ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINAND AGRICULTURE

QUANTIFICATION OF THE LEVEL OF AFLATOXIN CONTAMINATION IN DAIRYAND BEEF CATTLE COMPOUND FEEDS AND FEEDS INGREDIENTS IN BISHOFTU AND ADAMA DISTRICT, ETHIOPIA

BY

SISAY WOYESO NEBSO

JUNE, 2019

BISHOFTU, ETHIOPIA

QUANTIFICATION OF THE LEVEL OF AFLATOXIN CONTAMINATION IN DAIRYAND BEEF CATTLE COMPOUND FEEDS AND FEEDS INGREDIENTS IN BISHOFTU AND ADAMA DISTRICT, ETHIOPIA

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Animal Production

By

Sisay Woyeso Nebso

June, 2019

Bishoftu, Ethiopia

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Submitted by: Sisay Woyeso ____________ ____________

Name of Student Signature Date

Approved *for Submittal to a* Thesis Assessment Committee

1. Dr. Ashenafi Mengestu ____________ ____________

Major Advisor Signature Date

2. Dr. Fanos Tadesse ____________ ____________

Co- Advisor Signature Date

2. Mr. Yoseph Chernet ____________ ____________

Co- Advisor Signature Date

3. Prof. Berhan Tamre ____________ ____________

Department chairperson Signature Date

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Animal Production Studies

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by Sisay Woyeso titled **'Quantification of the Level of Aflatoxin Contamination in Dairy and Beef Cattle Compound Feeds and Feeds Ingredients in Bishoftu and Adama District, Ethiopia'** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Science in Animal Production.

________________________ ______________ ______________

Chairman (title and name) Signature Date

Dr. Gebeyew Goshu ____________ ____________

Internal Examiner (title and name) Signature Date

Dr. Etalem Tesfaye ____________ ____________

External Examiner (title and name) Signature Date

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Dr Ashenafi Mengestu ____________ ____________

Thesis advisor (title and name) Signature Date

Prof Hagos Ashenafi ________________ ______________

Assoc. Dean for Graduate Prog. Signature Date

DEDICATION

This thesis manuscript is dedicated to my wife Hawi Kebede and my sons Beniyam and Eyoram in appreciation for their unreserved help and love always.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my original work and that all source of material used for this thesis have been duly acknowledged. This thesis has been subtitled in partial fulfillment of the requirement for a postgraduate (MSc) degree at Addis Ababa Universiy College of Veterinary Medicine and Agriculture and is deposited at the University/Colleg e library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not subtitled to any other institute anywhere for the award of any academic degree, diploma or certificate.

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Name: Sisay Woyeso **Signature_________________**

College of Veterinary Medicine and Agriculture

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LIST OF ABBREVIATIONS

AFs	Aflatoxins
ANOVA	One Way Analysis of Variance
AOAC	AOAC-Association of Official Analytical chemists
BSA	Bovine Serum Albumin
CODEX	Codex Alimentarius Collection of Food standards
DON	Deoxynivalenol
ELISA	Enzyme-Linked Immune Sorbent Assays
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food Drug Administration
GAP	Good Agricultural Practices
GMP	Good Manufacturing Practices
HPLC	High Performance Liquid Chromatography
HSCAS	Hydrated Sodium Calcium Aluminosilicate
IAC	Immunoaffinity Clean-up
IEC	Ion Exchange
IARC	International Agency for Research on Cancer
KAP	Knowledge, Attitude and Practice
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
PACA	Partnership for Aflatoxin Control in Africa
TLC	Thin-layer Chromatography Plates
RIA	Radio Immune Assay
SEC	Size-Exclusion
USA	United State of America
WHO	World Health Organization

ABSTRACT

Aflatoxin is one of the groups of mycotoxins produced in animal feed by toxigenic fungi such as *Aspergillus flavus, Aspergillus parasiticus* and the rare *Aspergillus nomius*.These toxic fungal metabolites are found in feeds and foods. The aim of this study was to assess the knowledge, attitude and practice (KAP) of farmers and to quantify the level of aflatoxin AFG2, AFG1, AFB2 and AFB1 in Dairy and Beef cattle feeds and feed ingredients in Bishoftu and Adama districts. For this purpose a total of 60 livestock farmers and 60 livestock feeds of which 24 compound feed, 36 feed ingredients samples were purposively collected from Bishoftu and Adama districts. Aflatoxin analysis was performed by using high performance liquid chromatography with Immunoaffinity column clean up. The KAPs assessment revealed that awareness on factors that influence aflatoxin production, aflatoxin source and formation is very low among the dairy and beef cattle farmers. The contamination of aflatoxin AFB1, AFB2, AFG1 and AFG2 were found to be 20(83.3%), 17(60.8%), 62.5 and 17(60.8%), respectively in livestock compound feed whereas, the contamination of aflatoxin AFB1, AFB2, AFG1 and AFG2 in feed ingredients were found to be 20(55.6%), 23(63.9%), 21(58.3%) and 23(63.9%) respectively. The result of individual feed samples raveled that, all noug cake samples were contaminated with aflatoxin AFG2 with the range 1.09 to 114.49 µg/kg, AFG1: 6.34 to 735.98 µg/kg, AFB2: 2.69 to 151.71 µg/kg and AFB1: 16.85 to 980.28 µg/kg and total aflatoxin with range of 26.96 - 1953.14 µg/kg. The mean level of aflatoxin AFG2, AFG1, AFB2, AFB1 and total aflatoxin in noug cake were exceeded the maximum limit set by Food and Agriculture Organization, World Health Organization 20µg/kg and European Union 5µg/kg for dairy cattle feed. From this it can be concluded that noug cake feed was highly contaminated with all aflatoxins whereas wheat bran and wheat middling were less contaminated. These results suggest that extension education and risk mitigation should focus on mainly on noug cake and other feed ingredients to effectively reduce aflatoxin contamination in per-urban dairy and beef cattle feeds.

Key words: *Adama, Aflatoxine, Bishoftu, Compound feed, Cattle feed, Mycotoxin*

1. INTRODUCTION

Mycotoxins are highly toxic metabolites produced by fungi in food and feed stuffs that cause health problems in exposed humans and animal (Van Egmond *et al*., 2007). They are produced by moulds that contaminate various agricultural commodities either before harvest or under post-harvest conditions (Kabak, 2006). So far six groups of mycotocins (aflatoxins, patulin, ochratoxins, zearalenone, trichothencenes and ergot alkaloids) are known (Jestoi, 2008). Contamination with certain mycotoxins can reach levels lethal to man and animal (Gunterus *et al*., 2007).

Aflatoxins are group of mycotoxins that are produced during fungal infection of plants and plant products. Aflatoxins often occur in crops in the field prior to harvest, postharvest contamination can occur if crop drying is delayed and during storage of the crop (Martins *et al*., 2007). Humidity and low temperatures influence development of *Asperigillus* spp. and production of toxins (Ghorbanian *et al*., 2008). *Asperigillus parasiticus* is able to produce sclerotic from 25 to 35º C, with relative humidity between 65 and 80% (Giorni *et al*., 2007). The products have health hazard, and sub-symptomatic exposure is known to produce male reproductive system abnormality with several manifestations (Owino *et al*, 2007, Fardohan and Zoumenou, 2005).

