test

The recovery for individual aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) were conducted by spiking known concentration samples with aflatoxin standard and Percent recoveries of response factor (area/concentration) were calculated and the result were AFB_1=110%, AFB_2=110%, AFG_1=108% and AFG_2=105%. The result of recovery studies shown in the range between (105-110) % which is coincide with the standard recovery test (80-120) %.

4.4.Level of aflatoxin in traditional barley malt samples

The mean aflatoxin contamination in Debremarkos barley malt samples were 5.30 µg/kg and the range was 9.03-23.6 µg/kg. This was the second highest level of aflatoxin contamination compared with the three study site samples. From a total of eight samples taken for analysis three samples were highly contaminated by aflatoxin where as one was less contaminated that means less than limit of quantification (LOQ). All of the detected aflatoxins were aflatoxin G_1 and the other did not exist except the one that was detected which was aflatoxin G_2 and the average moisture content of the barley samples were 7.4 and the range was 5.4-9.8.

The mean total aflatoxin contamination levels of Samples from Finoteselam were 1.90 µg/kg and the range was 0.27-2.95 µg/kg. Among the eight samples, aflatoxin contamination was detected in five samples and two of them are less than limit of quantification and also the level of contamination was less than the other sites. In this site, all the four aflatoxin types were detected

and the average moisture content of the samples were 6.6 and the moisture content range was 5.2-8.6.

The mean aflatoxin contamination level of barley malt samples from Enjibarra were 6.72 µg/kg and the range was 0.36 -20.03 µg/kg. In this site the highest level of aflatoxin contamination was detected besides this the most carcinogenic aflatoxin for human that was AFB_1 was highly contaminate in this sites when comparing from the other sites. 75% of the samples were contaminated and the average moisture content was 9.7 and the range was 7.8-11.8.

As a result, samples from Debremarkos except aflatoxin G_1 all other types of aflatoxin were not detected while in Finoteselam and Engibarra all types of aflatoxin were detected. From the finding the highest contribution of the aflatoxin types for total aflatoxin level was G_1 and B_2 respectively, whereas G_1 which was detected and quantified in the entire study site while aflatoxin B_2 was detected in both Finoteselam and Enjibarra. As shown in Table 11 the overall grand mean positive content of traditionally prepared barley malt from the three study sites were 4.64 µg/kg.

Table 11. Aflatoxin level of traditional barley malt sample from different study sites

Site	Sample code	Aflatoxin level, (µg/kg)					Moisture content %
		AFG2(µg/kg)	AFG1(µg/kg)	AFB2(µg/kg)	AFB1(µg/kg)	Total afs (µg/kg)	
Debere Markos	DM1	ND	ND	ND	ND	ND	6.6
	DM2	ND	ND	ND	ND	ND	5.4
	DM3	ND	ND	ND	ND	ND	7.4
	DM4	ND	9.03	ND	ND	9.03	8.4
	DM5	ND	23.6	ND	ND	23.6	9.8
	DM6	ND	9.78	ND	ND	9.78	9.2
	DM7	<LOQ	ND	ND	ND	<LOQ	6.9
	DM8	ND	ND	ND	ND	ND	5.6
Total		ND	42.41	ND	ND	42.41	59.3
					Average	**5.30**	**7.4**

Finoteselam	FS1	ND	ND	0.27	0.3	0.57	7.2
	FS2	ND	ND	ND	ND	ND	5.2
	FS3	ND	ND	<LOQ	ND	<LOQ	6.0
	FS4	0.27	2.6	2.35	2.95	8.17	8.6
	FS5	ND	ND	ND	ND	ND	5.6
	FS6	ND	ND	ND	ND	ND	6.2
	FS7	0.4	2.2	2.49	1.3	6.39	7.2
	FS8	<LOQ	ND	<LOQ	ND	<LOQ	6.4
Total		0.67	4.8	5.11	4.55	15.13	52.4
Average						**1.90**	**6.6**
Enjibarra	EG1	ND	ND	ND	ND	ND	8.0
	EG2	<LOQ	2.29	1.94	2.27	6.50	10.6
	EG3	1.91	8.03	20.03	5.33	35.3	11.8
	EG4	<LOQ	ND	<LOQ	ND	<LOQ	8.8
	EG5	ND	ND	ND	ND	ND	7.8
	EG6	ND	ND	<LOQ	ND	<LOQ	9.2
	EG7	0.36	4.37	2.7	2.89	10.32	11.7
	EG8	<LOQ	ND	0.95	0.7	1.65	9.8
Total		2.27	14.69	25.62	11.19	53.77	77.7
Average						**6.72**	**9.7**
Grand mean of traditional barley malt						**4.64**	

