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

STUDY ON LEVEL OF AFLATOXIN IN DAIRY CATTLE FEEDS AND ASSESS KNOWLEDGE, ATTITUED AND PRACTICE OF FEED PRODUCERS, DAIRY FARMERS AND FEED TREADERS AROUND ADDIS ABABA, ETHIOPIA.

BY

MULUGETA FIKERE

ADVISORS:-EMANA GETU (Professor)
TILAHUN BEKELE (Assistant Prof)

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Declaration

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

Name: Mulugeta Fikere Signature: ----------Date: ----------

Advisors

1. EMANA GETU (PROFESSOR) Signature----------Date----------
2. Mr. Tilahun Bekele (Assistant. Professor) Signature----------Date----------

Examiners

1. External Mr. Adamu Zegeye Signature----------Date----------
2. Internal Dr. Kaleab Baye Signature----------Date----------

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List of abbreviations and acronyms

AFs-Aflatoxins

AOAC-Association of Official Analytical chemists

BSA-Bovine Serum Albumin

CSA- Central Statistical Authority

ELISA-Enzyme-linked immune sorbent assay

EU-European Union

FDA-Food Drug Administration

FMHACA-Food, medicine, health care administration and control authority

GC-MS-Gas chromatography coupled to mass spectrometry

HPLC-Higher performance liquid chromatography

HPLC-FLD-High-performance liquid chromatography with fluorescence Detector

HPLC-MS-High performance liquid chromatography coupled to mass spectrometry

IAC-Immunoaffinity Column

IARC-International Agency for Research on Cancer

KAP-Knowledge, attitude and practice

LC-Liquid chromatographic

LOD-Limit of Detection

LOQ-Limit of Quantification

MOA-Ministry of Agriculture

RSD-Relative standard deviation

SPE-Solid phase extraction

STD-Standard Deviation

TFA-Trifloroacetic acid

TLC-Thin-layer chromatography

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Abstract

Aflatoxins are naturally occurring toxic metabolites of Aspergillus flavus and Aspergillus parasiticus fungi. The aim of this study was to assess the level of aflatoxins G2, G1, B2 and B1 in dairy cattle feed around Addis Ababa. For this purpose twelve nug cake, twelve wheat bran and six atela samples were randomly collected from Debrezeyt, Sululta and Debrebrhan for aflatoxin analysis by using high performance liquid chromatography with Immunoaffinity column clean up. The result indicated that, from the total 12 samples 7(58.3 %) of nug cake were contaminated with all aflatoxins for G2 from 16.396 to 83.173 ng/g , for G1: from 128.235 to 981.122ng/g, for B2: from 18.089 to 944.271ng/g , for B1: from 149 to 887ng/g and total aflatoxin from 450 to 2320.53ng/g. The mean level of aflatoxin G2, G1, B2, B1 and total aflatoxin level in nug cake samples were 34.58ng/g, 334.15ng/g, 223.89ng/g, 347.9ng/g and 691.08ng/g respectively. In 3(25 %) of wheat bran samples only G1,B2 and B1were detected with the range of G1: from 0 to 24.229ng/gm for B2: from 0 to 22.435ng/g , for B1: from 0 to 35.318 ng/g and total aflatoxin from 0 to 61.947ng/g . The mean level of aflatoxin G1, B2, B1 and total aflatoxin level in wheat bran were 18.768ng/g, 18.52ng/g, 21.55ng/g and 59.0572ng/g respectively. Level of aflatoxin in atela samples about 3(50%) of the samples were investigated below the limit of quantification and one sample was below the limit of detection while, in two samples were not detected. The mean level of aflatoxin G2, G1, B2, B1 and total aflatoxin in nug cake were exceeded the maximum limit set by food and agriculture organization(FAO)/world health organization (WHO) 20µg/kg and European Union (EU) 5µg/kg for dairy cattle feed. In wheat bran only B1and total aflatoxins were exceed the limit of food and drug authority and food and agriculture organization(FAO)/world health organization(WHO)20µg/kg and the level of G2, G1, B2, B1 were exceed the limit of European Union(EU) 5µg/kg. Aflatoxin contamination in all atela samples were below the limit. The result of knowledge, attitude and practice assessment in this study revealed that, awareness of mold growth and formation of mycotoxin is very low among dairy farmers and feed traders. From the experiment it can be concluded that nug cake feed was highly contaminated with all aflatoxins and wheat bran was less contaminated. Implying that feeding atela to dairy cattle is the safest, while nug cake is the most dangerous in terms aflatoxin contamination.

Key words: Aflatoxin G2, G1, B2, B1, Total aflatoxin, Diary feeds, Immunoaffinity cleanup.

1. Introduction

1.1.Back-ground

Mycotoxins are the diverse groups of secondary metabolites of storage fungi. It can develop in many agricultural products and harmful for both animals and humans health when consumed mycotoxin contaminated feed and food staff (Reddy, 2010). Fungi can grow at the pre or post-harvest agricultural practices during seedling, harvesting, storage, transportation and feed preparation system (Whitlow, 2005).

Aflatoxins are a group of approximately 20 related fungal metabolites produced primarily by the storage fungi of A.flavus and A.parasiticus. It has four major naturally, produced aflatoxins are known as B1, B2, G1, and G2. “B” and “G” refer to the blue and green fluorescent colors produced under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds, respectively. Aflatoxin B1 is the most potent toxic of the group and cause liver cancer (Bennett, 2003).

Mycotoxin contaminations in agricultural commodity have significant economic implication. In the case of mycotoxin contamination, losses from rejected shipments and lower prices for inferior quality can devastate developing country export markets. Furthermore, livestock production includes mortality as well as reduction in productivity, weight gain, feed efficiency, fertility, and ability to resist disease. It is also causes a reduce of both quality and quantity of meat, milk and egg products in one country (Lopez-Garcia, 2001). Aflatoxin is a significant problem in cow’s milk when the ingestion of aflatoxin B1 contaminated feed by cow’s and aflatoxin B1 is converted or metabolites to aflatoxin M1 (Melkamu and Bezabih, 2014).

Several studies reported that, clinical signs resulting animals exposure to aflatoxin leads to weight loss, depression, hemorrhages, immune suppression or pulmonary edema as well as decreased milk production in dairy cattle (Fink-Gremmels, 2008).On the other hand, the presence of aflatoxin B1 in the feed of lactating animals can transfer to 4-hydroxylated metabolite in the liver and is excreted in the milk as aflatoxin M1and it is a major health risk

for humans when consumed, especially vulnerable groups, as it can be immunosuppressive, mutagenic, teratogenic and carcinogenic (Melkamu , 2014:Dawit, 2016).

In Ethiopia, oil seed cake, wheat bran, grass, cereal straw and seeds, and traditional beer waste are the most widespread sources of raw materials in the production of dairy cattle feed (Alemayehu 2003). However, oil seed cake, wheat bran and traditional beer waste in the study area mostly preferred by the dairy farmers for feeding to milking cows in the increase of milk volume. Most of the farmers produce animal feed from these basic raw materials without considering the exposure of mycotoxin development.

The study districts (Debrezyte, Sululta and Debrebrhan) are the potential source of milk production and supplying area for commercial purpose in the capital city of Ethiopia. However, the reduction of aflatoxin exposure in milk product in many countries has regulated the levels of aflatoxin B1 in feed and set the maximum permissible levels of aflatoxin M1 in milk product.

Currently, the legal limits of aflatoxin B1 in feedstuffs are highly variable from country to country, for example, USA Food and Drug Administration has established 20µg/kg for aflatoxin present in feed and 0.5 µg/L aflatoxin for milk (Khanafari, 2007). In addition to that, the European Union has set very stringent limit of the aflatoxin B1 in dairy cattle feeds present 5µg/kg and 0.05µg/L for milk (EU, 2003). Despite of this, aflatoxin contamination limit of diary feed in Ethiopia is not standardized. Therefore, the present study aimed to investigate the level of aflatoxin G2, G1, B2, B1 and total aflatoxin in dairy cattle from three districts by using high performance liquid chromatography with Immunoaffinity columns clean up.

1.2.Statement of the problem

The use of cultivated improved fodder crops for animal feed is not widely practiced because of an ever-expanding demand for farming land, even at the expense of permanent pastureland. According to MOA, a survey carried out by FAO in 1989 indicated that permanent pastureland has been declining by 1.1% over a 16-year period starting in the year 1973.

Dairy farmers in urban and pre-urban areas used mainly industrial byproduct such as different oilcakes, wheat bran and brewer's grain in combination with purchased hay or straw to feed their animals. The kinds of feed undergo to aflatoxin contamination through poor management and handling.

Now a day's the most alarming problem through time has been the presence of aflatoxin contaminated milk in the market. Some studies reported that there is a linear relationship between the amount of aflatoxin M1 in milk and aflatoxin B1 in feed, which is consumed by dairy cattle's (Dawit, 2016). Aflatoxin M1 in milk and milk products are considered to pose certain hygienic risks for human health.

On the other hand, aflatoxin can result in major economic losses and can negatively affect animal and human health. Economic losses from mycotoxicoses in agriculture are due to effects on livestock productivity, losses in crops, and the costs and effects of regulatory programs directed towards mycotoxin. Although the indirect exposure of humans to aflatoxin occurs through foods primarily milk, liver, and eggs derived from animals that consume contaminated feeds, handling of contaminated feed also results in exposure of mycotoxin through the skin and by inhalation (Schiefer, 1990).

1.3. Significance of the study

The findings of this study will be helpful to minimize aflatoxin contamination of milk and milk product, by determining the level of aflatoxin *G2, G1, B2 and B1* in dairy cattle feeds around Addis Ababa. The study can be used to ensure the safety of milk products for human

consumption as well as to safeguard the health of the consumers by increasing knowledge and awareness of mycotoxin contamination on animal feed.

The data obtained by this study will serve as a basis for setting aflatoxin regulatory limits in the country. Researchers and academicians of the field can use the findings of the study as reference materials. Generally, the result of this study will contribute to strengthen the development of food and feed safety in Ethiopia.

1.4. Research question

- What is the level of aflatoxin G2, G1, B2 and B1 in dairy cattle feeds (Noug cake, Wheat bran and *Atela*) at the study sites around Addis Ababa (Sululta, Debrezeyt and Debre brhan)?
- What is the knowledge, attitude, practice (KAP) of animal feed producer feed traders, and dairy farmers about aflatoxin contamination in dairy cattle feed?

1.5. Objective

1.5.1. General Objective

- To detect and quantify the level of aflatoxin G2, G1, B2 and B1 in dairy cattle feed, around Addis Ababa .

1.5.2. Specific Objective

- To determine the level of aflatoxin G2, G1, B2 and B1in selected dairy cattle feed (Wheat bran, Nug cake and *Atela*) around Addis Ababa.
- To compare the level of aflatoxins in dairy cattle feed between each selected study site.
- To evaluate whether the level of aflatoxin in dairy cattle feed samples around Addis Ababa are above the limit or below.
- To assess knowledge, attitude and practice (KAP) among feed producers, feed traders and dairy farmers

2. Literature Review

2.1. Livestock resource and milk production in Ethiopia

Ethiopia holds the largest cattle population in Africa estimated at about 53.4 million heads of cattle's (Saxena, 1997), which 10 million is dairy cows (CSA, 2011). Despite the large dairy cattle population of Ethiopia, the per capita milk consumption in Ethiopia 18.68 liters is very low as compared to the global average of 100 liters and even far below the average for Africa, 27kg/year (CSA, 2008).

Milk and milk products are economically important farm commodities and dairy farming is an investment option for smallholder farmers (Tsehay, 2001). In Ethiopia, urban and pre-urban dairy production has been emerging as important component of the milk production system. This system is contributing immensely towards filling in the large demand-supply gap for milk and milk products in urban centers, where consumption of milk and milk products is remarkably high (Gebrewold, 1998). Among livestock species, cattle have significant contributions to the livelihoods of the farmers. They serve as a source of draught power for the rural farming population, supply farm families with milk, meat, manure, serve as source of cash income, and play significant role in the social and cultural values of the society (Mekonnen., 2012).

Livestock also plays an important role in providing export commodities, such as live animals, hides, and skins to earn foreign exchanges to the country (Alemayehu, 2012). On the other hand, draught animals provide power for the cultivation of the smallholdings and for crop threshing almost all over the country and are essential modes of transport to take holders and their families long-distances, to convey their agricultural products to the market places and bring back their domestic necessities. Furthermore, livestock provides farmyard manure that is commonly applied to improve soil fertility and used as a source of energy.

2.2. Intra-urban and Pre-urban milk production

This system is known for its commercial purposes, although the scale of production is not large (Gebrewold etal, 1998). Dairy farms have been established in and around the big cities and they mainly dominate the informal milk market in the cities. The main feed sources of dairy cattle in the study area were agro-industrial by-products (oilseed cakes, bran, local beer waste etc.) and commercial hay (Mengistu, 1984). The dairy farmers keep exotic or hybrid cows, managed under a zero-grazing system (especially the in intra-urban dairies).

2.3. Large-scale commercial dairy farming

This system exists in the intra-urban, pre-urban and rural locations. It is a highly organized and intensive production system, and is defined as a farm, which keeps more than 30 dairy cattle as well as improved management systems (Geda, 2001). Those dairy farms that are located in the rural areas use their own transport facilities to supply milk to the processing plants. Some of the farmers even have their own small-scale milk processing equipment. There are many private dairy enterprises around Addis Ababa in Debrebrhan, Debrezeyt and Sululta. They are engaged in providing farm input (feed and veterinary drugs), animal health care, and milk processing (Zelalem, 2011)

2.4. Animal feed in Ethiopia.

Animal feed is a kind of natural or prepared product for animals such as oxen, cows, sheep, goat, etc. It contains protein, minerals and other nutrients, which are useful for beef and milk production as well as survival and growth of animals **(Seyoum, 2001).**

Natural grazing and browsing on plots of permanent grazing land and stubble following crop harvest make the main sources of animal feed in Ethiopia (Alemayehu , 2003). However, natural pasture is gradually declining when the rain is decreased during winter season, expansion of crop production into grazing lands, redistribution of communal lands to the landless and land degradation. Conserved natural hay and different crop residues such as *Teff*, barley and wheat straws, too, are important feeding strategies and feed components of ruminants in the highland areas. On the other hand, establishment commercial fattening and animal breeding enterprises have increase the demand for cattle feed

The use of cultivated improved fodder crops for animal feed is not widely practiced because of an ever-expanding demand for farming land, even at the expense of permanent pastureland. According to MOA, a survey carried out by FAO in 1989 indicated that permanent pastureland has been declining by 1.1% over a 16-year period starting in the year 1973. Dairy farms and feedlot operators in urban and pre-urban areas use mainly industrial byproducts such as different oilcakes, wheat bran and brewer's grain, in combination with purchased hay or straw, to feed their animals. The kind of feed used is largely determined by its availability rather than choice. Sometimes they also make use of formulated concentrate feed purchased from feed-processing plants.

The country faces severe feed shortage due to either the seasonality in the availability and the poor management and handling of animal feed. The current national animal feed demand is estimated at 95.8 million tons, whereas the supply is only to 65.6 million tons (ITAB 2010) there is unsatisfied huge gap. According to the report (Statistics, 2012), the total cattle population for the country is estimated to be about 52.13 million and the recommended rate of feed is 2 kg/head per day.

2.4.1. Natural Grazing

Natural pasture/ hay/ is produced in waterlogged areas and available in large amount until about (September- December) each year in the study area. However, the availability of these types of feed are decreased after this period, agricultural byproduct such as wheat, barley and *Teff* straws are used as the major source of animal feed throughout the year. Hence hay is widely used for dairy animals and livestock producers and dairy farmers are usually buy most of their hay requirements during (September-December) additional concentrate feed is needed (Tesfay, 2004). In the study area nug cake, wheat bran and *Atela*) are the most common concentrate feed for milking cow to increase the milk yield.

2.4.2. Industrial by-product

Agro-industrial by-products from different flour and edible oil mills grind mills and traditional beer waste (*Tella* and *Areke Attela*) are sold in all of the study areas. There are several flourmills and edible oil mills in the towns of Addis Ababa and around (Adama, Debrebrhan and Debrezeyt).These companies produce wheat bran and noug cake as a by-product and supply for dairy farmers as a feed.

2.4.3. Nug cake

Oilcakes are an excellent concentrate feed for ruminant livestock due to its nutrients .Ethiopia grows most of the temperate and sub-tropical oilseed plants such as linseeds, Niger seeds, groundnuts (peanuts), rapeseeds, sesame, sunflower and cotton. The processing of oilseeds is widely practiced at the household level, or in small village mills.

Noug cake is a byproduct of oil extraction from Niger seed and it can be stored and used as an animal feed supplement. However, some study reported that Niger seed has fewer diseases than other oilseeds; molds such as *Aspergillus Niger*, *A. flavus, Penicillium*sp, *Alternariaalternata*, *Rhizoctoniasolani*and*R. bataticola*are the major cause of safety and quality problem of Niger seed (Getinet, 1996). The meal remaining after the oil extraction contains about 24% proteins and 24%crude fiber. Niger meal from India contains higher protein (30%) and lower crude fiber (17%) levels than meal from Ethiopia (Singh, 1993).

The quality of seed cakes is affected by extraction method, number of extractions and adulteration. Modern and big edible oil mills use organic solvent extraction method which squeezes most of the oil and the result seed cakes are considered poorer in quality. The other extraction method is mechanical pressing method, which is not efficient in oil extraction. The cake produced from this method is considered of better quality.