Generally, tropical climatic conditions such as high temperatures and moisture, monsoons, unseasonal rains during harvest, and flash floods lead to fungal proliferation and mycotoxins production. Poor harvesting practices, improper storage and poor transport and marketing conditions also contribute to fungal growth and proliferation of mycotoxins. Among the mycotoxins, aflatoxins (Aflatoxin B1, B2, G1 and G2) raise the most concern and among the different forms of aflatoxin, aflatoxin B1 is found widely and in higher concentrations throughout the world in foods such as maize, peanuts and peanut cotton seed products. Outbreaks of aflatoxic hepatitis in humans have been reported in India, Kenya, and Malaysia (Ramesh and Siruguri, 2003).

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). Aflatoxicosis is primarily a hepatic disease; the susceptibility of individual animals to aflatoxins varies considerably depending on species, age, sex and nutrition (Abelwahed *et al*., 2008). Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth, such exposure is difficult to avoid because fungal growth in foods is not easy to prevent (Fardohan and Zoumenou, 2005). Afaltoxins are detected occasionally in milk, cheese, corn, peanuts, cotton, seed nuts, almonds, figs species and a variety of other foods and feeds (Ghorbanian *et al*., 2008). Aflatoxins loss to livestock and poultry producers from aflatoxins contaminated feeds include death and the more subtle effects of immune system suppression, reduced growth rates and losses in feeding efficiency (Fardohan and Zoumenou, 2005).

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). A recent study conducted in Ethiopia show that milk and dairy feeds in the greater Addis Ababa milk shed are highly contaminated with Aflatoxins in the range between 0.028 and 4.98 mg/L (Gizachew *et al.*, 2016).

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 dairy and beef cattle feeds in Ethiopia is not standardized and previous research in Ethiopia had paid little attention to feed contamination level particularly to aflatoxins. Therefore, the present study aimed to investigate the level of aflatoxin G2, G1, B2, B1 and total aflatoxin in dairy and beef cattle feeds with the following objectives:

- To quantify the level of aflatoxin G2, G1, B2 and B1 in Dairy and Beef cattle compound feeds and feeds ingredients, in Bishoftu and Adama district.
- To assess the knowledge, attitude and practices (KAP) among dairy and beef cattle producers.

2. LITERATURE REVIEW

Mycotoxins are secondary metabolites produced by several fungal species which severely affect the metabolic activities of plants, animals, and humans. So far, over 300 mycotoxins have been identified but the dominant types include aflatoxins, those produced by *Aspergillus spp*, *Ochratoxin* which are produced by *Penicillium spp, Fumonisins* produced from *Fusarium spp* and Ergot which are released by *Claviceps spp* (Derek, 2010) and these mycotoxins have been implicated in acute mycotoxicosis in both humans and farm animals (Ramesh and Siruguri, 2003).

2. 1. Fungi and Mycotoxins

Fungi are single cell or multicellular heterotrophic organisms with no chlorophyll which live by absorbing nutrients from organic matter. Fungi include mildews, molds, mushrooms, rusts, smuts, and yeasts (Sarah *et al*., 2008). Fungi are heterotrophic, and the molds form filamentous hyphae that produce spores and they can produce metabolites that are toxic to animals and humans (USDA, 1999). Reproduction in fungi occurs through the production of sexual and asexual spores and fungal infection begins from spores at any of the various stages of crop production and storage processes (Sarah *et al*., 2008).

Mycotoxins are highly toxic metabolites produced by fungi in food and feed stuffs that cause health problems in exposed humans and animal (Van Egmond *et al.*, 2007). Mycotoxins are substances produced by moulds that contaminate various agricultural commodities either before harvest or under post-harvest conditions (Kabak, 2006). There are six groups of mycotocins these are aflatoxins, patulin, ochratoxins, zearalenone, trichothencenes and ergot alkaloids (Jestoi, 2008)**.** Contamination with certain mycotoxin s can reach levels lethal to man and animal (Gunterus *et al.*, 2007).

Mycotoxins occur more frequently under tropical climate where high temperature and humidity are prevailing. In addition, diets in many tropical countries are more heavily concentrated on crops corn and nuts that are susceptible to mycotoxins; consequently, chronic health risks are particularly prevalent in such countries (Ramesh and Siruguri, 2003). Some mycotoxins are produced before harvest (DON, ERGOT); some occur following harvest (*fumonisin, ochratoxin*); and a few predominantly occur during storage (Ramesh and Siruguri, 2003).

Generally, tropical climatic conditions such as high temperatures and moisture, monsoons, unseasonal rains during harvest, and flash floods lead to fungal proliferation and mycotoxins production. Poor harvesting practices, improper storage and poor transport and marketing conditions also contribute to fungal growth and proliferation of mycotoxins. among the mycotoxins, aflatoxins (Aflatoxin B1, B2, G1 and G2) raise the most concern and among the different forms of aflatoxin, aflatoxin B1 is found widely and in higher concentrations throughout the world in foods such as corn, peanuts and cotton seed products. Outbreaks of aflatoxic hepatitis in humans have been reported in India, Kenya, and Malaysia (Ramesh and Siruguri, 2003).

The Food and Agriculture Organization (FAO) estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins (WHO, 1999). Mycotoxins contamination of foods and feeds has aroused significant public concern worldwide. Contamination of feeds with mycotoxins is considered several harmful effects on human and animal health, resulting economic losses as well as in undesirable trade barriers for raw materials and consumable products (Wu, 2006). A type of mycotoxin may be produced by different fungi and each one has its specific toxicity.

Table 1: Types of mycotoxins and corresponding toxigenic fungi

Mycotoxin	Fungus species
Deoxynivalenol (DON, VOMITOXIN),	*Fusarium graminearum*
Fusarium graminearum	*Fusarium culmorum*
T2 toxin and HT2 toxin	*Fusarium poae*
	Fusarium langsethiae
Fumonisins	*Fusarium verticillioides*
	Fusarium proliferatum
Aflatoxins B1, B2, G1 and G2	*Aspergillus flavus*
	Aspergillus parasiticus
Ochratoxin A	*Aspergillus ochraceus*
	Penicillium verrucosum
Ergot	*Claviceps purpurea*

Source: (Derek, 2010)

2.2. Aflatoxins

Aflatoxins are group of mycotoxins that have deleterious effects on humans and are produced during fungal infection of plants and plant products (Owino *et al.*, 2007). Aflatoxins are dietary mycotoxins which have health hazard, and sub-symptomatic exposure to aflatoxins is known to produce male reproductive toxic effects with several manifestations (Fardohan and Zoumenou*,* 2005).

Aflatoxins are group of mycotoxins and aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect (Ozay *et al.*, 2008). Aflatoxins was discovered in 1960 when more than 100,000 young turkeys on poultry farms in England died in course of few months from apparently new disease that was termed "Turkey x disease" (Cordova–Izquierdo *et al.*, 2007). Afaltoxins are classified in B1, B2, G1 and G2; metabolized to aflatoxins M1, and M2 (Boudra, *et al*, 2007). Aflatoxins B1 is a potent mutagenic and carcinogenic agent found in numerous agricultural and dairy products consumed by humans (Maridgal-Santillan *et al.*, 2007).