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

<LOQ: Aflatoxin is detected but it is less than the limit of quantification

DM: Debremarkos, FS: Finoteselam, EG: Enjibarra

4.5.Level of aflatoxin in industrially produced barley malt samples

Table 12 shows the level of aflatoxin in both imported and local industrial barley malt samples. The average aflatoxin level of local and imported industrial barley malt samples were 1.22 µg/kg and 1.69 µg/kg respectively. The average aflatoxin contamination level of imported barley malt was greater than locally produced this might be due to the transport condition during which time

product might be exposed to high temperature and moisture migration cooling conducive environment for toxin production. The grand mean of both industrial barley malt samples were 1.46 µg/kg and the average moisture content was 2.5 and the range 2.3-2.6, respectively.

Table 12. Aflatoxin level of industrial barley malt sample from different company

Site	Sample code	Aflatoxin level, (µg/kg)					
		AFG2(µg/kg)	AFG1(µg/kg)	AFB2(µg/kg)	AFB1(µg/kg)	Total AFs µg/kg	Moisture content
Industries	S01-local	ND	ND	ND	ND	ND	2.4
	S02-local	ND	ND	ND	ND	ND	2.3
	S03-local	0.28	1.97	0.21	1.21	3.67	2.5
	Total	0.28	1.97	0.21	1.21	3.67	7.2
					Average	**1.22**	**2.4**
	S01-import	ND	ND	ND	ND	ND	2.6
	S02-import	ND	ND	ND	ND	ND	2.4
	S03-import	0.43	2.67	0.35	1.61	5.06	2.6
	Total	0.43	2.67	0.35	1.61	5.06	7.6
					Average	**1.69**	**2.5**
					Grand mean of industrial barley malt	**1.46**	

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

S01: samples 01, S02: sample 02, S03: sample 03

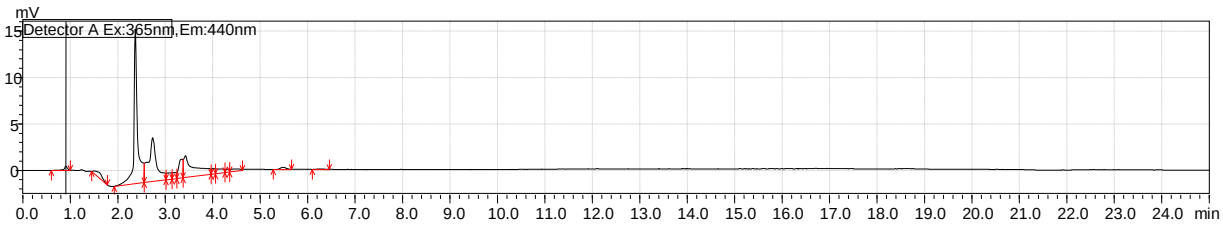

Figure 11. A chromatogram showing aflatoxin in barley malt sample not detected

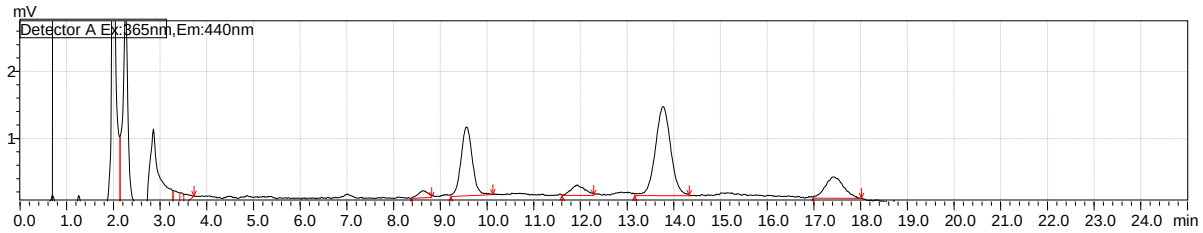

Figure 12. . A chromatogram showing detecting aflatoxin in barley malt sample.