The traditional Ethiopian means of extracting Niger oil, which includes a “combination of warming, grinding and mixing with hot water”, followed by hand centrifugation in a clay container. They also report that Niger seed is crushed in small cottage expellers and large oil mills (Raju, 2000). Seed cakes should contain some oil lest they become very rough for the animals to feed. On the other hand, farmer preferences for the types of seed cakes differ by

the purpose of feeding. For example, in Debrezeyt, linseed and cotton seed cakes are preferred to nug seed cake for fattening. However, fatteners in this area feed fattening animals with small amounts of nug cake at the last cycle of fattening because they believe that nug cake gives the fattened animal smooth and shiny hair, and results in red meat.

Dairy farmers prefer nug seed cake and cotton seed cake to linseed cake, because they believe that dairy cows feed with linseed and cotton seed cake become fat and milk yield decreases.

2.4.4. Wheat Bran

Wheat bran is a by-product of the dry milling of common wheat (*Triticumaestivum* L.) into flour it is one of the major agro-industrial by-products used in animal feeding. It consists of the outer layers (cuticle, pericarp and seed coat) combined with small amounts of starchy endosperm of the wheat kernel. Other wheat processing industries that include a bran removal step may also produce wheat bran as a separate by-product: pasta and semolina production from durum wheat (*Triticum durum*Desf.), starch production and ethanol production.

Milling yields variable proportions of flour, depending on the quality of the final product. The extraction rate (flour: grain ratio) goes from 100% for a whole meal flour to less than 70% for pastry flour.

Typical extraction rates range from 75% to 80%, resulting in 20 to 25% wheat offal's (Kent & Evers, 1994) In the industrial milling process, after a cleaning step that removes grain impurities, the grains are tempered (soaked to strengthen the outer layers and mellow the starchy endosperm in order to facilitate their separation) and then subjected to a series of grinding operations that produce finer and finer flour particles. The first grinding steps yield coarse particles of broken wheat and bran, and the later steps produce other by-products (Wheat Marketing Center, 2008). Milling by-products are traditionally named after their quality (fineness, color, etc.) and/or the stage of the process at which they arise, with considerable variations between languages, countries, regions, milling processes and even mills. In industrial countries, these products used to be sold separately (coarse bran,

fine bran, middling, second clear, thirds, etc.) but are now mixed together in variable proportions (Macdonald, 2002). Consequently, wheat milling form a range of products with a decreasing fiber: starch ratio, from the fibrous coarse barns produced by the first grinding steps to starchy feed-grade flours.

Wheat barns sold for animal feeding are typically mixtures of true coarse barns and finer products from the later grinding stages. In rural and traditional milling, flour is directly separated from bran in a one-step milling and screening. This type of bran has higher starch content and a higher nutritive value (Piccioni, 1965).

In Ethiopia, farmers prefer high-density bran since weight indicates that the bran contains more flour and thus higher energy (Gebremedhin, 2009). The situation is made even more complex by the existence of wheat bran's from other wheat species (durum) and wheat processing industries. Several studies reported that, Wheat bran is suitable for livestock feeding and very palatable to most classes of animals (Fuller, 2004). Wheat bran is a bulky feed that can be used to lighten dense, heavy feed mixtures. It can be readily incorporated into mashes. The qualities of bran depend on fair coating of flour and in the form of large, dry and non-adherent flakes.

2.4.5. Traditional Beer Waste (*Atela*)

Tella (Oroma: farso, Tigrinay:Sawa) is a traditional beer from Ethiopia and Eretria. It is brewed from various cerial grains, such as barley, wheat, or maize may be used and also the spices can be added. Dried and ground gesho leaves are used for fermentation. Due to the addition of bread and use of a fermentation vessel which has been smoked over dried olive wood or Abyssinian rose wood and Tella may have a smoky flavor. It may be offered in tella houses (Tellabet), usually in regular homes, where people meet, drink together and talk to each other.*Atela* is the byproduct of Tella and listed as the major feed resources in both agro ecological areas especially for dairy castles (Kassahun, 2015). Dairy farmers in the study area believed that *atela* for milking cow increase the milk volume. While in this study, *Attela* from *Tella* was analyzed for aflatoxin B1 and the total aflatoxin concentration.

2.5. Importance of milk

Livestock, milk, and milk products play an important role in the food security status in both highland and pastoral communities. Calcium and vitamin D are essential for bone growth and maintenance. Among the bone-forming minerals (Ca, P, Mg, Zn), dietary calcium supply is close to biological requirements and may be limiting in some parts of the world where there are few rich dietary sources of calcium, particularly for children and women during pregnancy and lactation (Prentice, 2006). Similarly, the vitamin D status of many women and young children is compromised by low UVB skin exposure and by factors that increase vitamin D usage, even in tropical countries with abundant sunshine (Mutebi1, 2015).

2.6. Traditional Storage of animal feed and mycotoxin contamination

Teff straw is stored in heaps of conical or pyramidal forms either at homesteads or in the fields. Mostly the heaps are stored usually on open areas, and rarely under shades. The heaps are made in such a way that they do not let rainwater percolate the heaps. As reported by farmers, a properly made heap (compact and tilted slope from the top to the foot) can stay for several years with minimum quality deterioration. Large traders and commercial livestock producers store baled straw under the shades (Gebremedhin, 2009).Commercial hay producers usually store hay for less than five months. However, as reported by the producers, hay can be stored for several years if it is protected from moisture and rodents. Stover's are usually first stored in heaps in the fields for a short time, after which they are transported to homesteads and stored in heaps again. The duration of field storage varies from area to area.

2.6.1 Wheat bran

Like other agro-industrial by-products wheat bran is sensitive to humidity. The shelf life of bran is lower than that of seed cakes. Feed traders reported that wheat bran can be stored for two months in the dry season while it can be stored only for one month in the wet season. Therefore the feed sealers and dairy farmers reported that they do not keep wheat bran for a period of more than two months (Gebremedhin, 2009).They are usually buy wheat bran enough for 1 to 2 weeks because of its short shelf life. The shelf life of bran is also affected by type of storage, rodent, moisture and humidity. Bran stored in ventilated stores can have longer shelf life than bran stored in non-ventilated stores, according to feed sealers.

2.6.2. Oilseed cakes

The cakes should be protected from moisture while being transported or stored. According to feed sealers, linseed, cotton and nug seed cakes have shelf life of about 5–6, 2–3, and 3 months, (Gebremedhin, 2009). According to feed sealers reported that, nug cake stored in piles over one another may be heated (create humidity) and its shelf life reduced. On the contrary, linseed and cotton seed cakes stored in piles do not easily get heated up.

2.7. Mold growth and formation of mycotoxin

Mycotoxins are secondary metabolites, which are produced by a number of different fungi (Bennett, 2003). Mold growth and the production of mycotoxin are usually associated with extremes in weather conditions. Environmental conditions– heat, moisture, and insect damage are cause of plant stress and predispose plants in the field to mycotoxin contamination. . Molds can grow over a temperature range of 10-40° C (50-104° F), a pH range of 4 to8, and above 0.7 aw (equilibrium relative humidity expressed as a decimal instead of a percentage) (Lacey, 1991).Because feedstuffs can be contaminated pre-harvest, control of additional mold growth and mycotoxin formation is dependent on storage management. After harvest, temperature, moisture content, and insect activity are the major factors influencing mycotoxin contamination of feed grains and foods (Bhat, 1988).

Molds can grow on feeds containing more than 12% to 13% moisture. In wet feeds such as silage, molds will grow if oxygen is available and the pH is suitable. Because most molds are aerobic, high moisture concentrations that exclude adequate oxygen can prevent mold growth.

Mycotoxins are toxic secondary metabolites produced by fungi that grow naturally in several agricultural commodities, causing a wide range of toxic effects in agricultural products (Whitlow, 2010). The species of Aspergillus, Fusarium and Penicillium molds are among the most important in producing mycotoxins detrimental to cattle. However, the mycotoxins of greatest concern include aflatoxin B1 in the feed contaminate the milk with aflatoxin M1, which are generally produced by Aspergillus mold, deoxynivalenol, zearalenone, T-2 Toxin, and fumonisin, which are produced by Fusarium molds; and ochre toxin produced by

Penicillium molds are affect castles. Several other mycotoxins such as the ergots are the most potent to affect cattle and may be prevalent at a times in certain feedstuffs. There are hundreds of different mycotoxin, which are diverse in their chemistry and effects on animals (Amare, 2015). It is likely that contaminated feeds will contain more than one mycotoxin.

2.8. Occurrence of mycotoxin in food and feed

The field and storage fungi can contaminate the food and feedstuffs in the different stages of production and transformation, from growth to transportation and storage (Negero, 2014). Field fungi, including *Fusariumgraminearum* and *F.Verticillioides* species and storage fungi, such as *Penicilliumverrucosum* and *Aspergillus flavus*. Dairy cattle feed may be naturally and simultaneously contaminated by several fungi that are able to produce different toxins (Wondimeneh, 2016). Most of the silage is made up from annual crops. The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxin.

Contamination of mycotoxin can occur when, growing crop or during storage, processing, or handling, and after the finished food products have been prepared for sale or consumption. Environmental stress conditions such as insect infestation, drought, cultivar susceptibility, mechanical damage, nutritional deficiencies, and unseasonable temperature, rainfall or humidity can promote mycotoxin production in growing crop (Alemayehu, 2014).

The most mycotoxins are chemically stable during storage and processing, even at high temperatures. Consequently, mycotoxin may also be found in dairy cattle feed resulting from the use of contaminated source of feed, other cereals, and agro industrial by-products (Dawit ,2016). They can also enter the human food chain via meat or other animal products such as eggs, milk, and cheese as a result of livestock eating contaminated feed. Mycotoxins can occur both in temperate and tropical regions of the world, depending on the species of fungi. For example, Aspergillus species find optimal conditions in tropical and subtropical areas, whereas Fusarium and Penicillium species are also adapted to Europe and US climates (FAO., 1995).

Mycotoxin concentration in the feed may vary from year to year (Alonso, 2013). Several species in the genera *Aspergillus*, *Penicillium*, *Alternaria*, *Trichoderma*, *Claviceps*, *Mucor* and *Geotrichum* make up the storage micro biota and are responsible for the production of mycotoxins found in grains and forage. The problem of food and feed contamination with mycotoxin has attracted great attention of the scientific community in the last decades, given the high risks to human and animal health and the negative economic impact. Studies have demonstrated the incidence of fungi and mycotoxin in several foodstuffs and silage (Alonso, 2013)

The presence of fungi in feed ingredients may cause general, unspecific problems for the animal production, such as reduced feed intake or feed acceptability (Melkamu and Yitbarek, 2014) .However, diseases caused by mycotoxin, known as mycotoxicoses, are the most important health problems caused by fungi contamination. They are characterized by diffuse syndromes, but with predominance of lesions in organs and tissues, such as liver, kidneys, epithelial and central nervous system, depending on the industry worldwide.

2.9. Toxic effect of aflatoxins in humans and dairy cattle's.

Aflatoxicosis is a disease caused by the consumption of aflatoxin, the mold metabolites produced by some strains of *Aspergillus flavus* and *Aspergillus parasitisus*. The four most common aflatoxins are B1, B2, G1 and G2. Contaminated grains and grain by-products are the most common sources of aflatoxins (Geremew, 2016). Corn silage may also be a source of aflatoxin, because the ensiling process does not destroy the toxins already present in silage.

Aflatoxins are metabolized in ruminants by the liver and excreted in the bile. Aflatoxin B1 is the most potent mycotoxin (toxic substance produced by a mold). Aflatoxin B1 increases the apparent protein requirement of cattle and is a potent cancer-causing agent (carcinogen). When significant amounts of aflatoxin B1 are consumed, the metabolite M1 appears in the milk within 12 hours. Research suggests M1 is not as carcinogenic or mutagenic as is B1, but it does appear to be as toxic as its parent compound.

Acute aflatoxicosis is characterized by quick deterioration of general status, loss of appetite, low feed conversion, interference with reproductive capacity, immunosuppressant, acute hepatitis, hemorrhage and death (Whitlow, 2010). The liver is the most importantly affected organ, with lesions caused by hemorrhagic necrosis, Centro lobular congestion, proliferation of cells of biliary ducts and fatty infiltration in hepatocytes (Oliveira ,2007). The early symptoms of hepatotoxicity from aflatoxicosis can include anorexia, malaise, and low-grade fever. Acute high-level exposure can progress to potentially lethal hepatitis with vomiting, abdominal pain, and death.

The effects of chronic toxicity are also characterized by hepatic lesions in a lower extent and include changes in growth of rumen microorganisms and genetic changes (Whitlow, 2010).In early stages of intoxication, mycotoxin cause relatively minor problems to dairy cows. The decline in performance may be negligible. Within days or weeks, the effect of continued mycotoxin consumption on performance (milk production or body weight gain) becomes more evident. Unspecific signs including loss of appetite, ketosis and displaced abomasums may be significantly increased by the ingestion of mycotoxin. Some mycotoxin can determine toxic effects in target organs, such as zearalenone, which causes estrogenic effects, swollen vulvas and nipples and vaginal prolepses. Reduced conception rates or abortion may also be related to mycotoxin consumption. The effects of mycotoxin are amplified by production stress: high-producing dairy cows are more susceptible to the effects of mycotoxin than low-producing ones (Abidin and Khatoon, 2012).

Aflatoxicosis in cattle may be classified in three distinct clinical forms:

(a) Primary acute disease, showing a variable picture as a function of sensitivity, generally after the intake of moderate to high amounts of AFB1, with lesions in liver, kidneys and central nervous system,

(b) Primary chronic disease, after the intake of moderate to low amounts of aflatoxin B1 that are unable to cause classical clinical picture, although there is a negative effect on health and reproduction, leading to delayed growth, loss in carcass quality and reduced milk yield (Whitlow , 2010) and

(c) Secondary chronic disease caused by the intake of small amounts of aflatoxin that are unable to cause evident clinical intoxication, but are able to predispose the animal to secondary diseases by immunosuppressant (Oliveira, 2007).

Besides the important impact on the health and performance of dairy cows, the intake of feed contaminated with aflatoxin B1leads to its biotransformation into aflatoxin M1 by liver enzymes of cytochrome P450. This hydroxylated derivative is water soluble and may be easily excreted in body fluids, such as urine and milk, which represents a serious risk to human health. Economic losses caused to milk producers due to the chronic toxic effects of aflatoxin may be less obvious than those caused by acute intoxications. While losses caused by mortality, decreased yield and milk contamination may be estimated, other less evident effects, such as infertility, reduced rumen and feed efficiency, immunological problems and other performance losses may be more difficult to be estimated.

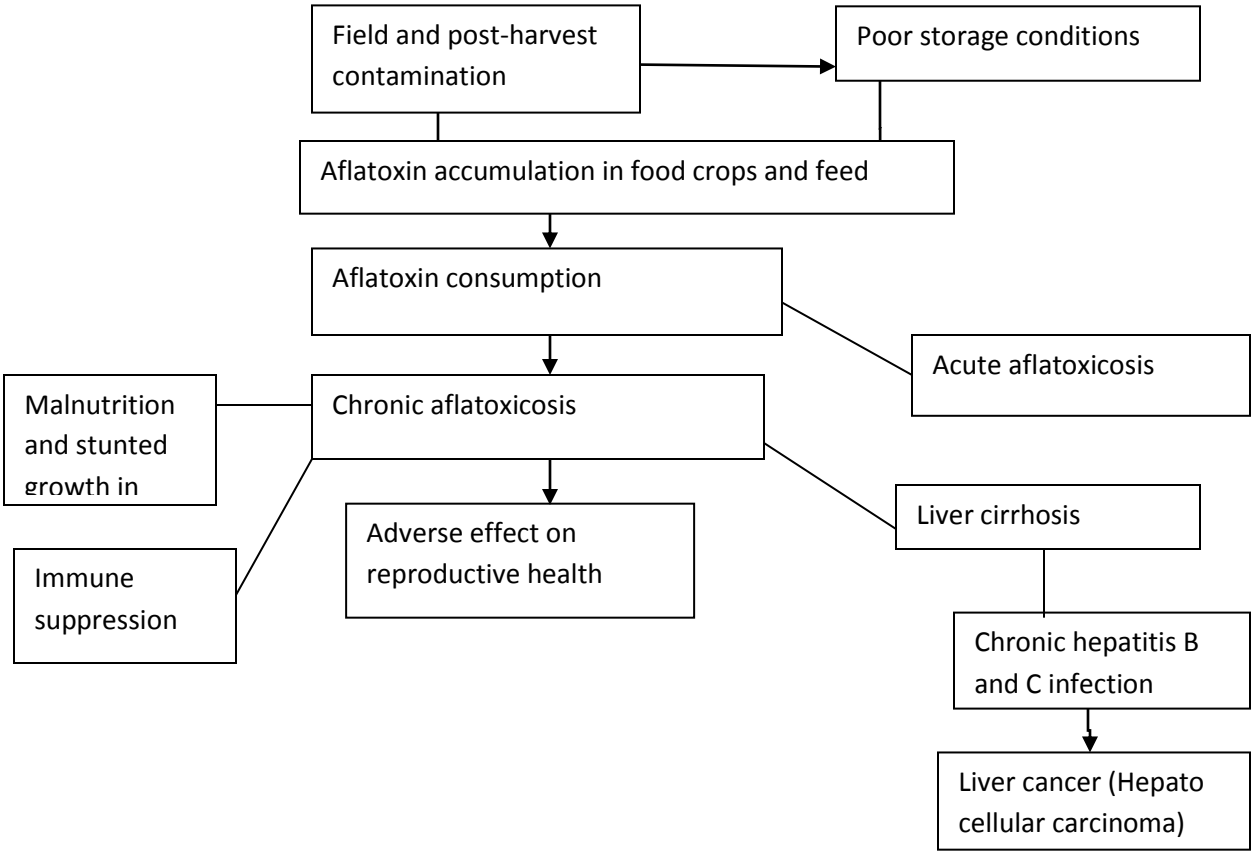

Figure 1. Aflatoxin disease pathways in humans and animals.