Aflatoxins don’t have flavour scent, they are fluorescent under the ultraviolet light and they are resistant to high temperatures (Cordova – Izquierdo *et al.*, 2007). Aflatoxins are produced by certain strains of fungi and *Asperigillus flavus* and *Asperigillus parasiticus* representing 93% of strains (Giorni *et al.*, 2007). The compound of aflatoxins B1, B2, G1, G2 are natural contaminants produced by *Asperigillus flavus* and/ or *Asperigillus parasiticus* when these fungi grow on different food products (Guzman de Pena, 2007).

Aflatoxin B1 is recognized by the International Agency for Research on Cancer as one of the most naturally occurring toxic and carcinogenic substances found in nature (Feddern *et al*., 2013). Aflatoxin producing fungi species and the moulds naturally originate in the soil and decayed vegetation in which risks of contamination begins with planting and can be worsened later during post-harvest practices through inappropriate harvesting, handling, storage, processing, and transport practices (Ephrem, 2015).

The degree of contamination during crop production and after harvest also depends on environmental conditions that are optimal for the growth of fungi. Aflatoxins are very slightly soluble in water (10–30 μg/mL), insoluble in non-polar solvents, freely soluble in moderately polar organic solvents such as chloroform and methanol especially in dimethyl sulfoxide (Feddern *et al*., 2013). They are unstable to ultraviolet light in the presence of oxygen, pH extremes (10) and to oxidizing agents. The lactones ring of aflatoxins is susceptible to alkaline hydrolysis and degraded by ammonia or sodium hypochlorite (Feddern *et al*., 2013).

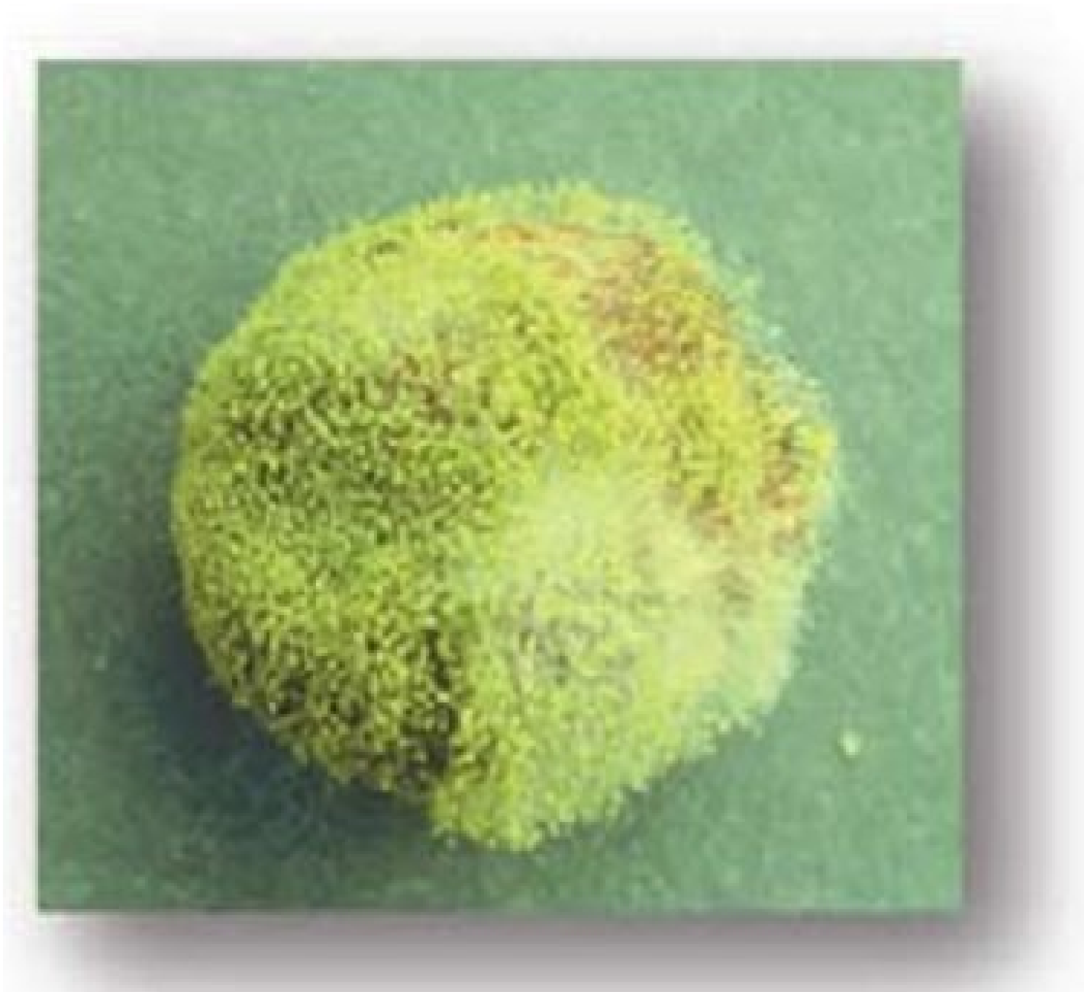
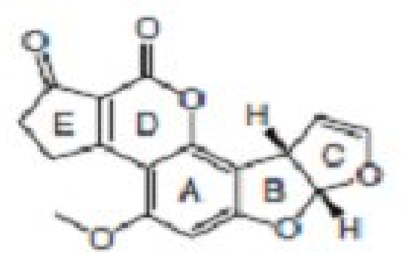


Figure 1: Yellow Mold Caused by *Asperigillus*. *flavus* and *Asperigillus*. *parasiticus*
Source: APSnet, Mycotoxins in Crops:

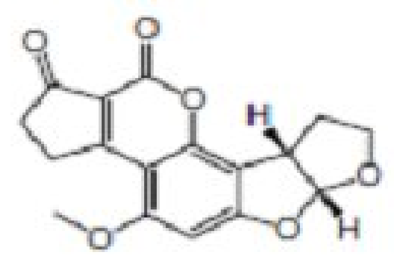
2.2.1. Structure of aflatoxins

Aflatoxins have closely related structures and form a unique group of highly-oxygenated heterocyclic difuranocoumarin compounds. The compound is made up of five rings, having a furofuran moiety (rings B and C), an aromatic six-membered ring (A), a six-membered lactone ring (D), and either a five-membered pentanone or a six-membered lactone ring (E) (Figure 2) and AFM 2 are hydroxylated products of aflatoxins AFB 1 and AFB 2, respectively, which bear a hydroxyl group at the junction of the two furan rings (Schuda, 1980).

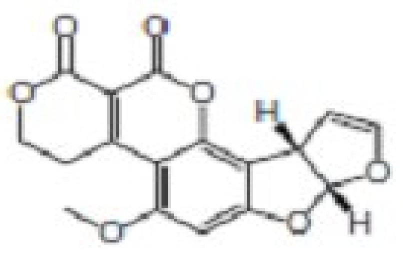
The minor aflatoxins have a hydroxyl group instead of a carbonyl group at ring E (AFR 0, AFRB1, AFRB 2 and AFH1). In others, the D-ring (AFB 1, AFRB 2) or the E-ring (AFB 3) is opened. Other structural analogs (similar molecular structure) include AFP 1 and AFQ 1, which are AFB 1 [metabolites found in urine and liver of rhesus monkeys, respectively (Sid *et al.,* 1974).