When we compare the aflatoxin contamination level of the traditional and industrial barley malt, traditional barley malts were more contaminated than industrial this was due to the malt producing process that means in traditional barley malt processing there was not a practice of cleaning, sorting, de-hulling and also in pre- harvest and post harvest stage or storage which was favorable condition of temperature and humidity. Where as in industrial barley malts there existed the above process and all the process or unite operation was done in a control manner. Among the traditional sample studying site Enjibarra was the highest aflatoxin contamination level followed by Debere Markos and Finoteselam.

On the other hand, the aflatoxin contamination level of industrial barley malt was slightly less this was due to good processing practice and the work was performed by educated personnel. The KAP study has indicated that the majority of the employees were skilled about the whole operation. From the industrial barley malt, imported barley malts were more contaminated than local malt which might be due to storage and transportation condition. To understand the average aflatoxin level of the entire samples collected sites were better to describe by the bar graph as shown below.

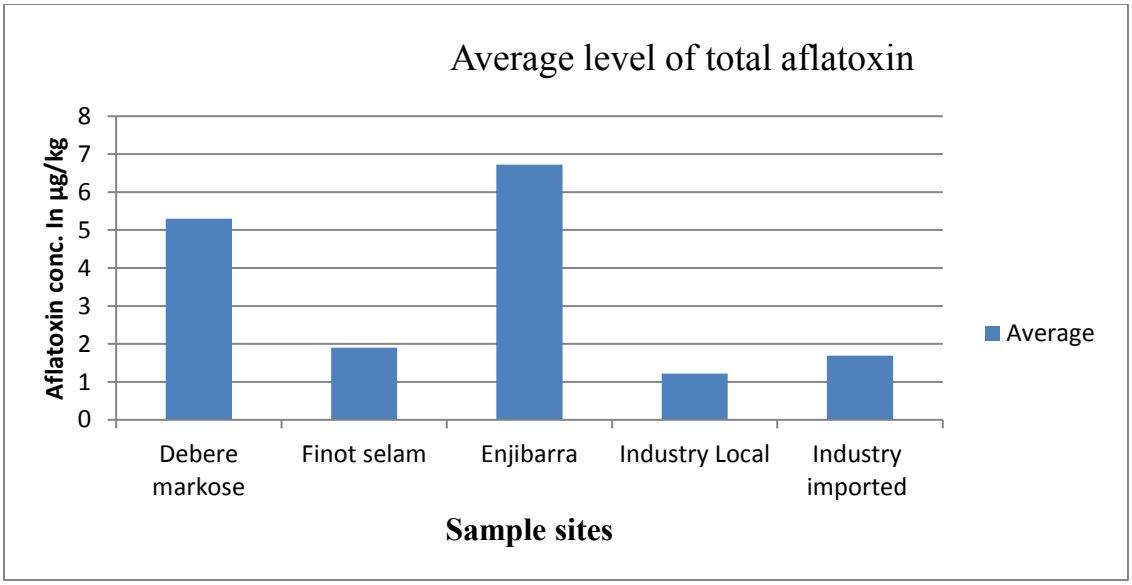

Figure 13. A bar graph shows the level of aflatoxin between traditional and industrial barley malt

When comparing this research finding with other studies conducted in different countries on traditional and industrial barley malt, there was little similar research conducted in this topic in Ethiopia and there was also limited on this area in the world. When it is compared to the level of aflatoxin obtained in malt samples with similar studies on traditional and industrial barley malt there is limited barley cereals study conducted in Ethiopia, a survey on aflatoxin contamination on cereals results show that Levels of total aflatoxin greater than 20 µg/kg, were most frequently encountered in all aflatoxin positive samples of corn, sorghum, wheat, red pepper and peanut followed by barley 17% and teff 13% (Habtamu and Kelebessa. 2001). And this paper finding showed that out of 24 traditional barley malt samples two samples have >20 µg/kg aflatoxin level. And another studies conducted in barley, sorghum and wheat deoxynivalenol (DON) levels at 40–2340 µg/ kg with an overall incidence of 48.8% among the 84 mainly suspected samples analyzed (Amare et al., 2006). To contrast with this finding there was less amount aflatoxin level occurred in this study.