(Adopted from Wu, 2010; USAID, 2012; WHO, 2011; Wu and Tritscher, 2011)

2.10. Residue of mycotoxin in milk

The aflatoxin M1 is detected in the milk few hours after the intake of the contaminated feedstuff. When the intake of aflatoxin B1 is finished, the aflatoxin M1 concentration in the milk decreases to an undetectable level after 72 h (Melkamu and Yitbarek,2014), disappearing from the milk two to three days after the feed stuff is removed from the diet (Oliveira, 2007). Therefore, the presence of aflatoxin M1 residues in milk and dairy products is a public health concern, taking into account their potential for additional aflatoxin exposure of humans through the diet. For this reason, several countries established maximum levels for aflatoxin M1 in milk and milk products, although, other mycotoxin may also be excreted into milk. All over the world and especially in the European Union, there are also strict regulations for the maximum tolerable concentration of aflatoxin B1 in feed ingredients and rations for dairy cows (Oliveira, 2007).

The presence of aflatoxin M1 in milk and dairy products is a worldwide concern, once these products are frequently consumed and milk is the one of the most important foods for children (Oliveira, 2007). Evidence of human exposure to aflatoxin M1 due to the consumption of milk and dairy products has been well demonstrated worldwide.

Some studies conducted in Ethiopia show that (Dawit, 2016) observed that all 110 milk samples (n=110) were contaminated with aflatoxin M1 with 26.3% of sample at the level above 0.05µg/l which is the limit adopted by European community. In another study in Ethiopia (Szonyi,2015) most of the sample had aflatoxin levels exceeding the food and agriculture organization(FAO)/world health organization(WHO)and European Union(EU) limits. In Kenya (Kang and EK, 2009) observed that A total of 830 animal feed and 613 milk samples were analyzed for aflatoxin B1 and M1 respectively using competitive enzyme immunoassay. 86% of the feed samples were positive for aflatoxin B1, 67% of these exceeded the FAO/WHO level of 5µ gKg-1, and 86% of the milk exceeded the WHO/FAO levels of 0.05µ g/Kg-1.

2.11. Economic impacts of aflatoxin.

Economic losses from mycotoxicoses in agriculture are due to effects on livestock productivity, losses in crops, and the costs and effects of regulatory programs directed toward mycotoxin (WU, 2006). According to the Food and Agricultural Organization (FAO) of the United Nations, up to 25% of the world's food crops have been estimated to be significantly contaminated with mycotoxin (Science, 1989)

Aflatoxin contamination can result in direct economic impact through export rejections from importers with contaminated product and low market value. Mycotoxins occur at concentrations high enough to cause major losses in health and performance of animals. However, mycotoxins are more usually at lower levels that result in interactions with other stressors to cause subclinical losses in performance, increases in incidence of disease and reduced reproductive performance. For animal producer, these subclinical losses are of greater economic importance than losses from acute effects (Wayne, 2007).

2.12. Prevention Strategy for mycotoxin in the diet of dairy cattle

Mycotoxin production by pathogenic fungi is greatly influenced by a number of physical, chemical and biological factors such as pre- and post-harvest temperature, CO2 and moisture levels but also nutrient availability, pesticide usage, physical damage or pest attacks (Tirado, 2010.).

2.13. Pre-harvest control methods

Using resistant varieties, field management, use of biological and chemical agents, harvest management, pesticide application in some cases and proper fertilization. Unfortunately, breeding for mycotoxin-resistant hybrids has been only partially successful. Fungicides have shown little efficacy in controlling pre-harvest aflatoxin contamination in corn (Duncan, 1994).

2.14. Post-harvest control methods

Improved drying methods, good storage conditions, use of natural and chemical agents (Ammunition), irradiation can apply for reduction of mycotoxin in food and feed stuff. However, mycotoxin-contaminated grains can be used for ethanol production, and in some cases mycotoxin-contaminated grains can be diluted with clean feeds (Kim, 2015). The FDA does not allow dilution of aflatoxin-contaminated feeds, which is considered as adulteration. The best strategy for postharvest control of mycotoxin is proper storage and handling of feed grains.

The inclusion of sorbent materials in feed or addition of enzymes or microorganisms capable of detoxifying mycotoxin has been reported to be reliable methods for mycotoxin prevention in feeds (Silvan, 2015).Certain feed additives can reduce mycotoxin exposure of animals and thus minimize their negative effects. Some additives may be beneficial in reducing mycotoxin formation because they are effective in reducing mold growth. Ammonia, propionic acid, microbial, and enzymatic silage additives have all shown some effectiveness as mold inhibitors. Mold growth inhibitors such as propionic acid may be helpful as a surface treatment when capping off the silo or daily after silage feed-out to reduce molding of the exposed silage feeding face (Huff, 1992).

If unacceptably high levels of mycotoxin occur, dilution or removal of the contaminated feed is preferable; however, it is usually impossible to replace all of a major forage ingredient. While dilution is sometimes a viable practice to reduce exposure, reduced feeding of silage could result in such a slow feed out, those mycotoxin problems within the silage increase. Ammunition of grains can destroy some mycotoxin, but there is no practical method to detoxify affected forages already in storage. Increasing nutrients such as protein, energy and antioxidant nutrients may be advisable (Marquardt, 1996).

Adsorbent materials such as clays (betonies) added to contaminated diets fed to rats, poultry, swine and cattle have helped reduce the effects of mycotoxin (Shi, 2005).

2.15. The Genus Aspergillus

Aspergillus species are a group of fungi exhibiting huge ecological and metabolic diversity. These include notorious pathogens such as *Aspergillus flavus*, which produces aflatoxin, one of the most powerful, naturally occurring, compounds known to humans and animals. These species are common and widespread (Machida, 2010). One include the aflatoxigenic species *A .Flavus, A. parasiticus* and more recently *A.nominus* which cause serious problems worldwide in agricultural commodities, and the other includes the non-aflatoxigenic species *A.oryzae, A.sojae and A. tamri*, traditionally used for production of fermented food in Asia (Kumeda, 2001). Aspergillus flavus is the name now used to describe a species as well as a group of closely related species. A. flavus is second only to A. fumigates as the cause of human invasive aspergillosis.

The genus *Aspergillus* includes over 200 species. Around 20 species have also reported as causative agents of opportunistic infections in human. Along with these, *Aspergillusfumigatus* is the most commonly isolated species, followed by *Aspergillus flavus* and *Aspergillusniger. Aspergillusclavatus, Aspergillususstus, Aspergillusglaucus group, Aspergillusnidulans, Aspergillusoryzae, Aspergillusterreus, and Aspergillusversicolor* are among the other species less commonly isolated as opportunistic pathogens (Benard *etal,* 2013). Among the different types of aflatoxins identified, the major members are Aflatoxins B1, B2, G1 and G2 aflatoxins are fluorescence under the UV light. Aflatoxin B1 and B2 emit blue fluorescence whereas aflatoxin G1 and G2 emit green fluorescence. The quantity and relative proportion of four compounds in culture extracts varies depending on mold strain, medium composition and culture conditions. Normally, aflatoxin B1 is present in largest amounts whereas B2 and G2 are produced in small quantity (Wogan, 1966).

Fungi, such as *A. oryzae,* involved in the industrial production of soy sauce and *A. Niger* used for the production of citric acid and enzymes such as glucose oxidize and lysozyme. Such is the interest in *Aspergillus* that, to date, the sequences of fifteen different *Aspergillus* genomes have been determined. They are among the most successful groups of molds with important roles in natural ecosystems and the human economy. In addition producing numerous useful extracellular enzymes and organic acids, these molds also produce secondary metabolites of

importance in biotechnology. Some *Aspergillus species* function as plant and/or animal pathogens. *Aspergillosis* is the name given to all animal diseases caused by growth of any member of the genus on a living host. Immunosuppression is generally a prerequisite for systemic *Aspergillus* infections in humans. The incidence of systemic *Aspergillosis*, the most serious form, is on the rise and imposes an increasing medical burden upon hospitals and physicians (Machida and Gomi, 2010).

Table 1. The color of the colony in various aspergillus species

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

Source, Rahul *etal.,* 2014

2.16. Aflatoxin

Aflatoxins are group of chemically related mycotoxin a family of extremely toxic, mutagenic, and carcinogenic compounds produced by *Aspergillus flavus* and *A. parasiticus* (Vijayanandraj, 2014). The names of aflatoxins B1, B2, G1, and G2 are based on their florescence characteristics. Aflatoxin B1 and B2 show strong blue fluorescence under UV light, whereas aflatoxin G1 and G2 exhibit yellow fluorescence (Baltaci etal.,2010) Among their order of toxicity aflatoxins are classified as B1 > G1 > B2 > G2. Letters 'B' and 'G' refer to its blue and green fluorescence colors produced by these compounds under UV light. Numbers 1 and 2 indicate major and minor compounds, respectively (Melkamu, 2014).

All aflatoxins have been classified as carcinogenic compounds for humans, but aflatoxin B1 has been tagged as the most dangerous, highly toxic, immunosuppressive, mutagenic, and teratogenic compound and its effects have been identified as well. Also, mal-absorption syndrome and reduction in bone strength may occur due to aflatoxin consumption. Aflatoxins not only have adverse effects on human health but also cause serious economic losses when tons of foods have to be dropped or destroyed for being contaminated with aflatoxins.

Aspergillus flavus and A. parasitic us colonize a wide variety of food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit (Strosnider, 2006). Aflatoxins of corn, peanuts, tree nuts, cottonseed, and other commodities is a continuing worldwide problem. Toxigenic *A. flavus* isolates produce aflatoxin B1, and B2 and toxigenic *A.parasiticus* isolates produce aflatoxins B1, B2, G1, and G2 (Ephrem, 2015). *A.flavus*is the predominant fungus in aflatoxin-contaminated corn and cottonseed while *A.parasiticus* is probably more common in peanuts than on corn (Ephrem, 2015). *A.flavus* and *A.Parasiticus* are temperature-tolerant fungi. The major target organ for aflatoxin in animals is the liver. These compounds have been found to be carcinogenic and teratogenic in animals, as well as the cause of impairment of protein formation coagulation weight gain and immunogenesis.

Aflatoxin can lower resistance to diseases and interferes with vaccine-induced immunity in livestock (Diekman and Green, 1992). Suppression of immunity by aflatoxin B1 has been demonstrated in turkeys, chickens, pigs, mice, guinea pigs, and rabbits (Guoqing Qiana, 2014). Swine, turkeys, ducks, and rainbow trout are very susceptible to aflatoxin. Broiler chickens are resistant compared to these, but much more susceptible to aflatoxin than layer-type chickens. Pale, friable, fatty livers may be evident in acute aflatoxicosis in poultry.

Symptoms of acute aflatoxicosis in mammals include: inappetance, lethargy, ataxia, rough hair coat, and pale, enlarged fatty livers. Symptoms of chronic aflatoxin exposure include reduced feed efficiency and milk production and decreased appetite (Whitlow,2010).

Humans are exposed directly to aflatoxin and other mycotoxin through consumption of contaminated foods. Handling contaminated feed can result in exposure of mycotoxin through the skin and by inhalation (Schiefer H. , 1990). Indirect exposure of humans to

aflatoxin occurs through foods primarily milk, liver, and eggs derived from animals that consume contaminated feeds. Aflatoxin B1 is excreted into milk of lactating dairy cows primarily in the form of aflatoxin M1 with residues approximately equal to 1.7% of the dietary level with a range of 1% to 3% (Melkamu, 2014). Aflatoxin appears in the milk within hours of consumption and returns to baseline levels within two to three days after removal from the diet (Melkamu, 2014). Dairy cattle convert dietary aflatoxin rather efficiently to aflatoxin M1 that occurs in milk. Aflatoxin at 30 ppb in feed will result in milk residue levels of less than 1 ppb.

To ensure the food's safety, the maximum level of aflatoxin in food has been set by international organizations. For each kind of aflatoxin a minimum quantity of concentration is allowed, for instance, European Commission Regulation 2010/165/EC established limits of 8 and 15µg/kg for AFB1 and total AFs respectively

Due to its carcinogenicity, aflatoxin B1 (AFB1) is the only mycotoxin with established maximum permitted level (MPLs) in feedstuffs under the Directive 2003/100/EC (amending Directive 2002/32/EC). Feedstuffs selected for immature animals and dairy cattle always have an action level of 20 ppb. With the following exceptions, all feedstuffs have an action level of 20 ppb. For cottonseed meal used as a feed ingredient for beef cattle, swine and poultry, the action level is 300 ppb. For corn grain and peanut products, action levels are tiered by usage and targeted at interstate shipments. When designated for breeding beef cattle, swine and mature poultry, the action level is 100 ppb. When designated for finishing swine (100ppb or greater) the action level is 200 ppb. When designated for finishing beef cattle the action level is 300 ppb. Blending contaminated ingredients with uncontaminated ingredients with the purpose of reducing aflatoxin concentrations is not allowed.

Several methods have been developed to determine AFs in foods, for instance: immunoassays techniques (Lee, 2004). Thin layer chromatography (TLC) (Stroka, 2000). High-Performance liquid chromatography (HPLC) with fluorescence detection (Bacaloni, 2008). Not long ago, analytical methods based on clean-up with immune affinity column and HPLC with post column derivatization and fluorescence detection have gained much popularity.

2.17. Chemical structure and physical properties of aflatoxin.

Aflatoxins are crystalline substances, which can be soluble in moderately polar solvents such as chloroform, methanol and dimethyl sulfoxide, and dissolve in water to the extent of 10-20 mg/liter. Aflatoxins B1, B2, G1 and G2, classified as their fluorescence color under ultraviolet light. The chemical structures of some aflatoxins are shown in figure 1. Crystalline aflatoxins are extremely stable in the absence of light and particularly UV radiation, even at temperatures in excess of 100ºC. A solution prepared in chloroform or benzene is stable for years if kept cool and in the dark (Wogan, 1966).

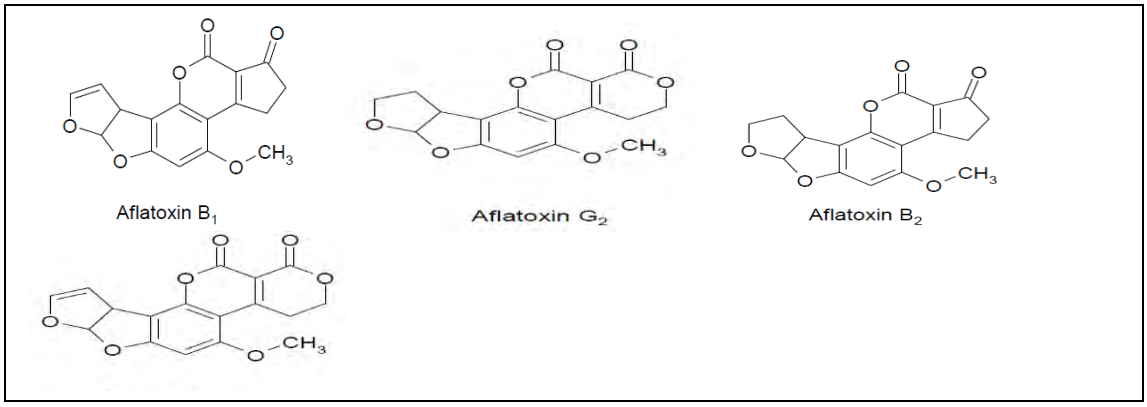

Figure 2.Chemical structure of aflatoxin

Source.*; Cole and Cox, 1981.*

Table 2. Summary of physical properties and spectral characteristics of aflatoxin.

Property aflatoxin	B1	B2	G1	G2	M1
Chemical Formula	C17H12O8	C17H14O8	C17H12O7	C17H14O7	C17H12O7
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

D1= Decomposition, Source: Cole and Cox (1981).

2.18. Health effects associated with aflatoxin

The species of *Aspergillus flavus* and *Aspergillus parasiticus* are of great concern due to production of aflatoxin and millions of people in Africa are chronically exposed to aflatoxin due to feeding on contaminated food. Aflatoxin problem is most serious in tropical and subtropical countries due to favorable climatic conditions for *Aspergillus flavus* and *Aspergillus parasiticus*. Human and animals are exposed to aflatoxin through diet (Chulze, 2010). Exposure to aflatoxin results in impairment of liver function and reduced feed intake, which might also explain the reduced milk production in dairy cattle exposed to aflatoxin. The impairment of hepatic functions might also account for the photosensitization associated with aflatoxin exposure (Miller, 1994).

The economic losses that result from contamination of crops and animal feeds with aflatoxin are also public health problems that result from ingestion of products contaminated with aflatoxin (Nigam, 2009).In many developed countries, there are stringent government regulations on aflatoxin than any other mycotoxin with very low threshold for tolerance (Doster, 2009). For animal feeds, European Commission has the maximum level for aflatoxin in animal feeds at 0.02mg/kg European Commission. (2003). A number of African countries still have to put in place regulatory mechanisms for aflatoxin. However, Kenya's limit for aflatoxin in products for human consumption is 20ppb (Kenya Bureau of Standards. (1988).