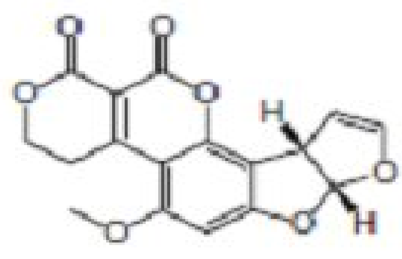
1 (aflatoxin B_1)



2 (aflatoxin B_2)



3 (aflatoxin G_1)



4 (aflatoxin G_2)

Figure 2: Chemical structure of major aflatoxins

Source: *Cole and Cox, 1981.*

2.2.2. Physical and chemical properties of aflatoxins

Aflatoxins B1, B2, G1, G2, M1, M2, B2A, and G2A have melting points of 268-269, 286-289, 244-246, 237-240, 299, 293, 240, and 190 degrees centigrade respectively (Reddy *et al*., 2000). Aflatoxin BI, B2, G1, and G2 have closely similar structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds. Their molecular formulas as established from elementary analysis and mass spectrometric determination are: B1:C17H12O6, B2:C17H14O6, G1:C17H12O7 and G2:C17H14O7.

Aflatoxin B2 and G2 were established as the dihydroxyl derivatives of B1 and G1 respectively (Table 2). Whereas M1 is tetrahydroxyl aflatoxin B1 and aflatoxin M2 is dihydroxyl aflatoxin B2 (Heathcote *et al*., 1978).

Extensive studies on the reactions of aflatoxins to various physical conditions and reagents have been conducted because of the possible application of such reactions in the detoxification of the materials contaminated with aflatoxins (Reddy *et al*., 2000). In the dry state, aflatoxins are heat stable up to melting point. In the presence of moisture however, and at elevated temperature, aflatoxins are destroyed over a period of time. Such destructions of aflatoxins occur in oil seeds, meals, roasted peanuts, or in aqueous solution at pH 7. However, it seems likely that such treatments lead to the opening of the lactose ring, with possible destruction of decarboxylation at elevated temperature (Reddy *et al*., 2000).

Table 2: Chemical and physical properties of aflatoxins

Aflatoxin	Molecular formula	Molecular weight	Melting point
B1	C17H12O6	312	268-269
B2	C17H14O6	314	286-289
G1	C17H12O7	328	244-246
G2	C17H14O7	330	237-240

Source: Feddern *et al*. (2013).

2. 2.3. Aflatoxin toxicity

Aflatoxins are a group of mycotoxins that have effects on humans and are produced during fungal infection of plants or plant products (Owino *et al.*, 2007). Aflatoxins classified as a class 1 carcinogen. Moreover, the hepatotoxic effects of aflatoxins depend on its binding DNA, the primary site of action (McKean *et al.*, 2006) of aflatoxins is on the metabolic system located in the mitochondria, particularly on dehydrogenases (succinic and glycerophosphate) and the electron transfer chain and oxidative phosphorlatation mechanisms (Cordova -Izquierdo *et al.*, 2007).

Aflatoxins have diverse biological and pathological effects, moreover the Aflatoxins M1 is the most noxious for the human consumption and it is the possible carcinogen for man besides other effects, which has great importance for the human health since it can act in way synergic additive and antagonistic (Jestoi, 2008).

Aflatoxins have the ability to contaminate agricultural commodities and can cause sickness or death in humans and animals. The risk of aflatoxin contamination of food and feed in Africa has increased due to environmental, agronomic and socio-economic factors (Ephrem, 2015). Tropical countries that are located between 40ºN and 40ºS latitude offer suitable growing conditions for mycotoxigenic fungi (Food Business Africa, 2017).

2.3. Factors Affecting Aflatoxin Production

Humidity and low temperatures influence development of *Asperigillus spp*. and production of toxins (Ghorbanian *et al.*, 2008). *Asperigillus parasiticus* is able to produce sclerotia from 25 to 35º C, with relative humidity between 65 and 80% (Giorni *et al.*, 2007). The season affects the level of toxins which produced from fungi. Moreover the levels of aflatoxins M1 were higher in winter and spring than in summer and autumn (Tajkarimi *et al.*, 2007).

Damage by pests, birds, mammals, and insects. Prolonged stresses due to drought and nutrient stresses during pollination can increase the chance of *Aspergillus* spores infection to crops (Derek, 2010). Heavy rain can also cause spores to splash onto fruits and grains. Aflatoxin M1 and M2 which are the hydroxylated metabolites of aflatoxin B1 and aflatoxin B2, respectively are found in milk from cows that ingest aflatoxin contaminated feed. Once aflatoxin is produced, it is stable to heat, cold and light and is difficult to detect because aflatoxins are colorless, odorless and tasteless and they exist in uneven distribution in grain bins (Cassel *et al.*, 2011).

According to Elsayed (1990) indicated that afaltoxin production was increased with advanced period of storage. Damaged pods were highly contaminated with *Asperigillus flavus* and accumulated large amounts of aflatoxins (Ozay *et al.*, 2008). However, sound intact pods recorded lower fungal contamination and were almost free of aflatoxins Hag Elamin *et al., (*1988) and Pazzi *et al., (*2005) reported that aflatoxins content of stored groundnut seeds increased with increasing period of storage.

According to Omer *et al.,* (2004) indicated that moisture is the major factor affecting the rate of deteriorationin storedgrains. Osman (1986) showed the absence of aflatoxins contamination in sound seeds of haricot bean, faba bean and maize as compared to significantly higher levels in damaged and mouldy seeds of the same crops. Moreover, Mixon and Roger (1973) reported that when the seed coat of the resistant seeds was picked with a needle, *A. flavusd* eveloped in the area of the injury.

2.4. Agricultural Products Susceptible to Aflatoxin Contamination

Aflatoxins often occur in crops in the field prior to harvest, postharvest contamination can occur if crop drying is delayed and during storage of the crop (Martins *et al.*, 2007**).**Aflatoxin producing fungi can contaminate several food commodities, including many of Africa's important staple cereal crops. Corn, nuts (peanuts), pistachio, Brazil nuts, oilseeds (cotton seed), copra, dried meat of coconut are some of the commodities with greater risk of 26 aflatoxin contamination (Cornea *et al*., 2011). This is because peanuts, cottonseed and copra are rich in edible oils which are very good substrates for growth of fungi (Idris *et al*., 2010).

Commodities which are moderately susceptible to aflatoxin contamination in the field include wheat, oats, millet, barley, rice, cassava, soybean, beans, pulses and sorghum. Other commodities such as cocoa beans, linseeds, melon seeds and sunflower seeds have been infrequently contaminated with mycotoxins with lower important rate compared to other commodities (Bankole *et al*., 2010). Corn silage is also a source of aflatoxins, because the ensiling process does not destroy toxins already present in silage (Cassel *et al*., 2011).

Afaltoxins are detected occasionally in milk, cheese, corn, peanuts, cotton, seed nuts, almonds, figs species and a variety of other foods and feeds (Ghorbanian *et al.*, 2008). Milk, eggs and meat products are sometimes contaminated because of the animal consumption of aflatoxins contaminated feed (Martins *et al.*, 2007).