In this study the result showed that the total aflatoxin contamination level was ranged from 0.21 to 23.6 µg/kg. This finding is less than the research conducted in Botswana on 46 malt samples of traditional sorghum malt, wort and beer analyzed for the presence of *Aspergillus flavus* was

isolated from malt samples, aflatoxins (B_1, B_2, G_1, and G_2) were not detected in any of the samples analyzed. When the malt, wort, and beer samples were analyzed for fumonisin B_1 and zearalenone, fumonisin B_1 was detected in three malt samples, with concentrations ranging from 47 to 1316 µg/kg, while zearalenone was detected with concentration in samples ranged from 102 to 2213 µg/kg in malt, 26 to 285 µg/l in wort and 20 to 201 µg/l, in beer (Nkwe *et al.,* 2005).

Another study conducted in Czech Republic on the determination of seventeen mycotoxins in barley and malt a total of 52 barley and malt samples were examined, none of the investigated samples contained any of the four aflatoxin nor ochratoxin A, while other mycotoxin like Fumonisin B_1, zearalenone and Enniatins were detected but the values did not exceed the maximum allowable limit for the selected mycotoxin in processed or un processed cereals set by the European Union (Bolechova *et al*., 2014).

Furthermore, a study conducted in Russia on barley malt from industry of 120 samples examined, 67 were contaminated with mycotoxin and among these four samples were contaminated by aflatoxin with a range from 5 µg/kg to 15 µg/kg, the remaining were contaminated by OTA, T-2 and ZEA as reviewed in the literature review (Volkova, 2013). When comparing this research finding with this paper there was less amount of aflatoxin existed in this finding.

Finally, comparing this result to the European legislation (Commission of the European Communities, 2010), setting the maximum allowable of 2 µg/kg for aflatoxin B_1 and 4 µg/kg aflatoxin total has been established in all cereals and all products derived from cereals with the exception of maize to be subjected to sorting or other physical treatment before human consumption for which a maximum level of 5 µg/kg for aflatoxin B_1 and 10 µg/kg for aflatoxin total has been established. So comparing this finding from the European legislation out of 24 traditional barley malt four samples had aflatoxin level above 2 µg/kg for AFB_1 which is the most carcinogenic among the other aflatoxin and eight samples had aflatoxin level above 4 µg/kg for total aflatoxin which was beyond the European standards where as in industrial barley malt out of six samples none of samples had aflatoxin level above 2 µg/kg for AFB_1 and also one sample had aflatoxin level above 4 µg/kg for total aflatoxin set by the European legislation.

Although, out of twenty four traditional barley malt samples five of them were below the limit of quantification.

Statistical analysis by SPSS was done and ANOVA results showed significance probability value (sig) = 0.539, and comparing to the p- value which was p= 0.539 > 0.05 this shows there was no significance difference between the study sites. Statistical analysis by paired samples T- test between the industries, between industries and study sites the p- values was 0.432 and 0.222 respectively and in both case the p- value was greater than 0.05 which shows there was no significance difference between them.

5. Conclusion and Recommendation

5.1.Conclusion

Barley malt which was an ingredient for both traditional tella and industrial beer was highly contaminated by aflatoxins. In traditional barley malt out of the total analyzed, 41.7% of the samples were positive to aflatoxin with the range between (0.27-23.6) μg/kg and 20.8% was less than limit of quantification. Among of the four types of aflatoxin, G_1 was the most prevalent in both traditional and industrial barley malt which was produced by *Aspergillus parasiticus*. The grand mean total aflatoxin types from traditional and industrial barley malts were 4.64 μg/kg and 1.46 μg/kg respectively. In traditional barley malt aflatoxin B_2 was the highest in both Finoteselam and Enjibarra followed by G_1 which was existed in all sites in the highest level. In both traditional and industrial barley malt samples, majority of them the total aflatoxin level were less than 4 ppb which is tolerable level in EU standard, setting for the sum (B_1, B_2, G_1 and G_2), and 4.0 μg/kg and also some of the traditional samples were above 2 μg/kg for AFB_1 which was the most carcinogenic.