Acute toxicity of aflatoxin is caused by ingestion of large amount of aflatoxin from highly contaminated food. This can causes different health problems such as decreased liver function and could lead to blood clotting mechanism, jaundice, a decrease in serum proteins that are synthesized by the liver, edema, abdominal pain, vomiting and death of affected person. This was the case in Kenya in 2004 where they were 317 cases and 125 deaths reported due to consumption of maize contaminated with aflatoxin (Probst C *etal*., 2010)identified the S strain of Aspergillus flavus as the causal agent of the outbreak. Epidemiological, clinical and experimental studies have indicated that exposure to large doses of aflatoxin causes acute toxicity but exposure to small doses for prolonged periods of time is carcinogenic. The liver is adversely affected by aflatoxin that cause snecrosis of liver cells and death (Maxwell & Wong, 1991).

The chronic toxicity is due to long time exposure to low aflatoxin concentration. The main symptoms are decreased growth rate that leads to stunted growth in children (Moss, 2002).In Togo and Benin, children who are underweight as a result of aflatoxins are also at higher risk for infections and diarrhea (Gong, 2002). Exposure of children to aflatoxin can be through contaminated milk containing Aflatoxin M1 that is a metabolite of aflatoxin B1. In animals, aflatoxin cause lowered milk or egg production and immune suppression that is caused by reactivity of aflatoxin with T-cell and a decrease in vitamin K activities including decrease in phagocytic in macrophages (Robens, 1992). It has been reported that there is a high risk among people with Hepatitis B and Hepatitis C carriers to develop cancer due to consumption of food contaminated with aflatoxin (William, 2003). Aflatoxin have also been linked to immune suppression and higher prevalence of hepato-cellular cancer has been reported in Africa (Strosnider , 2006)

2.19. Global regulation for aflatoxin in foods and feed

Mycotoxin is toxic to humans and animals when present in food and feed above tolerance limits. Reduction in mycotoxin exposure of humans and animals has necessitated regulatory interventions by some national governments and international agencies.

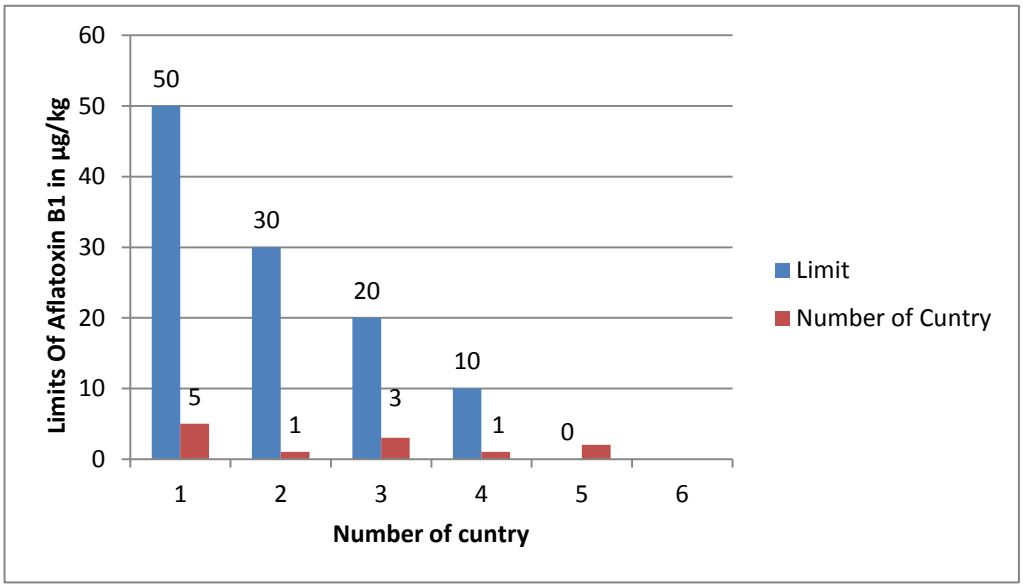

Figure 3.Worldwide limit worldwide limits for aflatoxin B1 in feed for dairy cattle.

However, different countries have different accepted levels for aflatoxin in foods and feeds. It is now realized by many developing countries that reducing aflatoxin levels in foods will present international trade advantages as well as offer long-term health benefit to the local population. The new European standards of aflatoxin level in groundnuts and cereals means that effective control must be found by developing countries for them to continue to export to the attractive European Union markets. For example, (Ephrem,2015),have proposed many solutions against aflatoxin production in food, and some of the strategies which may be applicable in the country by education and extension programs on radio and television discussion, food safety system, critical control (HACCP) system to produce food free of hazards. Strict food safety regulations implemented in most countries demand aflatoxin contamination less than 20 ppb. Despite availability of wide variety of diagnostic tools for estimation of aflatoxin, their utilization in most developing countries is limited due to high cost, difficulties with importation and lack of appropriate laboratory facilities and human skills.

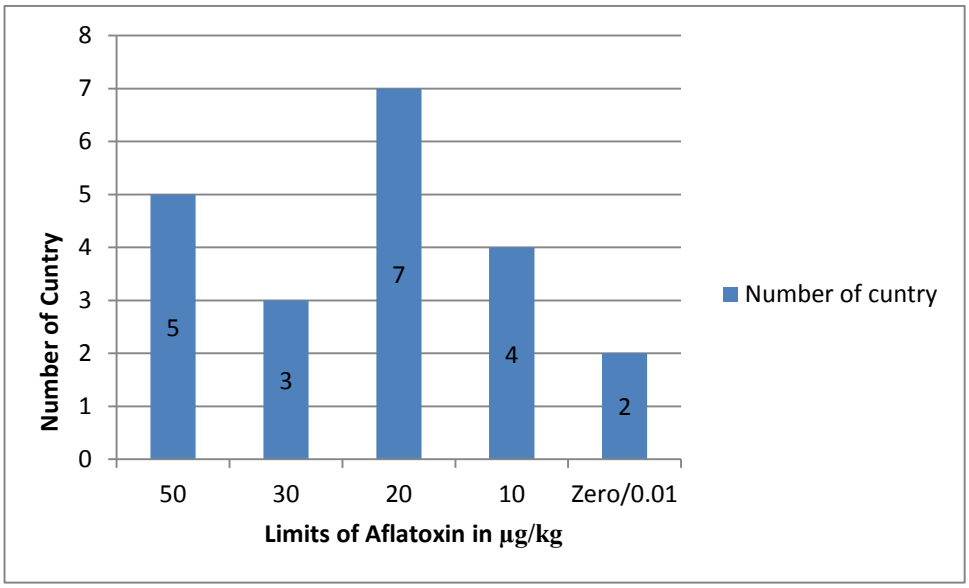

Figure 4.Worldwide limits for total aflatoxin in feed for dairy cattle.

Some countries have set permitted levels of aflatoxin in food and feed in order to control and reduce detrimental effects of these toxins. These levels are variable and depend on economic and developing status of the countries (Galvano, 1996). In US, Food and Drug Administration (FDA) has permitted a total amount of 20 ng/g in livestock feed and 0 .5 g/kg or 50 ng/l in milk (Ellis JA, 1995). In European countries, permitted levels of aflatoxin

M1 in milk, milk products and baby food are 0.005 mg/kg (Creppy, 2002), also, different countries have set different regulations for permitted levels of aflatoxin in livestock feed. For instance, European Union (EU) has set permitted levels of aflatoxin from 0.05 to 0.5 g/kg. Factors such as weather conditions are also effective in determining permitted levels of aflatoxin. However, according to US regulations the level of aflatoxin M1 in milk should not be higher than 500 ng/kg (Stoloff, 1991). There are thus differences in maximum permissible limit of aflatoxinM1 in various countries.

While food production, processing and marketing systems of developed economies have been largely successful in delivering mycotoxin safe products, regulations have been ineffective in most less-developed countries due to socio-economic, technical, institutional and policy factors. Agricultural products of the best quality are exported from less developed countries to addition export earnings, leaving poorer quality foods to be consumed locally, compromising the health of local populations.

Table 3. FDA action levels for aflatoxin in human food and animal feed ingredients.

FDA Action Levels for Aflatoxin in Human Food, Animal Feed and Animal Feed Ingredients	**FDA Action Levels for Aflatoxin in Human Food, Animal Feed and Animal Feed Ingredients**	**FDA Action Levels for Aflatoxin in Human Food, Animal Feed and Animal Feed Ingredients**
Intended Use	Intended Use	Intended Use
Human consumption	Foods, peanuts and peanut products, brazil and pistachio nuts	20 p.p.b.
Immature animals	Corn, peanut products, and other animal feeds and ingredients, excluding cottonseed meal	20 p.p.b.
Dairy animals, animals not listed above, or unknown use	Corn, peanut products, cottonseed, and other animal feeds and ingredients	20 p.p.b.
Breeding cattle, breeding swine and mature poultry	Corn and peanut products	100 p.p.b.
Finishing swine 100 pounds or greater in weight	Corn and peanut products	200 p.p.b.
Finishing (i.e.,feedlot) beef cattle	Corn and peanut products	300 p.p.b.

2.20. Overview of research on aflatoxin in Ethiopia review

Many studies conducted in Ethiopia shows that, the status of aflatoxin in Ethiopia widespread in the country, due to predisposing pre and post-harvest factors frequent end season drought (soil water stress), lack of resistant varieties, harvesting methods, storage facility and conditions (sanitary level, pest, moisture level), low or limited knowledge of aflatoxin by value chain actors, lack of regulation framework and monitoring facilities.

According to the study conducted by Alemayehu Chala in (2014), all the tested sorghum and finger millet samples were found to be contaminated by Fusarium and Aspergillus species. The result of the study was revealed that the average aflatoxins B1 and G1 concentrations in sorghum were higher than European standards.

Another study conducted by Amare Ayalew in 2010, showed that Aflatoxins were detected in 88% of the samples of Mize at 27µg kg-1 in one sample and less than 5 µg kg-1 in others. Fumonisin occurred in two samples from Diredawa at 700 and 2400 µg kg-1, and at 300µg kg-1 in one sample each from Adama and Ambo. Five samples contained DON at 50 – 700 µg kg-1. NIV was detected at 50, 130 and 210 µg kg-1. Further monitoring of mycotoxins in maize from different regions of the country was justified in order to conclusively determine the actual risks from mycotoxins and possibly low mycotoxin risk maize production areas.

Research thesis conducted by Eshetu Legesse Aschalew (2010), in Ethiopia From the total of 52 peanut (Arachishypogaea) samples analyzed, 38 (73.06 %) of the samples were positive for aflatoxin. The average levels of aflatoxins detected in the seed samples were between 0.57(from Babile new harvest sample) to 447.02 ppb (from Babile three month stored in pp bag). The higher level of toxicity is more than twenty times greater than the acceptable dosage (20 ppb: US Standards) in peanuts of three month stored after wet shelling. This research pointed out that the storage and shelling practice of farmers have effects in aflatoxin contamination in peanut despite enormous efforts to control this mycotoxin.

The study conducted by Dawit Gizachew in (2015) to detect and quantify the amount of AFM1 in raw cow's milk and AFB1 in dairy feed samples in the Greater Addis Ababa milk

shed using a value chain approach. The result of the study was showed that, all the feed samples were contaminated with AFB1 ranging between seven and 419 mg/kg. Overall, out of a total of156 feed samples collected, only 16 (10.2%) contained AFB1 at a level less than or equal to 10 mg/kg. the study was suggested that risk mitigation should focus on noug cake to effectively reduce aflatoxin contamination in per-urban dairy value chains in Ethiopia.

The study conducted by (Chala, ,2014) with the objectives to (i) identify Aspergillus species associated with groundnuts, (ii) determine the frequency of seed contamination, and (iii) survey agro-ecological conditions related to groundnut contamination by Aspergillus spp. About 270 groundnut samples were collected from farmers' storage, fields and local markets of three districts that is, Babile, Darolabu and Gursum of Eastern Ethiopia for mycological analysis in the year 2010. Results of the mycological analysis suggested heavy infestation of groundnut samples by various molds including Aspergillus niger, Aspergillus flavus, Aspergillus ochraceus, Aspergillus parasiticus and Pencillium species. At the district level, the incidence of infected groundnut kernels ranged from 50 to 80%. Within the district kernel infection varied between 36.3 and 100%. The common Aspergillus symptoms (yellowing or chlorotic leaves, wilting, drying and brown or black mass covered by yellow or greenish spores) were also observed in groundnut fields. The results were consistent with our earlier report of heavy aflatoxin contamination of groundnut from the same places, suggesting the urgent need to apply control measures against toxigenic fungi and associated mycotoxins.

The study conducted by Alemayehu and Tesfaye (Minota, 2016) on aflatoxins contamination of maize Results of fungal mycoflora evidenced the massive presence of Aspergillus species (75 %) followed by Fusarium (11 %), Penicillium (8 %) and Trichoderma (6 %) as characterized by biochemical and sporulation properties. Use of internationally developed biosensor for detection of fungal toxin in this work is the first approach that was utilized in the developing country like Ethiopia. In the end, we conclude that fungal contaminant and there metabolites are potential threat to the agricultural industry and require urgent intervention.

The occurrence of mycotoxins in barley, sorghum, teff (Eragrostistef) and wheat from Ethiopia has been studied by Amare Ayalew (Amare Ayalew, 2006) Samples were analyzed

for aflatoxin B1,ochratoxin A, deoxynivalenol (DON),nivalenol (NIV) and zearalenone (ZEN) using high performance liquid chromatography (HPLC) and for fumonisins (FUM) using enzyme linked immune sorbent assay (ELISA). AFB1 and OTA were detected in samples of all the four crops. Aflatoxin B1 was detected in 8.8% of the 352 samples analyzed at concentrations ranging from trace to 26µg/kg) OTA occurred in 24.3% of 321 samples at a mean concentration of 54.1µg/kg)1 and a maximum of 2106 lg kg) DON occurred in barley, sorghum and wheat at 40–2340 lg kg) with an overall incidence of 48.8% among the 84 mainly 'suspect' samples analyzed; NIV was co-analyzed with DON and was detected at 40 µg/kg) in a wheat sample and at 50, 380, and 490 µg/kg) in three sorghum samples. FUM and ZEN occurred only in sorghum samples with low frequencies at concentrations reaching 2117 and 32µg/kg), respectively. The analytical results indicate higher mycotoxin contamination in sorghum, which could be related to the widespread storage of sorghum grain in underground pits leading to elevated seed moisture contents.

A mycological survey carried out by (Dereje Assefa, 2012) for the first time, on freshly harvested groundnut kernels from the Northern Ethiopia, in three major groundnut growing woreda or districts of the Tigray Region in the 2009 harvest season, to detect the occurrence, severity of infection and distribution of Aspergillus species and to quantify aflatoxin contamination level. A total of 168 groundnut kernel samples, collected from farmers and research center fields were analyzed for prevalence of the Aspergillus fungi; and 141 smashed and grinded groundnut samples were analyzed for aflatoxin contamination. All samples were found 100 percent positive for Aspergillus species; however, there was significant ($P<0.05$) variation in infection severity among locations. Species of the genus Aspergillus flavus and A. niger were the two most prevalent component of the groundnut mycoflor in the region. Across the surveyed areas on average, 41.5% (range: 6.7 to 96.7%) and 12.3% (range: 0 to 90%) of the groundnut kernels were found to be infected by A. flavus and A. niger, respectively. Despite variations in contamination level among location, Aflatoxin B1 type was detected in all the samples. The detected aflatoxin concentrations were ranging from 0.1 to 397.8 ppb (mean: 28.7 and median 5.2 ppb). The highest level of Aflatoxin was detected in groundnut samples from the area (55.3 ppb). By All standards, according to the EU and FAO food safety guidelines for direct human consumption, before

processing and EU import limits, the qualities of the analyzed groundnut samples were very low, and significant amount of the samples were unsafe for human consumption as well as unfit for international market. The prevalence of the toxigenic fungi and associated extent of groundnut contamination in the region calls for urgent interventions of management practices to reduce the impact and awareness creation in the public.

A review conducted by Melkamu and Birhan in (2014) to evaluate mycotoxin and or aflatoxins in milk and milk products reviewed that, several research workers reported that there is a linear relationship between the amount of AFM1 in milk and AFB1 in feed which is consumed by dairy cattle and the conversion rate of AFB1 to AFM1 ranges between 0.5 and 6%. These metabolites are not destroyed during the pasteurization and heating process. Many countries standards limits of Aflatoxins M1 ranged between 0 to 0.5 ppb, in milk and dairy products.

A reviews Aflatoxin Contamination in Groundnut (Arachishypogaea L.) in Ethiopia and Its Management was conducted by Ephrem Guchi in (2015). Although groundnut has a huge potential as a cash crop to improve livelihoods of farmers and traders in various parts of Ethiopia, its market is declining and export of the crop has come to a standstill. This is due to aflatoxin contamination of the crop and the difficulty of meeting tolerance limits by importers and food processors, leading to rejection of the crop and reduction in market demand. Aflatoxin contamination is both a pre-harvest and postharvest problem. Therefore, management of aflatoxin contamination of groundnut in Ethiopia is very important using cultural practice such as habitat management, soil amendments and pre- and post-harvest managements, using physical control methods, using biological control methods, using resistance groundnut varieties and using chemical control methods.