The research done by Dickens and Pattee (1966) reported that under unfavorable climatic and storage conditions, oil seeds and many other agricultural products are subjected to invasion by toxigenic strains of *A. flavus* and *A. paracticus.* They added that although aflatoxins, has been found in variety of food-stuffs, the most pronounced contamination has been encountered in groundnut seeds, cakes and in the meals produced from these oil seeds. Maize samples in Sudan Savanna were associated with higher aflatoxins levels (Hell *et al.*, 2000).

2.5. Aflatoxins Occurrence in Processed Feeds

Corn is probably the commodity of worldwide concern because it is grown in climates that are likely to have potential contamination with afaltoxin (Ghorbanian *et al.*, 2008). Moreover, M1 contaminated milk and milk products, including skimmed milk, cheese and yoghurt (Van Eijkeren *et al.*, 2006) and pasteurized milk (Zinedine *et al.*, 2007).

Afllatoxins were detected in some cereals and leguminous seeds in the levels of the aflatoxins in these seeds were below the reported hazard threshold. Moreover, sound intact seeds contained low or no aflatoxins compared to significantly higher levels detectable in damaged and moldy seeds of the tested crop plants (Abdel-Rahim *et al.*, 1989).

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).

2.6. Aflatoxins Contamination in Dairy Products

The Aflatoxins B1 occur in peanuts, milk and animal feed (Offiah and Adesiyun, 2007). Transfer of aflatoxins B1 from feed to milk and from milk to curd and whey in dairy fed artificially contaminated concentrates was reported (Manetta *et al.*, 2005). Aflatoxins M1 in the lactating cow, B1 in feed of dairy cattle were detected (Van Eijkeren *et al.*, 2006). 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).

2.7. Risk factors that Predispose Crops to Aflatoxin Contamination

Field and postharvest practices can predispose crop produce to aflatoxin contamination. The risk of contamination is greater in developing countries where peasant farmers who constitute the majority face financial challenges and have little or no access to improved technology. The factors that influence mycotoxin production are either biological (biotic), environmental (abiotic) (Diener and Davis, 1966; Okelloetal., 2010). Some of the biotic factors include cultivar susceptibility and growth stage, insect and bird damage and presence of other fungi or microbes and strain variation in the fungus while abiotic factors include mechanical damage, moisture, temperature, pH and other crop stresses such as drought, soil type, suitability of substrate, excessive rainfall, gaseous exchange and gaseous environment and preservatives and crowding of plants (Suttajit, 1989). Nitrogen stress is another biotic factor which can also predispose crops to aflatoxin contamination.

Most of the factors enumerated above are beyond the control of farmers in developing countries. For instance, unpredictable rainfall which is worsened by climate change makes crops grown in developing countries more prone to water stress and therefore a higher risk of aflatoxin contamination. Also, due to lack of access to improved technology, farmers in developing countries cannot test soils to determine their physicochemical characteristics before cropping (Okelloetal, 2010).

2.8. Mold Growth and Formation of Mycotoxin

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. Mold s can grow over a temperature range of 10-40° C (50-104° F), a pH range of 4 to 8, and above 0.7 (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 majorfactors influencing mycotoxin contamination of feed grains and foods (Bhat, 1988).

Temperature and humidity influence which fungi infect damaged crops. Aflatoxin production are favored by warm conditions; thus, global warming, particularly in currently temperate climates, poses a potential problem is this regard. Developing crops are frequently very resistant to infection by *A. flavus* and subsequent aflatoxin contamination, unless environmental conditions favour fungal growth and crop susceptibility. Wounding by insects, mammals, birds, mechanical processes and/or the stress of hot dry conditions all result in significant infections during the pre-harvest period. Furthermore, climate directly influences host susceptibility (Ghorbanian *et al*., 2008).

2.9. The Status of Aflatoxin Contamination in Ethiopia

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 and 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.
(Alemayehu, 2014).

Aflatoxins were detected in 88% of the samples of corn 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. Further monitoring of mycotoxins in corn 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 (Amare, 2010).

In Ethiopia 73.06 % of the samples were positive for aflatoxin ranging 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 (Aschalew, 2010).

Quantified 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 7 and 419 mg/kg. 10.2%contained AFB1 at a level less than or equal to 10 mg/kg (Dawit, 2015). 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 (Minota, 2016).

Freshly harvested groundnut kernels, 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. 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 (Dereje, 2012).
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). 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 (Dereje, 2012).

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 (Melkamu and Birhan, 2014). 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, physical and biological control methods, resistance groundnut varieties and using chemical control methods (Ephrem, 2015).

2.10. Toxic Effects of Aflatoxins in Humans and Dairy Cattle

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).

2.10.1. Aflatoxicosis and livestock production

Aflatoxicosis is primarily a hepatic disease; the susceptibility of individual animals to aflatoxinss varies considerably depending on species, age, sex and nutrition (Abelwahed *et al*, 2008). Mycotoxins can occur in livestock feed and if the toxins are consumed by animals they can channel into milk or meat and become a food safety hazard for human being (Ramesh and Siruguri, 2003). The problem of aflatoxin has resulted in reduction of livestock and poultry productivity (Bennett and Klich, 2003) and it has also led to higher susceptibility to infectious diseases in livestock and kidney and liver cancers in human beings (Rios *et al*., 2013).

Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproduction performance, and reduced feed utilization efficiency, anaemia, and jaundice. Young and nursing animals may be affected as a result of the conversion of aflatoxin B1 to the metabolite aflatoxin M1 excreted in milk of dairy cattle. Aflatoxins cause liver damage, decreased milk and egg production in livestock and poultry respectively. Aflatoxin also causes 29 infections due to immune suppression and embryo toxicity in animals consuming low dietary concentrations (Dhanasekaren *et al*., 2011).

Aflatoxin B1 is considered by the International Agency for Research on Cancer (IARC) as having produced sufficient evidence of carcinogenicity in experimental animals to be classified as a carcinogen (IARC, 1993). The problems associated with aflatoxins also have negative implications on domestic and export trades. The overall losses due to food and feed spoilage, reduction in livestock productivity, morbidity, mortality and reduced selling prices of contaminated meat, milk, and egg devastate the domestic and export market and pose an economic loss (Bhat and Vasanthi 2003). In Indonesia, Philippines and Thailand, 5% of the maize and peanuts produced were discarded due to contamination of aflatoxins (Bhat and Vasanthi 2003).

The annual cost of aflatoxin contamination and other molds in these countries in terms of product spoilage, human health effects, and losses in the poultry and pork sectors was calculated to be 477 million dollars (Bhat and Vasanthi, 2003). In the United States estimated value of maize lost due to aflatoxin contamination was $225 million per year (Betran and Isakeit, 2003). In the 1980s' in Malawi, the share of groundnut exports collapsed from 64% to 0.2% due to aflatoxin contamination (Rios *et al*., 2013).

2.10.2. Aflatoxins in human health

Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth, such exposure is difficult to avoid because fungal growth in foods is not easy to prevent (Fardohan and Zoumenou, 2005). Even though heavily contaminated food supplies are not permitted in the market place in developed countries, hence concern still remains for the possible adverse effects resulting from longterm exposure to low levels of aflatoxins in the food supply (Umarani *et al.*, 2008). Also Aflatoxins recognized as a cause of liver cancer and they have additional important toxic effects in farm and laboratory animals (Jonathan *et al.*, 2004).