The result obtained in industrial barley malt samples none of them were the aflatoxin level for B1 was beyond than 2 μg/kg and the mean aflatoxin level was 1.69 μg/kg which was less contaminated than traditional one. Moreover, comparing the aflatoxin content of traditional and industrial barley malt, traditionally produced barley malts were more contaminated, so the contamination level of both traditional and industrial barley malt has influence on both traditional and commercial beer. On the other hand the knowledge, attitude and practice towards mycotoxin specifically aflatoxin in traditional barley malt producer was low and also they did not concern the presence of mould in the cereals where as in industrial barley malt producer majority of them know the risk of aflatoxin. There for given awareness creation and training how to handle their products to the producer.

5.2.Recommendation

The research result indicates that there is a high level of aflatoxin in barley malt on both industrially and traditionally produced malts. To mitigate the problems I suggested the following recommendations.

- As compared to the industrially produced barley malt the traditionally barley malt was found with high level of aflatoxin contamination. To minimize the aflatoxin contamination of the traditionally produced malt there must be intensive aflatoxin management practices at post-harvest level by exercising in time harvesting, good way of transporting and storage of barley grain and the next researcher find a solution how to handle the problems.

- Both traditional and industrial malt producers must monitoring and controlling of the malting process, this process induces moisture to the barley and this moisture shall be well managed and controlled before favoring aflatoxin contamination and proliferation. The drying process using either sunlight or smoke must follow appropriately and adequately.

- The industrially produced malt also found with considerable level of aflatoxin contamination therefore the industries must establish adequate quality control laboratory to select and procure aflatoxin free barley and they shall implement good housekeeping and good manufacturing practices (GMP) to prevent contamination of barley during malt processing in their premises.

- The result revealed that traditional malt producer's knowledge, Attitude and Practice was poor enough in the course of aflatoxin management and control. Therefore, The Stakeholders must coordinate themselves and provide adequate awareness creation and trainings for both barley growers and malt producers. As aflatoxin contamination would result from either the grain barley or during malt processing and after.

- The regulatory bodies shall strengthen the regulation activities to make malt produced free from aflatoxin. The federal and regional regulatory bodies shall produce or adopt aflatoxin the standard and enforce it so that the malt producers will not only aware of it but also make them to comply with the standard.

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Annexes

Questioner form

I am Addisu Alebie who is a post graduate student of Addis Ababa University center for food science and nutrition, conducting a study to determine the level of Aflatoxins between traditional and industrial barley malt in Ethiopia. The purpose of my visit is to know the knowledge, Attitude and practice (KAP) on aflatoxins for industrial producer. If you are willing to participate in the study, I will ask you few questions for about 20 minutes. Your honest answers to these questions will help me for a better understanding of the topic, and will eventually help in designing and implementing appropriate interventions to alleviate aflatoxin related problems.

Position in the Company: ----------

Qualification: ----------

Date: ----------

Part I Socio- demographic characteristics and KAP questioner on industrial barley malt producer

Question number	Question	Response	Instruction
1	Age		
2	Sex	Male	
		Female	
3	Marital status	Single	
		Married	
		Divorced	
4	Educational status	High school	
		BSC degree	
		MSC degree	
5	Family income	----------Birr	
6	Occupation	Company employer	
		Company owner	

	Knowledge		
7	What ingredient used to prepare malt?	Barley----------	
		Wheat----------	
8	From where the ingredient is found?	Unions	
		Farmers	
9	Where the finished malt product store?	Silo	
		Warehouse	
10	Do you hear about moulds?	Yes	
		No	
11	The colour of the moulds	Black	
		White	
12	Problems related to moulds	Yes	
		No	
	Attitude		
13	Do you have knowledge about food toxin like mycotoxin?	Yes	
		No	
14	Do you think that mould cereals used for malt preparation has a health impact?	Yes	
		No	
	Practice		
15	Have you taken training with related to food safety system?	Yes	
		No	
16	Have you put your ingredients for a long time before preparing the malt?	Yes	
		No	
Thank you!!!			