2.21. Analytical methods for aflatoxin determination in food and feed.

Many analytical methods have been developed and are available for estimation of aflatoxin in agricultural commodities. These include: thin-layer chromatography plates (TLC), high performance liquid chromatography (HPLC), Liquid Chromatography (LC), enzyme-linked immune sorbent assays (ELISA), spectro-photometricaly, or by other techniques chromatography.

2.21.1. Enzyme-Linked Immunosorbent Assays (ELISAs)

Any type of assay involving Ab-Ag reaction, where one of the reactants is conjugated with an enzyme, is considered as an ELISA. Amplification and visualization of Ab-Ag interaction area were achieved by this enzyme conjugation. ELISA is the most used immunoassays used in food aflatoxin detection.

Antibodies or antigens are immobilized on a solid-phase matrix by linking them, either through adsorption or covalently. Reactants are usually adsorbed on to the wells of 96- or384- micro liter plate of polystyrene, where a strong hydrophobic binding and slow dissociation rate characterize adsorption. After this coating process, the residual protein binding capacity of solid matrix is blocked by exposing it to an excess of unrelated protein (e.g. gelatin or bovine serum albumin "BSA"). The next step is the addition of a test solution, which may be serum with an unknown concentration of antibodies against the immobilized antigen. After incubation and washing, binding of specific antibodies is visualized by the addition of anti immunoglobulin-enzyme conjugate followed by a substrate, generating a colored product when hydrolyzed. This change of color is proportional to the amount of antibodies bounded and may be recorded visually or spectro photo metrically. In case of an antigen measurement, the process is the same but may be done by using competitive- or sandwich-type assays. When using microarray format, ELISA may detect other toxins, such as aflatoxins in a sample (Lamberti, 2009).

2.21.2. Liquid Chromatography (LC).

The principle of liquid chromatography is the separation process which is based on the distribution between two phases. The sample is propelled by a liquid which percolates a solid stationary phase. Thus a variety of stationary phases can be used in liquid chromatographic systems. The liquid chromatographic process and the separation of the sample may be achieved, both, in low and high-pressure systems. The correct selection of the separation mode stationary phase and mobile phase may be straight (normal) phase, reversed phase and size-exclusion (SEC) or ion exchange (IEC) liquid chromatography respectively.

2.21.3. Thin-Layer Chromatography (TLC).

Thin-layer chromatography is a very commonly used technique in syntactic chemistry. This technique identifies compounds by determining the purity and progress of a reaction. Such reaction is fast and only requires a small quantity of the compounds. In TLC the mobile phase is liquid and the stationary process is a solid adsorbent. Several factors determine the efficiency of a chromatographic separation. The adsorbent should show a maximum of selectivity toward the substances that are being separated so that the differences in rate of elution will be large. For the separation of any mixture, some adsorbents may be too strongly adsorbing or too weakly adsorbing.

With the improvement of extraction and isolation method as well as the application of new reagents, the TLC detection becomes a simple and widely used analysis method (Akiyama, 2001).

2.21.4. High performance liquid chromatography (HPLC).

Recently HPLC is the most common used chromatographic technique for a detection of a wide variety of mycotoxin, especially for aflatoxin (De Rijk, 2011). The analysis sample cleanup can be performed by liquid-liquid partitioning, solid phase extraction (SPE), column chromatography, Immunoaffinity clean-up (IAC) columns, and multifunctional clean columns (Rahmani, 2009). Recently the IAC columns have become very popular because of its high selectivity. The IAC columns can be used for sample preparation before HPLC analysis either in off-line or in-line mode (Cichna, 2001). A chromatographic process can be defined as separation technique, which involves mass-transfer between stationary and mobile phase. HPLC utilize liquid mobile phase to separate the components of a mixture.

3. Materials and method

3.1. Description of the study area

Noug cake, Wheat bran and Atela samples were collected from Sululta, Debrezeyt and Debrbrhan towns. In these towns the noug cake, wheat bran and Atela are the common concentrate feed for dairy cow. Sululta is located23 km away from Addis Ababa in Oromiya Regional State Surrounding Finfine, Oromiya Special Zone and located at: - 09º 17' 84'' N & 38º 75' 79'' E DebreZeyt is located in Oromia National Regional State, East Shewa Zone at a distance of 47 Km from Addis Ababa. It isolated at 8º 43'- 8º 45' N & 38º 56'E. Debrebrhan is located in the Semen Shewa Zone of the Amhara Region, it is 130 kilometers away from Addis Ababa located at 9° 41′N 39° 32′E.

3.2. Sample collection and sampling

Animal feed samples were collected and information was gathered from the study area by using semi structured questioner (annex- 1) for feed producers, traders and dairy farmers to capture their knowledge, attitude and practice (KAP) on aflatoxin contamination. The questioner was focused on their awareness on mold growth and formation of aflatoxin on animal feed.

In this study a total of 30 samples of dairy feed (12 wheat bran, 12 noug cake and 6 *Atela*) samples were randomly collected from (Debrezeyt, Debrebrhan and Sululta). In this case the main road was used as a reference for each study site as the road divides the study sites in to two parts. By using the road as a reference, each study sites was categorized into 4 villages and 4 samples were collected randomly from each site.

Since molds and aflatoxin occur in an extremely heterogeneous fashion in feed and food commodities, stratified random sampling was used to make a composite sample consisting of subsamples from every part of a store, sack, or unit of feeds. During sampling the feeds ware groped into 4 parts depending on the layers and height of the bags. Then choose a simple random sample from each group and 100g of sample was weighed and zipped with plastic bag.

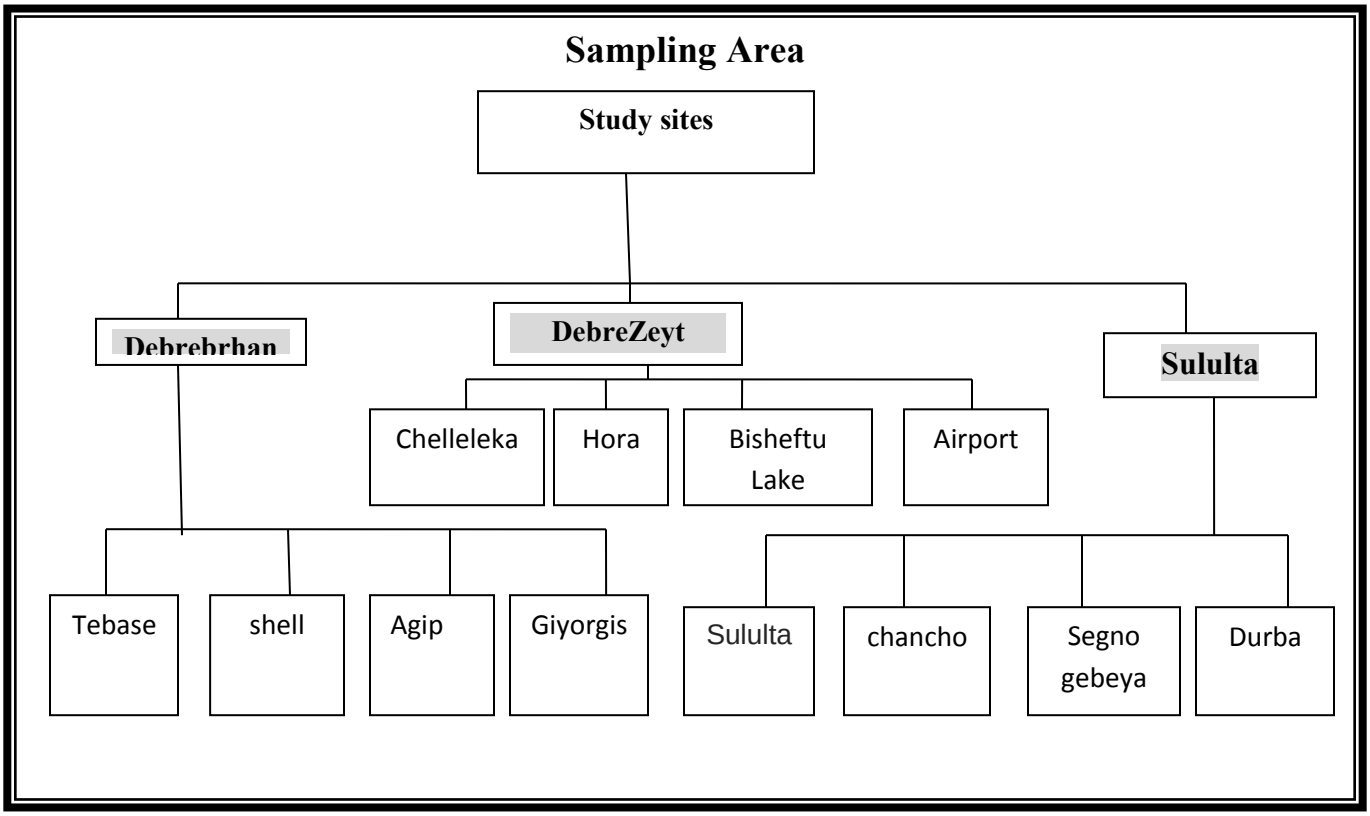

Figure 5. Flow chart for sampling area

3.3. 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 105ºC 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 105ºC for 3 hours and after cooling to room temperature in desiccators it was again weighed (W3). The moisture content was determined by using Eq.(2).

$$\textbf{Moisture content in } \% = \frac{\mathbf{W2 - W3}}{\mathbf{W2 - W1}} * \mathbf{100} \ldots\ldots\ldots\ldots\ldots\ldots (\mathbf{2})$$

3.4. Chemicals and reagents:

Acetonitrile and methanol were HPLC grade, distilled water and phosphate buffered saline (PBS:NaCl 8g l-1, KCl 0.2 g l-1, NaHPO 1.15 g l-1, KHPO 0.2 g l-1; and adjust pH to 7.4 using 0.1M HCl or 0.1M NaOH) were supplied by Ethiopian food medicine and healthcare administration and control authority (EFMHACA).

3.5 Immunoaffinity columns.

LC Tech Afla PERP Immuno-affinity column was used for sample clean-up for the Aflatoxin analysis with a maximum loading capacity of 100ng aflatoxin B1 and selectivity against aflatoxin B1, B2, G1 and G2 were purchased (imported) from, LC Tech GmbH Bahnweg 41 D-84405 Dorfen.

3.6. Standards.

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

3.7. Mobile phase.

The mobile phase was a water-methanol-acetonitrile (60:25:15, v/v/v) isocratic method was applied for better resolutions of the chromatographic peak. The mobile phases were filtered by applying vacuum in a filter unit and degassed.

3.8. Apparatus.

Cartridge, blender jar, Miller, Glass funnels, Timer, Immuno affinity column, 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, Wash bottle, Micropipettes, Micropipette tips, Millipore Filter, Electronic balance, syringes(5ml and 10ml), Paraffin, Sample label, Vials with screw cap. HPLC system setup contains auto sampler, injector, oven, column; Link, Degasser, fluorescence detector and desktop computer with chromatography software were used.

3.9. Instrument.

The model 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 C^{O} temperature and 1.2ml min-1 f low rate was used. The run time was 15 minutes, injection volume 20µl, diluents methanol and Needle wash (Water: Methanol 90:10 v/v).Aflatoxins were detected at 365 nm excitation and 440 nm emission wavelengths.

3.10. Analytical procedure.

3.10.1. Method adaptation.

To evaluate the performance of analytical instrument and validity of this method; the LOD, LOQ, precision, recovery, linearity and the working range were primarily identified.

3.10.2. Identification.

Identification of aflatoxin were determined by retention time of individual and total aflatoxin (AFG2, AFG1, AGB2 and AFB1) injecting at the same condition and its precision determined by percent relative standard deviation (%RSD)

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

LOD was determined by injecting (0.01, 0.2, 0.01, and 0.2) ppb of individual aflatoxin G2, G1, B2 and B1 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 aflatoxin G2, G1, B2 and B1 respectively to obtain the lowest amount of analyte which can be reproducibly quantities above the baseline noise, that gives S/N >10.

3.10.4. 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.

3.10.5. Linearity.

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

3.10.6. Recovery.

Recoveries were determined by spiking the sample with the known concentration of total aflatoxin (equimolar mixture of B1:B2:G1:G2) was applied in 10 mL of 10% v/v methanol in water and spiked in feed sample and injected in duplicate and finally purified through Immuno affinity column. The column washed with PBS (2 ml) and AFs was slowly eluted with methanol (2 ml) into a graduated glass vial. The extract was injected in HPLC system for recovery and accuracy taste.

3.10.7. Standard preparation.

Aflatoxin standards obtained from Sigma Aldrich (St. Louis, MO, USA). From the stock solution aflatoxin mixed standard which have a concentration of (2, 5, 10, 20, 30, 50, and 100) ppb were prepared for method validation. Standards solutions prepared in 10ml volumetric flasks using HPLC grade Methanol as a diluents. The prepared standards transferred to vials and stored at 4 C^0 and protected from light to avoid deterioration of the aflatoxin in the solution.

3.10.8. Sample preparation and cleanup.

3.10.9. Sample preparation.

Sample preparation was conducted using the method of AOAC Official method 950.02 for animal feed .To achieve the maximum particle size reduction and thoroughness of the mix the entire lots of samples were ground through hammer mill and passed through number 14 sieve split sample sequentially in sample splitter. The coarse portions were regrind of the remain and Weigh 50gm for wheat bran and traditional beer waste and 20gm for noug cake from each sample for aflatoxin estimation.

3.10.10. Extraction.

Prior to detection and quantification of the aflatoxin, they must be isolated from the rather complex and variable sample matrices. The purpose of this step was to dissolve the analyte quantitatively in the solvent, with as few little additional compounds as possible in order to avoid interferences. The choice of solvent is an important question in consideration with the polarity of the analyte and the type of sample (matrix). In this study 3type of feed samples (Wheat bran, Local beer waste and Noug cake) were extracted for aflatoxin analysis. Extraction solvent of 100mL methanol: water (8:2) for wheat bran and Local beer waste and 100mL methanol: water (8:2) plus 50 mL n-hexane for Noug cake was used.

Wheat bran and *Atela*.

50 g of sample and 5 g of salt (NaCl) was weighed and transferred into a blender jar and 100 mL of 80% methanol / 20% of DI water was added. The mix was blended at high speed blender jar for two minutes and the blended contents were filtered through what man No.4 filter paper into a 500 mill beaker. The 2mL of filtrate was diluted with 14-mLof phosphate buffer saline (PBS) and the diluted filtrate (equivalent to 1gm of sample) was passed through the column at the flow rate 2 mill per minutes. The column was washed by passing 20 mill of PBS (pH 7.2) and the toxin was eluted by 1 mill of 100% pure methanol at a flow rate of 1 drop per second and collected within an amber vial, and following pass 1mL of water through the column and collect the same vial for HPLC injection.

Nug cake/Niger seed cake/

20g of sample and 20 g of salt (NaCl) was weighed and transferred into a blender jar and 100 mL of 80% methanol/20% DI water plus 50 mL of n-hexane was added. The mix was blended at high-speed blender jar for five minutes and the blended contents were filtered through what man No.4 filter paper into a 500 mL beaker. From the filtrate, 14mL was taken and diluted with 86-mLof phosphate buffer saline (PBS). The diluted filtrate (equivalent to 1gm of sample) was passing through the column at the flow rate 2mL per min. The column was washed by passing 10mL of water and the toxin was eluted by 1mL of 100% pure methanol two times at a flow rate of 1mLdrop per second and collected within an amber vial for HPLC injection.

3.10.11. Immuno affinity Clean-up

Principle

The affinity column should contain antibodies raised against aflatoxin B1, B2, G1, and G2.The detection for aflatoxin is based on antibody-antigen reactions. Since different kinds of aflatoxin molecules can be considered as antigens, it is possible to detect them by developing antibodies against the compounds. After extraction from the sample matrix, the aflatoxin have to be further isolated from any co-extracted matrix constituents. This step consists in removal of the substances, which may interfere with the detection of the analyte. A number of alternative clean-up techniques for mycotoxin have been described in the literature. Here were used Immunoaffinity columns (AFLAPREP^{R}) which meet the requirements for a quick, simple and specific procedure as follows:

Enrichment Step IAC.

The column was preconditioned by PBS (10 mL) prior to sample application and kept under buffer during the experiment. The mixture was allowed to pass through column by gravity or at a flow rate of 5 ml/min. The column contains monoclonal antibodies to aflatoxin bound to a solid support. By passing the diluted extract through column any aflatoxin present in the sample were bound to the antibody within the column.

Washing

After the whole diluted filtrate of the samples were passed through the gel, the latter was washed with 10 ml of distilled water through the column and the remaining liquids in the gel was removed by applying pressure from the top of the column.

Elution

Sample reservoir on top of the (AFLAPREP^{R}) column was removed and an appropriate 2mL amber vial was placed below the affinity column. The bound toxins were eluted by using a total of 2ml of methanol as elution solvent. The elution process was performed in two steps to ensure complete release of analyte. First, a volume of 1ml elution solvent was applied and

after that, volume has passed through column, 5minutes was left before the second portion of 1ml of elution's solvent for effective release of toxin from the gel of the column. The remaining solvent solutions were eluted by application of slight overpressure on the top of the column.

Dilution

The final elutes were diluted with 1.5 mill of pure methanol before HPLC injection to adjust the concentration with the working range.

3.10.12. HPLC determination and calculations.

Injection

The elute aflatoxin (B1, B2, G1 and G2) methanol solution were determined at parts per billion (µg/kg) levels in the feed sample by Immuno affinity column cleanup and high performance liquid chromatography with fluorescence detection and calculated according to the following equation.