Evidence of acute aflatoxicosis in humans has been reported from many parts of the world, namely the third world countries, like Taiwan, Uganda, India, and many others (Jonathan *et al.*, 2004). The syndrome is characterized by vomiting, abdominal pain, pulmonary edema, convulsions, coma and death with cerebral edema and fatty involvement of the liver, kidneys, and heart (Umarani *et al.*, 2008). Conditions of the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that flavor fungal development in crops and commodities and lack of regulatory systems for aflatoxins monitoring and control (Abelwahed *et al.*, 2008).

According to IARC (1988), Aflatoxins B1 placed on the list of human carcinogens. This is supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and liver cell cancer (Fardohan *and* Zoumenou, 2005).

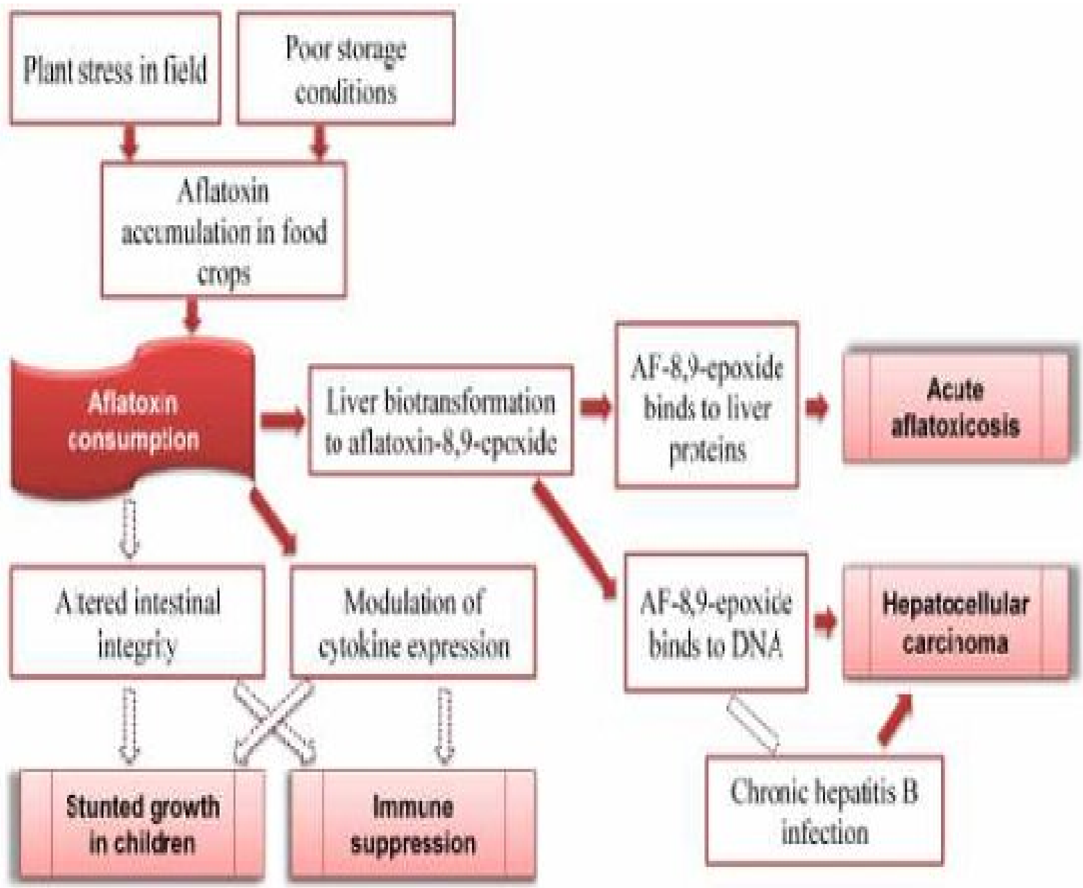


Figure 3: Aflatoxin disease pathways in humans and animals

Source: Wu and Tritscher, (2011)

2.10.3. Diagnosis and treatment of aflatoxicosis

Aflatoxicosis is a herd rather than an individual cow problem. If aflatoxicosis is suspected, the ration should be analyzed immediately. If it is present, the source should be eliminated immediately. Diagnosis of aflatoxicosis is often difficult because of the variation in clinical signs, gross pathological conditions and the presence of infectious diseases due to the suppression of the immune system (Caseal *et al*., 2011). On the farm, more than one mold or toxin may be present in the contaminated feed, which often makes definitive diagnosis of aflatoxicosis difficult. Treatment should be directed at the severely affected animals in the herd and further poisoning prevented. Levels of protein in the ration and vitamins A, D, E, K and B should be increased as the toxin binds vitamins and affects protein synthesis. Good management practices to alleviate stress are essential to reduce the risk of secondary infections that must receive immediate attention and treatment (Caseal *et al*., 2011).

The tolerance of animals for aflatoxin infection depends on the age and sex of the animal, its health status, and overall management level of the farm (Caseal *et al*., 2011). To avoid contamination of milk, lactating dairy cattle should not consume greater than 20μ/kg in the total ration to prevent from passing the limits of 0.5μ/kg in milk set by Food and Drug Administration of the United Nations (Caseal *et al*., 2011). Consequently, calves should not receive milk from cows that are fed with feeds containing > 20 μ/kg.

This does not suggest that feeding at these levels or below will reduce or eliminate the potential for aflatoxicosis for there are no clear cut safe feeding levels as it varies with individual animals (Caseal *et al*., 2011). Ingestion of aflatoxins at levels even lower than those listed in the guidelines may cause some undesirable side effects and depends on such factors as age, sex, and general health of the animals (Caseal *et al*., 2011). Monitoring animal health closely and discontinuing the use of contaminated feed immediately if undesirable effects are noticed are important practices to tackle the problems of aflatoxicosis (Caseal *et al*., 2011).

2.11. Economic Loss Due to Aflatoxin in Livestock and Crops

The economic impact of aflatoxins drive directly from crop and livestock losses as well as indirectly from the cost of regulatory programs designed to reduce risks to animal and human health (Martins *et al.,* 2007). The Food and Agriculture Organization estimates that 25% of the world's food crops are affected by mycotoxins of which the most notrious are aflatoxinss. Aflatoxins loss to livestock and poultry producers from aflatoxins contaminated feeds include death and the more subtle effects of immune system suppression, reduced growth rates and losses in feed efficiency has been particularly useful in minimizing aflatoxins contamination peanuts (Fardohan and Zoumenou, 2005).

2.13. Prevention and Management of Aflatoxin Contamination

The control of management of aflatoxins requires an integrated approach. A combination of technology solutions, effective regulations and standards, and enabling environment could bring about mitigation and control of aflatoxin on the continent. A number of strategies for reduction and control of mycotoxins have been considered in different African countries such as prevention of mould growth in crops and other feed stuffs, decontamination of mycotoxin-contaminated foods, continuous surveillance of mycotoxins in agricultural crops, animal feed stuffs and human food (Darwish *et al*., 2014).

It is important for producers to realize that good agricultural practices (GAP) represent the primary line of defense against contamination of cereals with mycotoxins, followed by the implementation of good manufacturing practices (GMP). Better post-harvest storage practices to ensure that cereals are cooled quickly and evenly to prevent wet spots and should be maintained at a moisture content that does not promote mould growth (Finnegan, 2010). Control measures across the entire grain supply chain can increase the accessibility of aflatoxin-free foods to households for both domestic consumption and international market. During post-harvest, screening and decontamination of grain can be employed using color or density sorting in order to remove contaminated grains from a grain lot before they are used for food processing (Ephrem, 2015).The use of plant essential oils and atoxigenic strains of *Aspergillus flavus* have been reported to reduce aflatoxin levels (Derek, 2010).