ቃለ መጠየቅ

ሰላምታ ; ስሜ አዲሱ አለቤ እባላለሁ በአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋኩልቲ የምግብ ሳይንስና ኒውትሪሽን ተማሪ ነኝ::እኔ ከዚህ አካባቢ በሚገኙ የብቅል ምርቶች ላይ የአፋላ ቶክሲን ብክላ ጥናት ለማድረግ ቃለ-መጠይቅ እና ምርቶችን እየሰበሰብኩ እገኛለሁ:: አፈላ ቶክሲን ማለት ፈንገስ በተባለ በተለይም አስፐርጂለስ በተባለ የሚፈጠር መርዛማ ነገር ሲሆን በሰዎች ጤና ላይ ጉዳት የሚያመጣ ሲሆን በተጨማሪም በምርታማነት ላይ ጉዳት ያመጣል:: ስለሆነም እርስዎ የሚሰጡን እውነተኛ መረጃ ወደፊት አፍላቶኪሲን በብቅል ምርቶች ላይ ለሚደረጉ ጥናቶች ጠቃሚ ይሆናል::

ቃለ-መጠይቁን የሞላው ስም----------

ፊርማ ----------

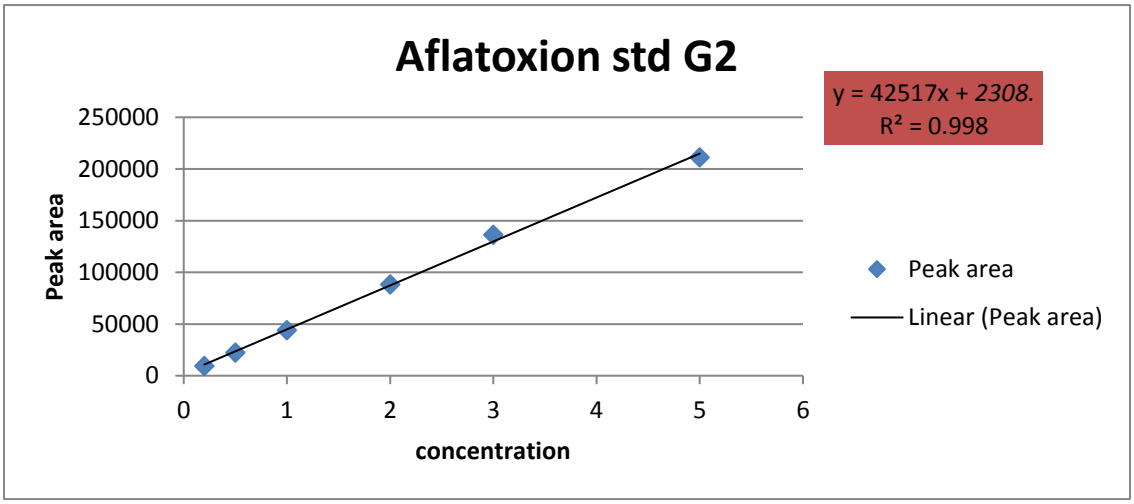