Calculation

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

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

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

Calculation of the calibration curve (function) obtained by linear regression: y = ax+ b

$$\mathbf{C_{contam}}\left(\frac{ng}{g}\right) = \frac{\mathbf{Csample} * \text{Solvent*Elution}}{\text{Wt*Aliquot}} * F = \left[\frac{ngxmlxml}{mlxgxml}\right]$$

Where :-

Wt (g) = sample material taken for analysis

Solvent (mill) = solvent taken for extraction;

Aliquot (mill) = aliquot taken for immune affinity clean-up;

Elution (mill) = final volume collected after elution from IAC;

F= Dilution factor

C_{smp}(ng/mL) = concentration of aflatoxin calculated from linear regression;

C_{contam}(ng/g) = contamination of sample material with aflatoxin;

Signals (units) = area of aflatoxin peak obtained from the measured solution.

3.10.13. Experimental Design.

Completely randomized experimental designs were followed to see the level of aflatoxins in dairy feed in around Addis Ababa Ethiopia.

3.10.14. Study design for knowledge attitude and practice (KAP).

The study design computed for the survey is a purposive sampling technique and Semi-structured questionnaires used to get more information. The questionnaire evaluation carried out for KAP assessments related to dairy feed handling and formation of fungi (shegata) on the feed. Based on the purposive sampling 50 participants were engaged on the survey. The response were classified in three parts; the first part was general information about the formation of fungi, the second part contained selection criteria for quality dairy feeds and the final part was KAP related to the transfer of mycotoxin in milk and other animal products .

3.10.15. Statistical data analysis.

For data analysis, Excel Microsoft office 2013 and IBM SPSS Statistics version20 software were used. In the SPSS method, one-way analysis of variance (ANOVA) was performed to

evaluate the levels of total aflatoxin mean comparison between the study sites. A P-value of less than 0.05 ($P < 0.05$) was considered to show statistical significance. Assumptions of ANOVA were checked. As there was one dependent variable one-way ANOVA used. The dependent variable level was aflatoxin concentration and the independent variable study site in the study area. As the independent variable, the study sites were used to test if environmental conditions affected for the possible mean aflatoxin level difference.

4. Results and Discussion

4.1. Aflatoxin survey result.

Socio-demographic Characteristics among feed traders, dairy farmers and feed producers ware observed .As demonstrated below in table 4 from the summary, the majority of feed producers, feed traders and dairy farmers participated in the study were above the age of 30 years, mostly married or widowed, and 20% of them were illiterate. Their economic class was categorized in the poor and medium economic class where the majority 40% has a family income of ≤ 2000 birr per month on the average. The majority of dairy farmers were married, and illiterate. Although, a better mix of educational status was distributed among feed producers and feed sealers grouped in this limited survey. The economic status can be regarded as low class for the majority feed sealers and medium among feed producers and dairy farmers.

A semi-structured questionnaire result shows KAP (Knowledge, attitude and practice) assessments related to mold growth and formation of aflatoxins among dairy farmers, feed producers and feed traders based on purposive sampling techniques. On this survey, a total of 50 participants (20 dairy farmers, 25 feed traders and 5 feed producers) were participated and the response grouped in knowledge, practice and attitude in table-4 below.

Table 4. Socio demographic characteristics of dairy farmers feed sealers and feed.

Statement	**Response**		
	Frequency		**Percentage**
Age	20-30	25	50
	30-50	20	40
	>50	5	10
Sex	Male	30	60
	Female	20	40
Marital status	Single	15	30
	Married	20	40
	Diverse	10	20
	Windowed	5	10
Educational	Illiterate	10	20

status	Elementary school	30	60
	College Diploma	10	20
Family income	<2000	20	40
	>2000	15	30
	>5000	15	30
Occupation	1. House Wife 2. Government Employee 3. Private Employee 4. Merchant 5. Daily Laborer 6. Other specify…	Private =50	100

As summarized in table 5 below a total of 50 participants (20 dairy farmers, 25 feed sealers and 5 feed processors) were interviewed for their knowledge, attitude and practice (KAP) regarding aflatoxin contamination in dairy feed. According to the result of (KAP) assessment, awareness of mold growth and formation of mycotoxin is very low among the dairy farmers and feed sealers. About five feed producers (10%) respondents reported that they were aware of mold growth and formation of mycotoxin the remaining 45 feed sealers and dairy farmers (90%) of respondents were not. On the other hand, 20% of respondents were answered that moisture and heat are the favorable conditions for mold growth (shagata) on animal feed.

About 35 dairy farmers and feed sealers (70%) respondents were reported that they not aware on cleaning, drying, ventilation, paste control and visual inspection prior and after storage of the feed. This practice is critical problems in feed handling due to the storage fungi can grow easily and the result is formation of aflatoxin.

According survey result, over 50% of dairy farmers and 40% feed sealers did not know that milk could be contaminated with aflatoxin among the dairy feed. Feed processors were more aware of aflatoxin than others.

About 35 dairy farmers and feed sealers (70%) of respondents were reported that the food that not use for human consumption due to mold growth (shagata), damage or expired can be used as animal feed.

With respect to location of the feed storage system, about 50% of the dairy farmers store their dairy feed in the field out of their home, 30% stored in the shade and 20% stored in house.

All urban dairy farmers in the study site were used concentrate feed every day to feed milking cow to increase milk volume. The most common ingredients in concentrate feeds in the study site were wheat bran, noug (*Guizotiaabyssinica*or Niger seed) cake and Atela.

The majority of dairy farmers about 60% are prefer nug cake, 30% wheat bran and 10% traditional beer waste/ *Atela*/ relatively lower levels due to its low availability.

Table 5. Summary of aflatoxin KAP among dairy farmers, feed traders and feed producers.

Statement	**Response**		
Knowledge		**Frequency**	**percentage**
Knowledge on mold growth and formation of mycotoxin.	Yes	5	10
	No	45	90
Knowledge on Conditions that favorable for mold growth on animal feed	Yes	20	40
	No	30	60
Do you know the effect of mold contaminated feed on dairy cows and dairy products?	Yes	10	20
	No	40	80
Knowledge on how aflatoxin transfers from dairy cows to their milk and milk product.	Yes	5	10
	No	45	90
Do you know the symptoms of Aflatoxin infection in dairy cow?	Yes	15	30
	No	35	70
Knowledge on Selection criteria for quality of dairy feed.	Color	25	50
	Odor	15	30
	Color, odor and moisture	10	20
Practice			
Cleaning and drying of the storage prior to feed storage.	Yes	15	30
	No	35	70
Post storage activities like visual inspection, moisture and pest control ventilation.	Yes	16	32
	No	15	30
Type of feed prefer for milking cow to increase milk volume	Nug cake	30	60
	Wheat bran	15	30
	Traditional beer waste	5	10
Where do you store your dairy feed?	Open field	25	50

	In shade	15	30
	In house	10	20
Attitude			
Do you prefer the lower quality with lower price to purchase dairy feed?	yes	25	50
	No	25	50
Is there a problem the food that not uses for human consumption, due to mold growth, damage or expire used as animal feed?	Yes	20	40
	No	30	60
The food that not use for human consumption, due to mold growth, damage or expire used as animal feed?	Yes	35	70
	No	15	30
Type of feed prefer for milking cow to increase milk volume	Nug cake	30	60
	Wheat bran	15	30
	Traditional beer waste	5	10
Where do you store your dairy feed?	Open field	25	50
	In shade	15	30
	In house	10	20

4.2. Result of the level of aflatoxin G2, G1, B2 and B1in dairy cattle feed.

To determine the percent of moisture content in the samples of dairy feed were conducted based on the method of (AOAC, 2000). Out of twelve nug cake samples analyzed in the present study the moisture content was range 9.25- 23.6%. The result was relatively higher than the moisture content analyzed by the same method in wheat bran with the range 4.2-15.7%.

A total of 30 dairy feed samples were collected for aflatoxin G2, G1, B2 and B1analysis. The samples were included the common dairy concentrate feed in the study site such as Niger seed (*Noug*) cake n=12, wheat bran n=12 and traditional beer waste n=6. All 30 dairy feed samples were analyzed and determined the level of aflatoxin G2, G1, B2 and B1by using HPLC with immune affinity column clean up.

According to the result of individual feed samples raveled that, all nug cake samples were contaminated with aflatoxin G2 with the range 0 to 83.173 ng/g ,G1: 128.235 to 981.122ng/g, B2: 0 to944.271ng/g and B1: 149 to 887ng/g and total aflatoxin with range of 450- 2320.53ng/g (Table-6).

The mean of aflatoxin G2, G1, B2, B1 and total aflatoxinsin nug cake were 34.58ng/g, 334.15ng/g,223.89ng/g,347.9ng/g and 691.08ng/g respectively.

Table 6. Level of aflatoxin G2, G1, B2, B1 and total aflatoxin in nug cake

Sample Code	B1(ng/g)	B2 (ng/g)	G2 (ng/g)	G1 (ng/g)	Total AFs(ng/g)	Moisture content (%)
NUG SUA12	152.445	105.364	24.35	168.235	450	14.15
NUG SU B12	178.475	226.7	24.735	128.235	558	10.41
NUG SU C12	887.646	944.271	83.173	405.44	2320.53	23.6
NUG SUD12	415.954	ND	N/D	152.401	568	12.9
NUG DBZ A12	335.298	36.635	16.396	981.122	1369	12.7
NUG DBZ B12	380.626	ND	ND	192.415	573	15
NUG DBZ C12	345.47	ND	ND	401.923	747	9.25
NUG DBZ D12	302.96	ND	ND	385.641	688.6	10.8
NUG DB A12	355.455	18.089	ND	427.904	801	10.8
NUG DB B12	367.149	ND	ND	249.256	616.4	10.9
NUG DB C12	304.301	60.319	ND	318.793	683.39	11.45
NUG DB D12	149.048	175.87	24.279	198.382	547.58	13.05
Average	347.9ng/g	223.89ng/g	34.58ng/g	334.15ng/g	691.08ng/g	12.917

Note: - ND= Not detected

LOD= Limit of detection

LOQ= limit of quantification

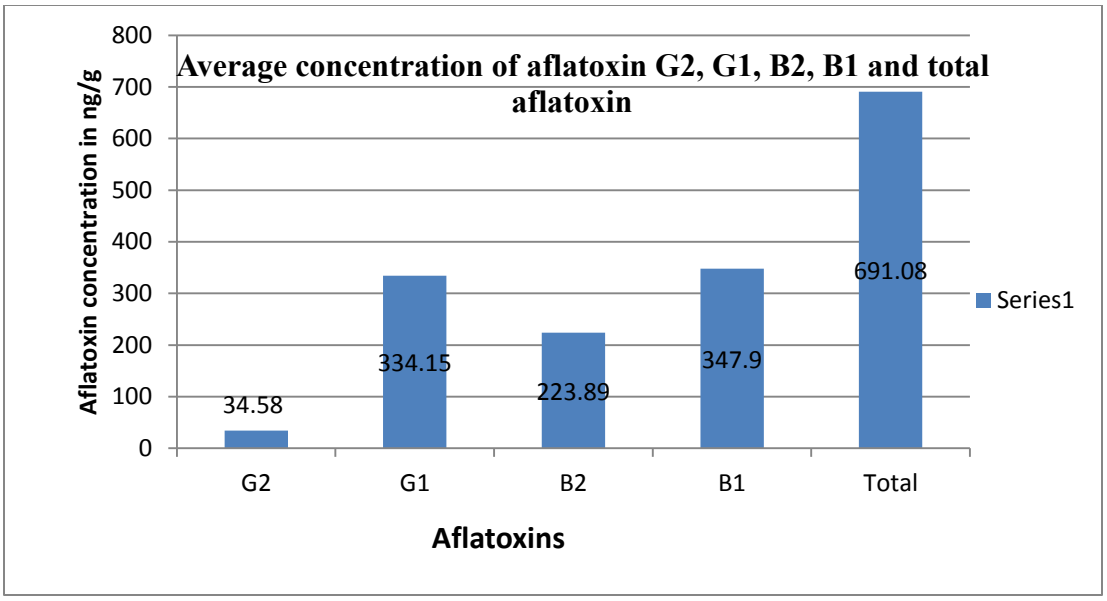

Figure 6. Average level of aflatoxin G2, G1, B2, B1 and total aflatoxin concentration in nug cake samples.

As summarized in table 7 level of aflatoxin B2 and B1 in nug cake samples in Debre brhan is lower as compare to the two sites Debre zeyt and Sululta. The reason lower level of aflatoxin found in Debrebrhan is the environmental conditions. Debre brhan is located astronomically; at 9°41' North latitude and 39°40' East longitude and characterized by cool temperate climate with average temperature of 14.5C^{o} (Solomon, 2016). Aflatoxins are found in many countries of the world, especially in tropical and subtropical regions where the warm and humid weather provides optimal conditions for the growth of moulds at a temperature for the growth of the moulds is 24 – 35C^{0} (Odoemelam, 2009)

Table 7. Average level of aflatoxins in nug cake based on each study site.

Sample Site	level of aflatoxin in ng/g				Level of total aflatoxin in ng/g	A.V moisture content
	G2	G1	B2	B1		
Sululta	33.06	213.58	319	408.63	974.27	15.265
Debrezeyt	16.396	490.3	36.64	302.96	846.3	11.93
Debrebrhan	24.279	298.53	63.57	293.988	680.37	11.55

According to the result of individual samples in wheat bran about 6(50%) of the samples were contaminated with G1: from 0 to 24.229ng/gmB2: from 0 to 22.435ng/g, B1: from 0 to 35.318 ng/g and total aflatoxin from 0 to 61.947ng/g (Table-8). The mean of aflatoxin G1, B2 , B1 and total aflatoxin level in wheat bran were 18.768ng/g, 18.52 ng/g, 21.55 ng/g and 59.0572 ng/g with respectively. Aflatoxin G2 in wheat bran samples was not observed in two samples and in 10(80%) of the samples below the limit of quantification.

Table 8. Level of Aflatoxin G2, G1, B2, B1 and total aflatoxin in wheat bran

Sample Code	B1 (ng/g)	B2 (ng/g)	G2 (ng/g)	G1 (ng/g)	Total AFs (ng/g)	Moisture content (%)
WB SU A12	15.281	22.435	<LOQ	24.229	61.947	15.7
WB SU B12	35.318	<LOQ	<LOQ	<LOQ	35.318	7.5
WB SU C12	14.046	17.798	<LOQ	17.036	48.881	7.2
WB SU D12	<LOD	<LOD	<LOQ	ND	<LOD	4.2
WB DBZ A12	<LOD	ND	ND	ND	ND	6.9
WB DBZ B12	<LOD	ND	ND	ND	ND	6.9
WB DBZ C12	<LOQ	15.34	<LOQ	18.061	33.4	9.8
WB DBZ D12	<LOD	<LOD	<LOQ	ND	<LOD	10.8
WB DB A12	<LOD	<LOD	<LOQ	ND	<LOD	7.2
WB DB B12	<LOD	<LOQ	<LOQ	15.745	15.745	6.6
WB DB C12	<LOD	ND	ND	ND	ND	7
WB DB D12	<LOQ	<LOQ	<LOQ	<LOQ	LOQ	6.2
Average	21.55 ng/g	18.52 ng/g	LOQ	18.768ng/g	59.0572ng/g	8

Note: -

ND= Not detected

LOD= Limit of detection and

LOQ= limit of quantification.

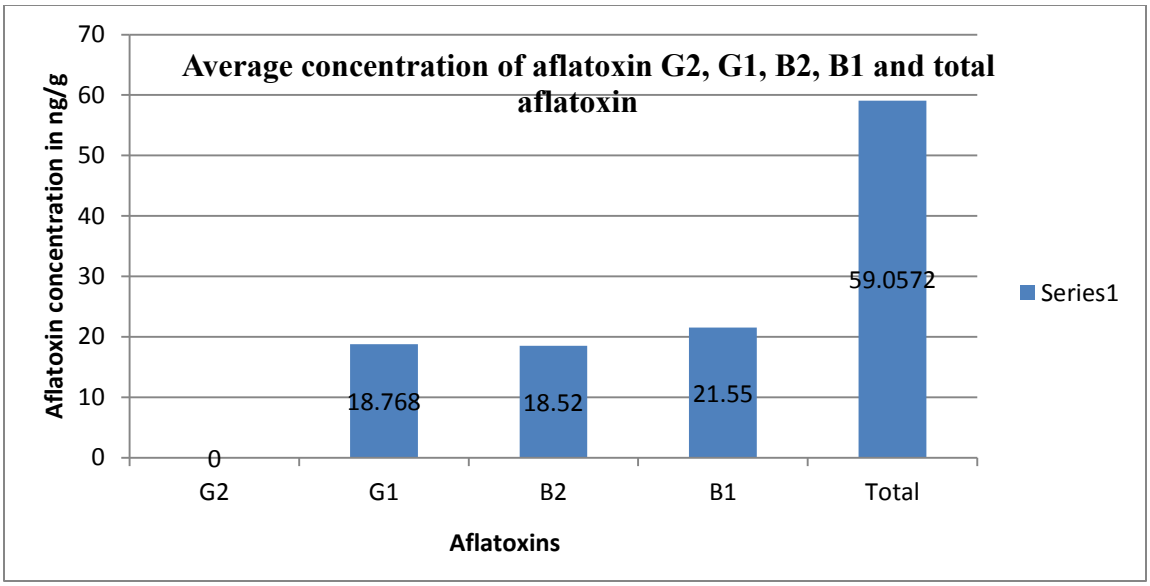

Figure 7. Average level of aflatoxin G2, G1, B2, B1 and total aflatoxin concentration in wheat bran samples.