Application of ash was found to be effective ways to reduce aflatoxin (Kirui , 2016). From Kenya reported that, maize cob ash was effective in reducing the aflatoxin level of maize and maize products.

Currently a bio-control method of aflatoxin mitigation is being tried in Africa. The bio-control product for aflatoxin management currently under testing in Kenya is Aflasafe KE01. Aflasafe KE01 is a mixture of spores of the bio-control atoxigenic strains (non-toxigenic indigenous *Aspergillus flavus*) that are coated onto sterile grain (sorghum), which serves as carrier and food for the fungi.

The atoxigenic strains grow and multiply on, and disperse from, the carrier to initiate displacement of aflatoxin-producers in the field. The product is applied 2–4 weeks prior to crop flowering. It is broadcasted on the crop and soil at an application rate of 10 kg/ha. In Kenya, field testing for an even lower rate of 5kg/ha is underway, translating to a direct cost benefit to the farmer through reduced pricing as a farm input (Bandyopadhyay, *et al*., 2016).

This method has several advantages including modifications to fungal communities caused by application of bio-control strains carry over through the value chain, discouraging contamination in storage and transport, even when conditions are very favorable to fungal growth. Positive influences of atoxigenic strain applications carry over between crops and provide multi-year benefits, with a single application of atoxigenic strains benefiting not only the treated crop but 32 also rotation crops and second season crops that miss a treatment and that because fungi can spread, as the safety of fungal communities within treated fields improves, so does the safety of fungal communities in areas neighboring treated fields (Derek, 2010).

The net effect is that the crop is protected throughout the supply chain (Bandyopadhyay, *et al*., 2016). It is not possible to completely eliminate fungal infection and mycotoxin contamination (Derek, 2010). Aflatoxins in cereals are fairly resistant to further food processing and the most effective way to reduce the contamination is to prevent it before happening in the process of crop production. To minimize the negative effects of mycotoxin toxicity on animals that consume contaminated feeds, utilization of aflatoxin binders (adsorbents) was also reported to be advisable. These compounds are often added to animal feeds and inhibit intestinal absorption of mycotoxins and thereby prevent their deleterious effects in animal production. The most common binders include hydrated sodium calcium aluminosilicate (HSCAS), sodium or calcium bentonites and zeolites.

These binders have great impact on improving animal production and health by providing quality products to consumers of animal products (Feddern *et al*., 2013). The rapid progress in genomic research of both host plants and fungal pathogens could lead to a better understanding of the mechanisms of aflatoxin formation, pathogencity of the fungus, and crop-fungus interactions (Guo, 2009).

Efforts are being made to understand fungus biology and the mechanism of aflatoxin biosynthesis using genomic tools. Thus, developing novel strategies to control aflatoxin contamination is the ultimate goal for scientist in the future. This will contribute greatly to achieve the goal of devising novel strategies to eliminate pre-harvest aflatoxin contamination resulting in a safer food and feed supply (Guo, 2009).

2.14. Legislations to Control Aflatoxin Contamination

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. Recently, there are different international organizations including the U.S.A., E.U., CODEX alimentariuos, ISO, FAO/WHO, FDA, IDF that can establish international standards and limits of mycotoxin levels to ensure safety and quality of foods and feeds. For example EU general food safety legislation 178/2002 (EU 178/2002) imposes legal obligations for food business operators (including growers and food and feed manufacturers) to ensure that the food and feed they sell should comply the limits set by the standards setting organizations. It also requires them to identify, review and control critical points in their processes for food safety.

According to EU Regulations 1881/2006 (EU 1881/2006), the maximum contents of aflatoxin B1 and total aflatoxins (B1, B2, G1 and G2) in cereals and cereal products allowed are at 2µg/kg and 4µg/kg, respectively. The maximum limits of aflatoxin B1 and total aflatoxin in maize grain subjected to sorting before human consumption are 5µg/kg and 10µg/kg respectively.

In order to minimize human exposure to these toxins, EU regulations on its directive of 2002/32/EC has set maximum limits for aflatoxin B1 in animal feed, which are in the range of 5-30 µg/kg (Derek, 2010). Total aflatoxin implies the sum total of B1, B2, G1 and G2, in which the product under investigation is detected only for its status of aflatoxin positive or negative which means the detection does not go for further detecting the different forms of aflatoxins B1, B2, G1 and G2.

There are also national standards established by different states and countries which are set for enforcement in their own state and can be implemented in courtiers that have trade relations with them. For example the Regulations of England (regulation 2009, No. 1223) guides and controls the contaminants status in foods in England and food business operators are not allowed to mix or dilute cereal lots containing mycotoxins above the standard already established for their own nation.

Table 3: FDA maximum level of aflatoxin in animal feeds and food

Species	**Commodity**	**Maximum level (ppb)**
Dairy animals	All feeds and feed ingredients	20
Human	Milk	0.5
Human	Any food except milk	20
Poultry & dairy animals	Corn and other grains	20
All species	Animal feed other than corn or cottonseed meal	20
Breeding beef cattle, breeding swine or mature poultry	Corn and other grains	100
Finishing swine of ≥100 lbs	Corn and other grain	200
Finishing beef cattle	Corn and other grain	300
Beef cattle, swine, poultry	Cotton seed meal	300

Source: Carlson and Ensley (2003).

2.12. Methods of Detection of Aflatoxins

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 immuno sorbent assays (ELISA), spectro-photometric, or by other techniques chromatography.

Thin-Layer Chromatography

Thin-Layer Chromatography (TLC), also known as flatbed chromatography or planar chromatography is one of the most widely used separation techniques in aflatoxins analysis (Suzana *et al.*, 1993). Since 1990, it has been considered the AOAC official method and the method of choice to identify and quantitate aflatoxins at levels as low as

1ng/g (Stroka and Anklam, 2000). The TLC method is also used to verify finding by newer, more rapid techniques (Grosso *et al.*, 2004).

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).

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.

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

The study was carried out in Bishoftu and Adama districts. Bishoftu is a town found at 9°N latitude and 40°E longitude and at an altitude of 1850 meters above sea level. It is located in Oromia region, East Shewa Zone about 47.9 km Southeast of Addis Ababa, the capital city of Ethiopia with a bimodal rainfall pattern, having a main rainy season from June to September. The annual average rainfall and temperature are 866 mm and 20^{o}C respectively (NMSA, 2010). Bishoftu district is a place where large commercial dairy farms, different feed mill produce feeds for sale and rest produces primarily for own consumption, feed suppliers and feed traders are located. Hence this area has a greater potential of livestock production and accessible for livestock feeds.

Adama is in Oromia regional state in East Shoa zone at about 99 km south east of Addis Ababa (39.17°E and 8.33°N) with an altitude of 1622 meters above sea level in the rift valley. Its annual rainfall ranges from 400 mm to 800mm and has a temperature of 13.9°C to 27.7°C (NMSA, 2006). Adama district a place where large feedlot, different feed mill produce feeds for sale, different factories, feed suppliers and feed traders are located. Hence this area has a greater potential of beef production and accessible for finishing feed and feed ingredient for cattle.