ቀን ----------

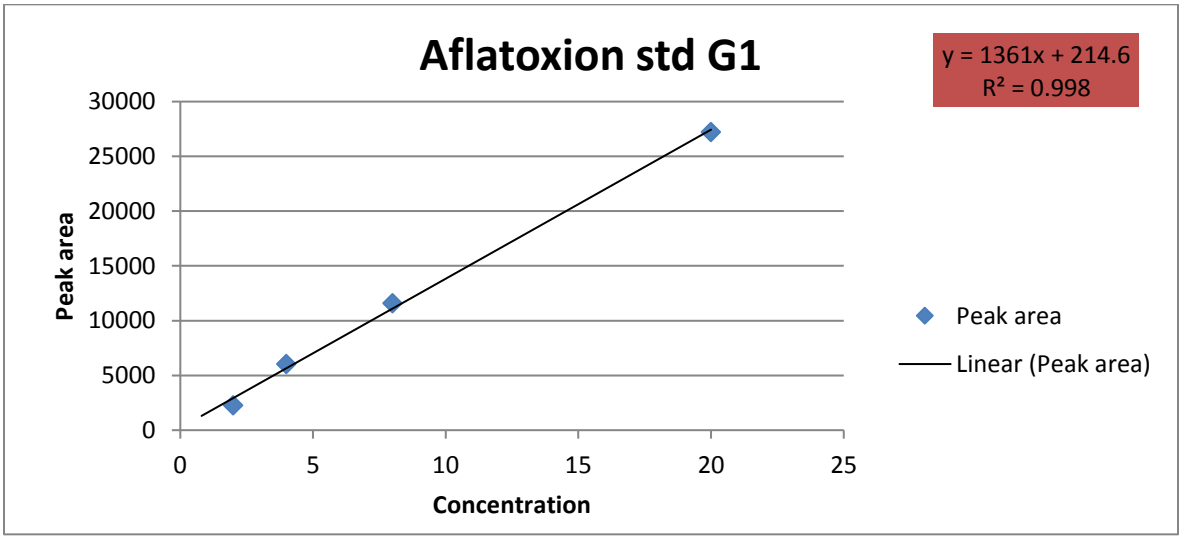
Part II. ሶሾ-ዲሞግራፊ ባህሪ እና የእውቀት፡ የአመለካከትነና የአጠቃቀም ሁኔታ በተመለከተ በባህላዊ ብቅል አምራቾች ላይ የተዘጋጀ መጠየቅ

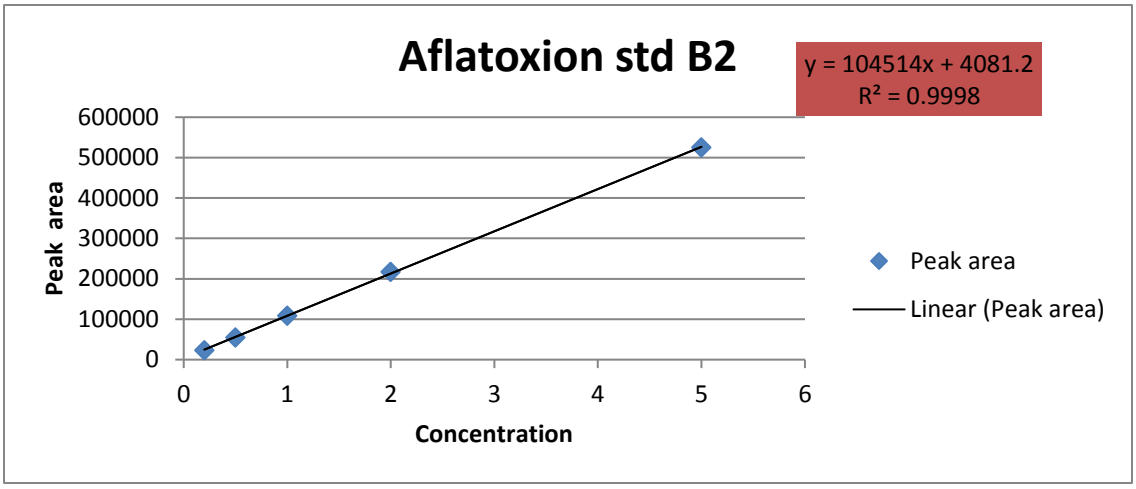
ተ.ቁ	ጥያቄ	ምላሽ	አስተያየት
1	እድሜ		
2	ጾታ	ሴት ወንድ	
3	የትዳር ሁኔታ	ያላገባ	
		ያገባ	
		የፈታ	
		ባሏ የሞተባት	
4	የትምህርት ሁኔታ	ያልተማረ/ች	
		ማንበበና መጻፍ የሚችል/የምትችል	
		አንደኛ ደረጃ ተማሪ	
5	ወርሀዊ ገቢ	በብር----------	
6	የስራ ሁኔታ	የግል ሰራተኛ	
		ተቀጣሪ ሰራተኛ	
	ያላቸው የእውቀት አድማስ		
7	ብቅል ለማዘጋጀት የሚጠቀሙት ጥሬ ዕቃ	ገብስ	