According to the result shown in table 9, level of individual aflatoxins in each study sites were below the limit of FDA action level and FAO/WHO limit 20µg/kg in dairy cattle feed. The reason for lower aflatoxin in wheat bran is the average moisture content in wheat bran was below 10% and mostly moisture content 14-15% is suitable for mold growth. The milling process of wheat cleaning and drying of wheat grain may be contributed to lower concentration of aflatoxin in the bran.

Table 9. Average level of aflatoxins in wheat bran based on each study site.

Sample Site	Average level of aflatoxin in ng/g				Level of total aflatoxin in ng/g	A.V moisture content
	G2	G1	B2	B1		
Sululta	<LOQ	10.316	10.058	16.161	36.535	8.65
Debrezeyt	<LOQ	18.06	15.34	<LOD	33.4	8.6
Debrebrhan	<LOQ	15.75	<LOQ	<LOD	15.75	6.75

While from the total of all *Atela* samples as shows in (Table-**10)**, three samples were detected below the limit of quantification (<LOQ), and 2 samples were not detected and 1 sample was below the limit of detection (<LOD).

The reason for lower levels of aflatoxin in *Atela* feed samples the processing activities like pickling of damaged grain, roasting and fermentation may be contribute to decrease the level of aflatoxin concentration in *atela* samples

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Table 10. Level of Aflatoxin G2, G1, B2, B1 and total aflatoxin in Atela samples.

Sample	**B1**	**B2**	**G2**	**G1**	**Total AFs**
Code	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)
TBWSUA12	ND	ND	ND	ND	ND
TBWSUB12	ND	ND	ND	ND	ND
TBWDBZA12	<LOD	ND	<LOD	ND	<LOD
TBWDBZB12	<LOQ	ND	<LOD	<LOQ	<LOQ
TBWDBA12	<LOQ	ND	<LOD	<LOQ	<LOQ
TBWDBB12	<LOQ	ND	<LOD	<LOQ	<LOQ

Note: - ND= Not detected; LOD= Limit of detection ; LOQ= limit of quantification.

4.3. Discussion.

Analysis of individual feeds revealed that nug cake, was highly contaminated with aflatoxin G2, G1, B2, B1 and total aflatoxin and wheat bran had relatively low levels of aflatoxin contamination as compared to nug cake, while aflatoxin contamination in *Atela* too much lower than nug cake and wheat bran.

As compared to the recent study conducted in Ethiopia (Dawit, 2016) observed that level of aflatoxin B1 in nug cake samples were ranging from 290 to 397µg/kg, where as in the present investigation aflatoxin B1 in nug cake was range from 149 to 887µg/kg which means much higher than the previous findings

In another study conducted by a commercial enzyme-linked immune sorbent assay (ELISA) in Addis Ababa milk shade (Barbara Szonyi, 2015) the report similarly agree that the presence of nug cake in the feed increase aflatoxin concentration in both feed and milk.

The study conducted in Kenya (Kang, 2009) also similarly observed that a total of 830 animal feed samples were analyzed for aflatoxin B1 using competitive enzyme immunoassay and 86% of the feed samples were positive for aflatoxin B1, 67% of these exceeded the FAO/WHO level of 20µ gKg-1 and EU 5µ gKg-1

In comparison of the present investigation with different international standards the average level of aflatoxin G2, G1, B2, B1 and total aflatoxin in nug cake were 34.58ng/g, 334.15ng/g, 223.89ng/g, 347.9ng/g and 691.08ng/g respectively was exceeding the acceptable limit of US Food and Drug Administration (FDA) 20µg/Kg, food and agriculture organization (FAO)/World Health Organization (WHO) 20µg/Kg and European Union (EU) 5µg/Kg for dairy feed.

The reasons for high Aflatoxins content in noug cake samples could be insect damage, improper storage management and moisture content can increase the risk of mycotoxin formation and aflatoxins contamination. However, some study reported that Niger seed has fewer diseases than other oil seeds, molds such as *Aspergillus Niger*, *A.flavus, Penicillium*sp, *Alternariaalternata*, *Rhizoctoniasolani* and *R. bataticola*are the major cause of safety and quality problem of Niger seed (Getinet and Sharma 1996)

The traditional Ethiopian means of extracting Niger oil, which includes a “combination of warming, grinding and mixing with hot water”, followed by hand centrifugation in a clay container as a result to which encourages mold growth due to moisture and heat.

The high nutrient content and moisture content in nug cake may be support mold growth and formation of aflatoxin.

The result of this study in wheat bran samples were shown that from the total twelve samples of wheat bran 6 (50%) of the samples were contaminated with aflatoxin G1, B2 and B1.

In comparison the level of aflatoxin B1 in wheat bran with the recent study conducted in Addis Ababa and its surroundings by Dawit Gizachew, 2016, reported that the level of aflatoxin B1 in wheat bran was range from 9- to 31 µg/kg and in the present investigation found from 0 to 35.318 µg/kg which is much higher than the previous findings.

In comparison of the present investigation with different international standards the average level of aflatoxin G1, B2 ,B1 and total aflatoxin in wheat bran were 18.768ng/g, 18.52 ng/g, 21.55 ng/g and 59.0572 ng/g respectively, and only aflatoxin B1 and total aflatoxin were exceed the limit of FDA action level 20µg/kg, food and agriculture organization (FAO)/World Health Organization (WHO) 20µg/Kg. Levels of aflatoxin G1 and B2 in wheat bran were exceed the limit of EU 5µg/kg for dairy cattle feed.

According to the moisture content determination data below 10% was found in wheat bran, most of the samples had lower moisture content when we compare with moisture content of nug cake samples and it has a longer shelf life up to 6 months than the nug cake, which has three months of shelf life.

According to the result shown in (Table 10) from the total of 6 *Atela* samples analyzed for aflatoxin contamination, three samples were detected below the limit of quantification (<LOQ), and 2 samples were not detected and 1 sample was below the limit of detection (<LOD). While the finding of this study, conclude that aflatoxin concentration in *Atela* was not exceed the limit of FAO/WHO, FDA action level and EU standard for dairy cattle feed.

The reason for the lower level of aflatoxins concentration in *Atela* could be the processing activities in traditional beer preparation, like sorting of grain for the malt and *asharo* making, washing and roasting may be contribute to decrease the lower level of aflatoxin concentration in the sample. On the other hand, fermentation process also contributes to decrease level of aflatoxin. According to some study reported that lactic fermentation could decrease aflatoxin B1. Lactobacillus strains could remove more aflatoxin B1 than *Pediococcus* and *Leuconostoc* strains and the reduction of the initial amount of aflatoxin B1 ranged from 1.80 to 44.89% (Abdelah Zinedine, 2005).

Overall, the high levels of aflatoxin contamination of the feed should be of concern for the dairy sector, because aflatoxin can seriously reduce livestock productivity. In livestock, consumption of very high levels of aflatoxin causes acute toxicities and death, while chronic consumption of lower levels can cause liver damage, gastrointestinal dysfunction, and decrease in appetite, reproductive function, growth, average daily gain, body weight and production (Wayne,2007).The presence of aflatoxin M1 in milk and dairy products is a worldwide concern, once these products are frequently consumed and milk is the one of the most important foods for children (Oliveira, 2007). On the other hand, the economic losses due to chronic exposure of cattle to aflatoxin could be significant to the pre-urban dairy industry in Ethiopia.

The ANOVA results revealed that, there was no significant mean differences in aflatoxin contamination of nug cake and wheat bran among the three study sites (Sululta, Debrezeyt and Debrebrhan) at a significance Probability- value of (Sig) = 0.74 for nug cake and 0.072 for wheat bran. Taking alpha (α) level of significance 0.05. $P = 0.74 > 0.05$ for nug cake and $P = 0.072 > 0.05$ for wheat bran. In conclusion there was no significant mean difference in total aflatoxin among noug cake and wheat bran in the three study sites.

5. Conclusion and Recommendation

5.1. Conclusion.

The result of this study shows that, the levels of aflatoxin G2, G1, B2, B1 and total aflatoxin in noug cake were exceeded the limit of FDA action level and European Union in around Addis Ababa (Sululta, Debrezeyt and Debrebrhan). According to the result of this study, the level of aflatoxin B1and total aflatoxin in wheat bran samples was exceeded the limit of FDA action level 20µg/kg and all aflatoxins was exceeded to the limit of EU 5µg/kg for dairy cattle feed. According to the present study, aflatoxin contamination in *Atela* samples was not exceeded the limit set by FDA and EU for dairy cattle feed.

Consumption of feed contaminated by aflatoxin leads to different problems in reproductive, digestive and respiratory tracts of dairy cattle's and causing contaminated milk production. More over the consumption of infected milk by human incur to health and economic problems to the society.

Therefore, in order to prevent from the presence of aflatoxin M1 into food chain, aflatoxins should be controlled effectively during feed production, storage and distribution.

The result of knowledge, attitude and practice (KAP) assessment in this study revealed that, awareness of mold growth and formation of mycotoxin is very low among the dairy farmers and feed traders.

5.2. Recommendation

- Training program on pre and post harvest preventive strategies of aflatoxin contamination in dairy cattle feed is recommended to increase knowledge attitude and practice of feed producers, dairy farmers and feed traders.
- Regulatory body should be provide regulatory limit of aflatoxin in dairy cattle feeds and also conduct post marketing surveillance related to aflatoxin contamination of feed and milk.
- Due to aflatoxins are produced on livestock feed in appropriate moisture and temperature conditions for mold growth, proper drying, good storage conditions, regular visual inspection and cleaning are recommended to minimize contamination of the feed.
- National and regional government, academicians, research institutes should be conduct assessment on control methods of aflatoxin contamination in dairy feeds.
- Another study is needed to evaluate another type of mycotoxin in animal feed.
- Based on the result of these study dairy farmers should be recommended wheat bran and *Atela* feed for their milking cow rather than feeding nug cake due to its high level of aflatoxin contamination.

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7. Annex

Annexes-1. Questioner.

Questioner form

I am Mulugeta Fikere who is a postgraduate student of Addis Ababa University center for food science and nutrition, conducting a study on the level of aflatoxin in dairy cattle feeds and assess knowledge, attitude and practice of feed processors, dairy farmers and feed traders around Addis Ababa Ethiopia. The purpose of my visit to know the knowledge, Attitude and practice (KAP) on mould growth and formation of aflatoxin in animal feed. 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 related problems.

Name of the interviewer Mulugeta Fikere

Signature _____

Date of Interview _____

QUESTIONNAIRE

PART I SOCIO-DEMOGRAPHIC CHARACTERSICS

Question Number	Question	Response	Instruction
1	Age (Years)	1. 20-30 2. 30-50 3. >50	
2	Sex	1. Male 2. Female	
		1. Single 2. Married	

3	Marital Status	3. Divorced 4. Widowed	
4	Educational Status	1. Illiterate. 2. Elementary Education (1-6) grades 3. Secondary Education (6-12) grades 4. Higher Institution, 5. Other Specify……..	
5	Monthly Family Income	…….Birr	
6	Occupation	1. Feed trader 2. Dairy farmer 3. Animal feed processor 4. Other Specify……	

PART II

KAP Questioner for dairy farmers and feed traders

Question Number	Question	Response	Instruction
Knowledge (K)			
1	What is mould? Can you describe it?	1- Yes 2- NO 3- Don't know	
2	Do you have information about how mould can develop?	1- Yes 2- No 3- Don't know	
3	Do you have selection criteria for quality of animal feed?	1. Yes 2. No	**If yes go to Q 5**
4	What is your selection criteria?	1. Color 2. Odor 3. Color, odor and moisture 4. Other specify---------	
5	Which storage conditions are favorable for mould growth on animal feed?	1. Moist 2. Hot and moist 3. Dry and cold 4. Don't know 5. Other specify----------	
6	Do you get a problem when you are store animal feed for a long time?	1-Yes 2-No	If yes go to Q 9
7	what type of problem	1. Insects 2. Mould 3. Other specify----------	

Attitude (A)			
1	Do you think Foods not used for human consumption due to mould growth can apply for animal feed?	1- Yes 2- NO 3- Don't Know	
2	Type of feed you prefer for dairy cattle?	1. Noug cake 2. Wheat bran 3. Traditional beer waste 4. Composite of noug cake and wheat bran 5. Other specify----------	If, yes Q-3,4,5
3	Do you think animal feed can contaminate with aflatoxin?	1- Yes 2- No 3- Don't know 4- Other specify----------	
4	Do you think moldy feed can affect animal health?	1- Yes 2- No 3- Don't know 4- Other specify----------	
5	Do you prefer to consume traditional beverages because of cultural reasons?	1-Yes 2-No	
6	Do you think mould growth can create on animal feed?	1-Yes 2-No 3-Don't know	
Practice (P)			
1	Where do you get animal feed?	1-From industrial waste supplier 2- From feed processors 3 – From farmers 4 –other specify----------	
2	Do you have store your animal feed for a long time?	1. Yes 2. No	
3	Do you have the habit to clean the store before storing of animal feed?	3. Yes 4. No 5. Other specify-------	
4	Where do you store animal feed for a long time ?	1- Open space 2- In the shade 3- In the house 4- Other specify---------	
5	Do you dry animal feed before storage?	1. Yes 2. No	**If yes go to Q6**
6	How do you dry animal feed?	1. Using sun drying 2. Other drying technology 3. Other specify----------	

PART III

KAP QUESTIONER ON FEED PRODUCERS

Question Number	Question	Response	Instruction
Knowledge (K)			
1	From what source of raw materials produce commonly animal feed?	1. Wheat bran 2. Nug cake 3. Other specify----------	
2	Do you harvest your own cereals used animal feed production?	1. Yes 2. No 3. Other specify----------	
3	Where do you think dairy farmers store animal feed?	1- Open space 2- In home 3- Don't' know 4- Others Specify……..	
4	Do you exclude moldy raw materials for the production of animal feed?	1- Yes 2- No	
5	What factors you think animal feed to be moldy?	1- In the field 2- Storage Conditions 3- Transport 4- processing 5- Do not know	
Attitude (A)			
1	In your opinion what makes animal consume moldy feeds?	…….	
2	Do you recommend using expire and or damaged items of the food as animal feed?	1 – Yes 2 - No	
Practice (P)			
1	How do you store the finished animal feed?	……	
2	Do you have protection mechanism of mould growth on animal feed?	…….	
3	Do you have the habit of cleaning the store before storing animal feed?		

Annex-2. Validation of the chromatographic method

Identification

The retention time of individual and mixed aflatoxin as shown in (Annex-2) gives a good precision having a range between (0.3–1.08) percent RSD, which is acceptable according to FDA standard, which is less than 2%RSD. The elution order of individual aflatoxin was in the order of AFG2, AFG1, AGB2 and AFB1 with 10.818, 13.197, 15.109 and 19.082 retention times respectively. In addition to the retention time chromatographic result for Blank (diluents), individual (AFG2, AFG1, AGB2 and AFB1) and mixed aflatoxin shown in Figure 6 demonstrates the qualitative aspect of identification test is more defined and acceptable.

Table 1. Summary of retention time (RT) for individual and mixed aflatoxin standards in identification of aflatoxin.

Aflatoxin	Aflatoxin 100ppb Injection Retention time(Min)		N	Mean	Std.Devation	%RSD
	For single run	For mixed run				
AFG2	10.818	10.586	2	10.702	0.16	1.532
AFG1	13.197	13.277	2	13.237	0.0566	0.43
AFB2	15.109	15.354	2	15.23	0.17325	1.144
AFB1	19.082	19.487	2	19.2845	0.281	1.46
Valid N			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 parts per billion for aflatoxin G2, G1, B2 and B1, 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 as shown in annex-2.

The limits of quantification for individual aflatoxin (G2, G1, B2 and B1) were 0.05, 0.8, 0.05 and 0.8 parts per billion respectively. 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 2. Limit of Detection (LOD) and Limit of Quantification (LOQ).

Aflatoxin	LOD		LOQ	
	PPb	Signal to Noise Ratio (S/N>3)	PPb	Signal to Noise Ratio (S/N>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

Precision

As verified in (annex-2) 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), on the same day, under the same experimental conditions. It shows an acceptable %RSD which had a values of <5% and < 3.0% for peak area and retention time respectively. A precision criterion the instrument precision (repeatability) 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 3. Precision

No	Aflatoxin	N	%RSD for peak Area	%RSD for Retention time
1	AFG2	10	3.042349276	1.0378300083
2	AFG1	10	3.801215574	1.849923829
3	AFB2	10	4.255234401	1.070212234
4	AFB1	10	2.827238321	1.085623759

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. The International Conference on Harmonization (ICH) guidelines specified a minimum of five concentration levels, along with certain minimum specified ranges. Regression equation was found by plotting the peak area (y) versus the aflatoxin concentration (x) expressed in ppb as presented in annex-3, the acceptability of linearity data is usually determined by examining the correlation coefficient and y-intercept of the linear regression line for the peak area versus concentration plot. As shown in (annex-3) the demonstration coefficient (R^2) obtained for the regression line demonstrates the excellent relationship between peak area and concentration of aflatoxin and its coefficient of correlation (R^2) sited on 0.998. The regression coefficient (R^2) is$\geq$0.998 is generally considered as evidence of acceptable fit of the data to the regression line on the FDA standard.

The range was resulting from the 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 in annex-3, the data obtained during the linearity studies was used to assess the range of the assay method.