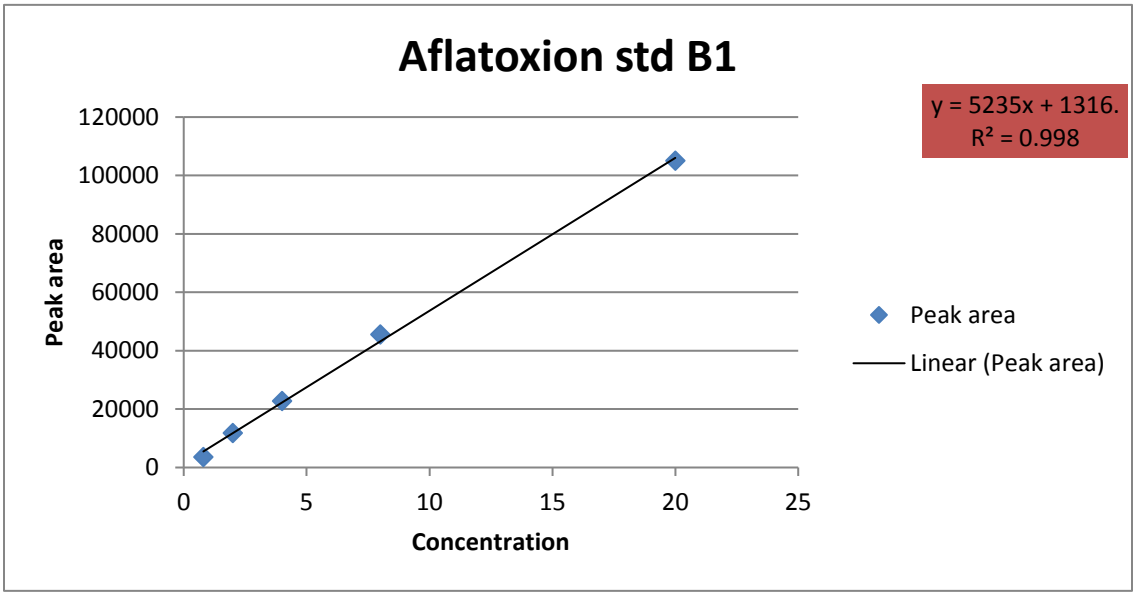
		ስንዴ	
8	ጥሬ እቃውን ከየት ያገኛሉ?	ከገበሬዎች	
		ከቸርቻሪዎች	
		ከማህበራት	
9	ጥሬ እቃ አቅራቢዎች ምርቱን የሚያሰቀምጡበት ቦታ	ጎተራ ውስጥ	
		አላውቅም	
10	ስለ ሻጋታ ሰምተው ያውቃሉ?	አዎ	
		አላውቅም	
11	የሻጋታው ቀለም ምን ይመስላል?	ጥቁር	
		ነጭ	
12	ሻጋታ የሚያስከትለውን ችግር ያውቃሉ?	አዎ	
		አላውቅም	
	የአመለካከት ሁኔታ		
13	የሻገተን እህል ቆሎ ከመስራት ይልቅ ለብቅልነት ይጠቀማሉ?	አዎ	
		አልጠቀምም	
14	ከሻገተ እህል የተዘጋጀው ብቅል የጤና ችግር እንዳለው ያውቃሉ?	አዎ	
		አላውቅም	
	የአጠቃቀም ልምድ		
15	ሻጋታ ያለው እህል ከገበያ ቢያገኙ ይገዛሉ?	አዎ	
		አልገዛም	
16	ጥሬ እህሉን ብቅል ከማዘጋጀትዎ በፊት ለብዙ ያስቀምጣሉ?	አዎ	
		አላስቀምጥም	
17	ያመረቱተን ምርት ወይም ብቅል የት ያስቀምጣሉ ?	ጣራ ላይ	
		መሬት ላይ	
			አመሰግናለሁ

Part III Calibration curve for aflatoxin G_2, G_1, B_2 and B_1






Aflatoxion std B2
y = 104514x + 4081.2
R² = 0.9998
Peak area
600000
500000
400000
300000
200000
100000
0
0
1
2
3
4
5
6
Concentration
Peak area
Linear (Peak area)


Aflatoxion std B1
y = 5235x + 1316.
R² = 0.998
Peak area
120000
100000
80000
60000
40000
20000
0
0
5
10
15
20
25
Concentration
Peak area
Linear (Peak area)