Table 4. Linearity check.

Aflatoxin	N(point)	Calibration Curve Equation	R^2
AFG2	7.0	Y=42517X+2308	0.9982
AFG1	7.0	Y=1361X+214.6	0.998
AFB2	7.0	Y=10594X+5031	0.9982
AFB1	7.0	Y=5235X+1316	0.998

Accuracy and recovery.

The accuracy of this analytical method was obtained by standard additions, which can also be used to determine recovery of spiked analyte. The recovery specification was conducted based on the company requirement R-Bipolar Rhone Ltd for the Immunoaffinity column.

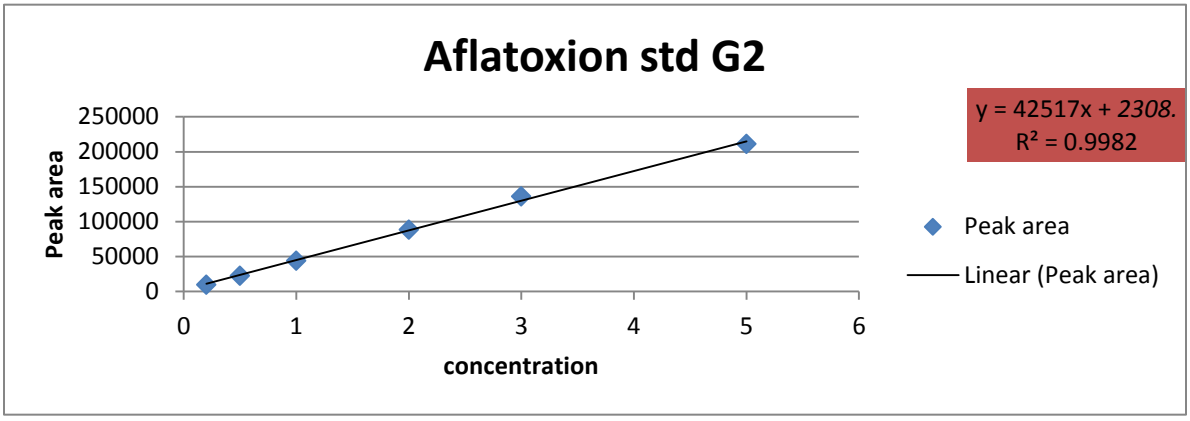
Greater than or equal to 85% and less than or equal to 110% of aflatoxin B1, B2, G1, and greater than or equal to 110% of aflatoxin G2 should be recovered with standard solutions. This was exceeds the specification required by AOAC method 999.07

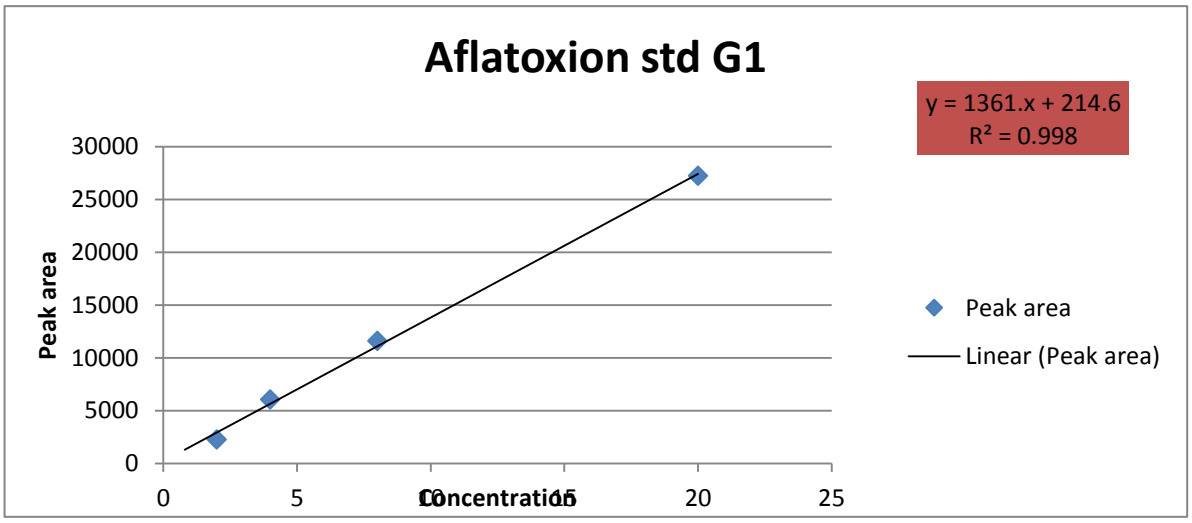
The recovery for individual aflatoxin (B1:B2:G1:G2) were conducted by adding two known concentration of aflatoxin standard to the spike feed sample and injected in duplicate to HPLC. Percent recoveries of response factor (area/concentration) were calculated and the result was AFG2=105%, G1=108%, B2=110% and B1=110%.The results of recovery and accuracy studies shown in the range between (105-110) % and it are evident that the method is accurate within the desired recovery range.

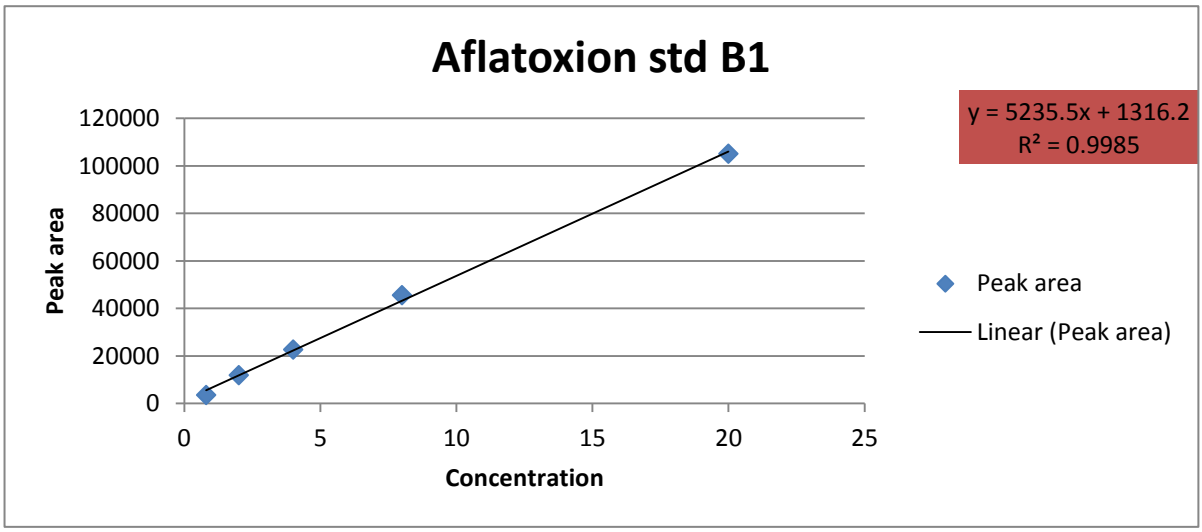
Table 5. Working Range

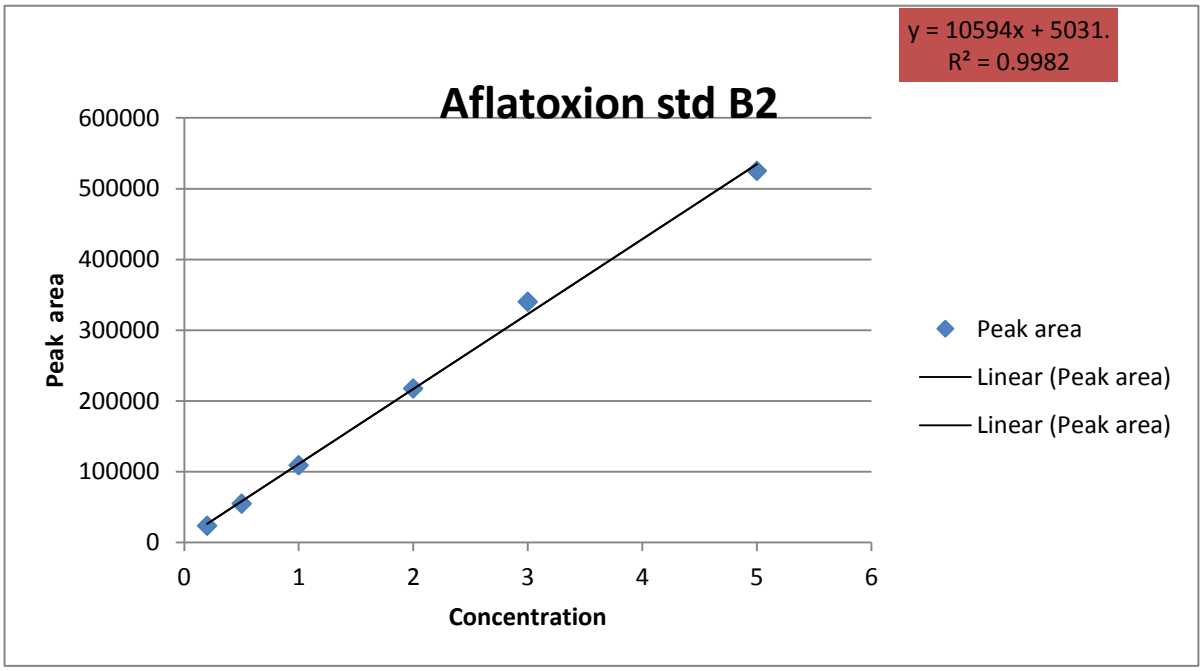
AflatoxinsumPPb	Aflatoxin (PPb)			
	AFG2	AFG1	AFB2	AFB1
2	0.2	0.8	0.2	0.8
5	0.5	2	0.5	2
10	1	4	1	4
20	2	8	2	8
30	3	12	3	12
50	5	20	5	20
100	10	40	10	40

Annex-3. Calibration curve.



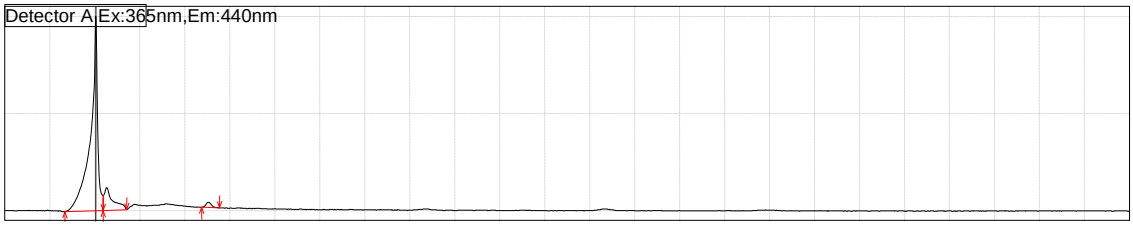


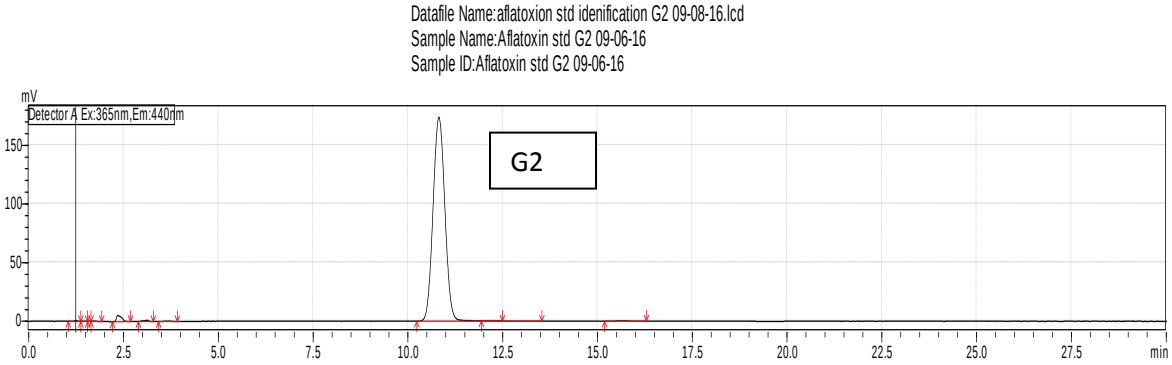

Aflatoxion std B1
y = 5235.5x + 1316.2
R² = 0.9985
Peak area
Concentration
120000
100000
80000
60000
40000
20000
0
0
5
10
15
20
25
Peak area
Linear (Peak area)

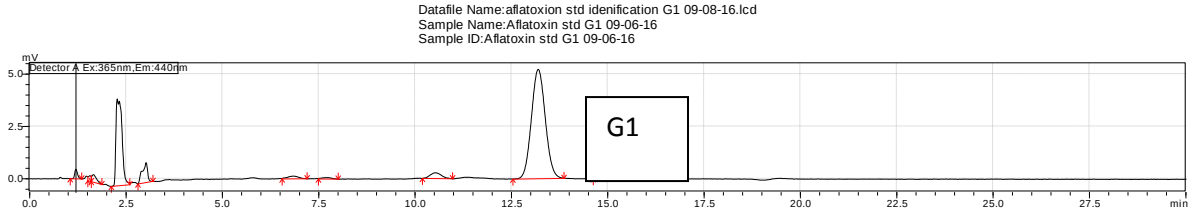

Aflatoxion std B2
y = 10594x + 5031.
R² = 0.9982
Peak area
Concentration
600000
500000
400000
300000
200000
100000
0
0
1
2
3
4
5
6
Peak area
Linear (Peak area)
Linear (Peak area)

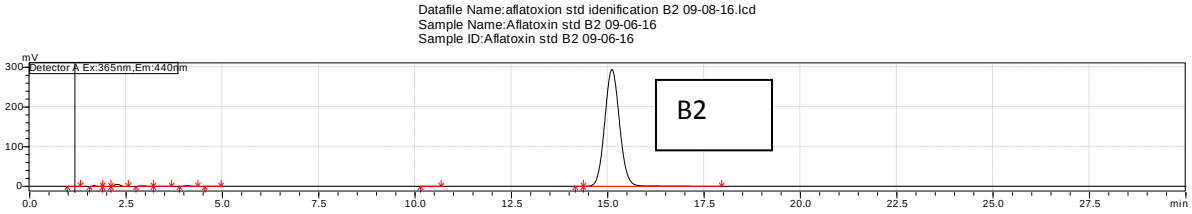
Annex-4. Chromatogram of blank, individual and mixed aflatoxin runs in HPLC

BlankRun









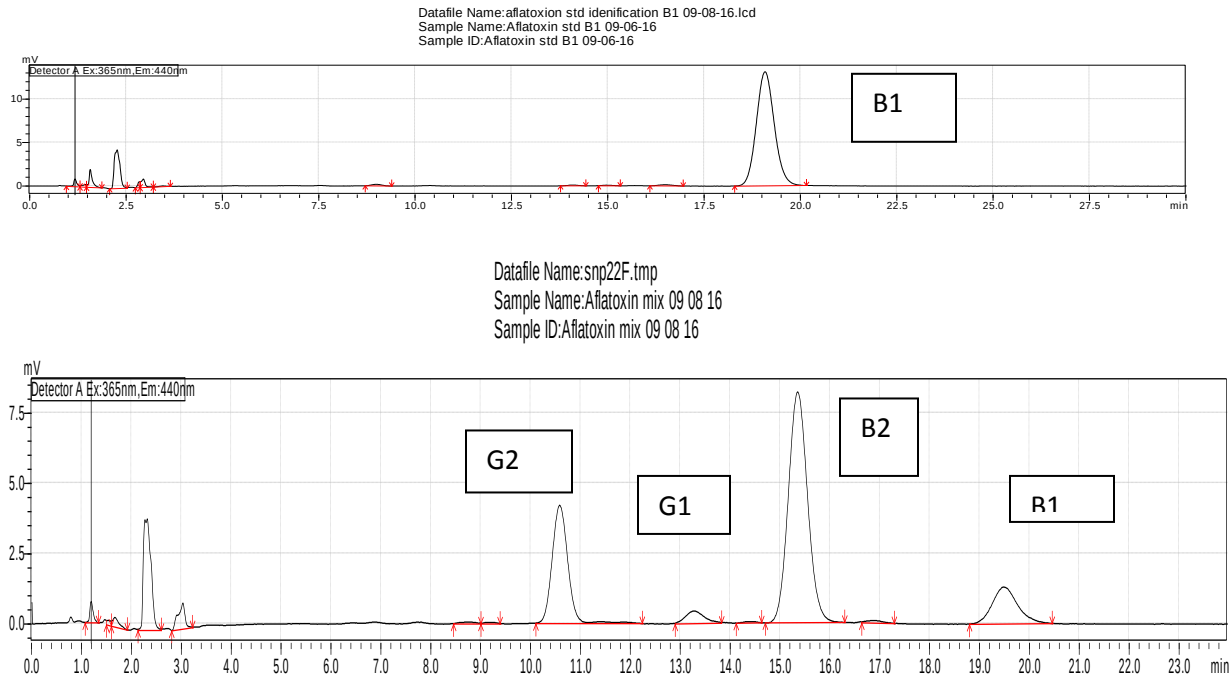


Figure. Chromatograms of individual and mixed aflatoxin standard solution for Identification Test

Annex-5. Photo of taken during sample collection and analysis.

Figure 1. Feed sample in a zipper bag assigned a code for each Type

Figure 2. A Photo of Sample preparation and clean up obtained during laboratory analysis.

Figure 3. Powder Dairy feed samples

Figure 4. Image of noug cake obtained from oil extraction from Niger seed.