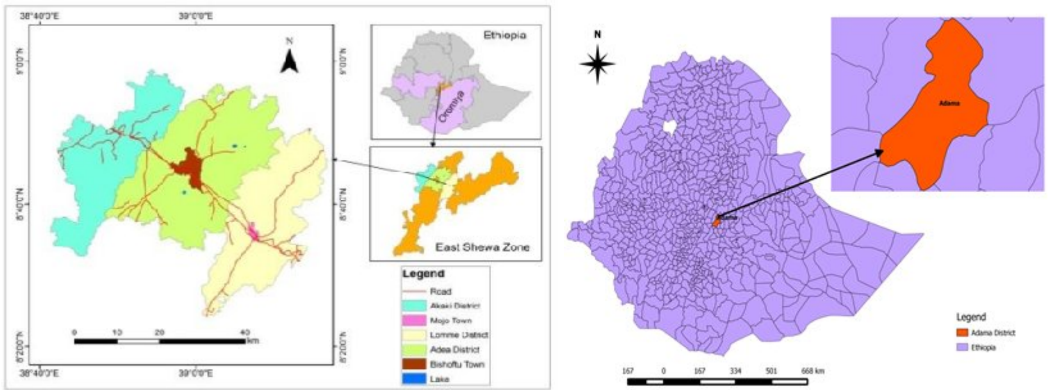


Figure 4: Map of Study Area Bishoftu and Adama

3.2. Study Design

A cross-sectional design was used in seven selected kebeles of Bishoftu and Adama town. For this, purposively four Bishoftu town kebeles and three Adama town kebeles were selected. The selection of kebeles was based on their proximity, availability of cross dairy breed, feedlot, feed processing industries and factories and feed production as well as owners' willingness.

3.3. Questionnaire Survey

The study design computed for the survey is a purposive sampling technique and Semi structured questionnaires and was pre-tested used to get more information. The questionnaire evaluation carried out for KAP assessments and association of risk factors related to dairy and beef feed handling and formation of fungi '*shegata*' on the feed. The response was classified in three parts; the first part was general information, the second part contained socio-demographic characteristics and the final part was KAP related to mycotoxin in livestock feed and feed ingredients.

3.4. Quantitative analysis of Aflatoxins Levels in Feeds

3.4.1. Sample size determination

Purposive sampling technique was applied to collect all the necessary feed samples. To determine sample size 90% standard error, 0.83 Probability of occurrence, design effect equals 1 and desired confidence of 0.1was used. An average prevalence of 83% aflatoxin in feeds in around Addis Ababa by Dawit, (2016) was used. Sample size was determined using the method described by Fisher *et al*., 1998 as detailed below.

$N = z^2pqD/d^2$

Where:

N =Sample size to be calculated.

Z=1.96 at 90% standard error.

P = Probability of occurrence 0.83, q = 1-p

D =design effect equals 1 (around Addis Ababa City)

d2 =Desired confidence of 0.1

Therefore $N = 1.96^2 x\ 0.83x0.17x1/0.1^2$

54 feed samples and 54 Questionnaires.

Total number of samples collected was 108 samples.

Purposive sampling technique was applied to collect all the necessary samples.

3.4.2. Sampling

Livestock feed and feed ingredient samples were collected and information was gathered from the study area by using semi structured questioner (Annex- 1) for dairy and beef cattle farmers to capture their knowledge, attitude and practice (KAP) on aflatoxin contamination. A questionnaire survey was focused on their awareness on mold growth formation of aflatoxin on animal feed and the risk factors for aflatoxin contamination.

In this study a total of 60 samples of livestock feed and feed ingredients were (24 Dairy and Beef compound feed and 36 feed ingredients samples (wheat bran, wheat middling, *noug* seed and linseed cake and corn) samples were purposively collected from four Bishoftu and three Adama town kebeles. Moulds and aflatoxins occur in an extremely heterogeneous fashion in feed and food commodities. It is thus crucial that sampling was carried out in a way that ensures that the analytical sample is truly representative of the consignment. Failure to do this may invalidate the subsequent analysis. So care was taken to obtain a sample of grain which is as representative as possible of the whole bulk. 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 were grouped into 4 parts depending on the layers and height of the bags. Since majority of the farmers either buys one bag of animal feed (100Kg), or in small convenient amounts for a few days use, a sample of about 200gm were taken and weighed and zipped with plastic bag from each bag/ household for analysis after mixing thoroughly the contents in the bag.

The feed ingredients like Noug seed cake, wheat middling, and corn and wheat bran samples were collected from four Bishoftu and three Adama town kebeles. In these towns wheat bran, *noug* (*Guizotia abyssinica* or Niger seed) cake, pea hulls and maize grain widely available and are common feeds source of farmers. Dairy farmers also widely used agro-industrial by-products including Brewer’s dry yeast from beer factories are the common concentrate feed for dairy and beef cattle.

3.5. Laboratory Analysis

3.5.1. Moisture content determination

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

Principle of Detection of aflatoxin using High Performance Liquid Chromatography (HPLC)

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 CO 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.

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.

3.5.2. Sample Preparation

Homogenization

As the distribution of mycotoxin is extremely non homogenous and hence the amount of aflatoxin is not uniformly distributed throughout the feed stuff, laboratory samples should be homogenized by grinding the entire laboratory sample as homogenization reduces particle size and disperse the contaminated particles evenly through the comminuted lab sample. Sample preparation should be different from analytical area. Grinding the sample is performed to the required sieve size. For this purpose 50gm of feed samples were grinded by pestle and mortar.

Sample extraction procedure

First weigh about 20gm of grounded sample from randomly taken lab sample into a beaker and add 2.0gm of NaCl (lot 0000266187) to 20gm of sample (for fatty feeds only) then extracted with 100 ml methanol/water (lot 79345(132299)) (4/1, v/v) and 50 mL of n-hexane (Lot 121005) (for fatty feeds only) in a blender jar at high speed for five minutes after extracting pass the extract through a plaited filter. To accelerate phase separation the extract could be centrifuged at 4000xg for 5 min using centrifuge (Hermle labortechnik GmbH). The separation phase from this was used for the lower phase of the extract in a separator funnel for the following steps (Figure 5).

Figure 5: Laboratory extraction processs

Add 14 mL of the purified extract to 86 mL PBS buffer (pH 7.2). PBS prepared by dissolves potassium chloride (0.20g), potassium dihydrogen phosphate (0.20g), Sodium Dihydrogen Phosphate Dodecahydrate (2.92 g), and Sodium Chloride (8.00 g) in 900 mL deionized water. Alternatively lower sample volumes (e.g. 7 ml extract + 43 ml PBS buffer can be used and mixed thoroughly the sample was filtered using a Buchner funnel and a filter paper or syringe filter of 0.24 micrometer sieve size. Multiple filtrations with syringe filter are advantageous to get clearer sample that is easier to pass later on AflaCLEAN columns. After this calibration curve should be prepared using the working calibration solutions described. These solutions cover various ranges of aflatoxin concentrations. Make the calibration curve prior to analysis according to the method and check the plot for linearity. To establish a calibration curve, we have prepared 8 calibration points of a mixed aflatoxin standard containing various concentrations per injection solvent (0.5, 1.25, 2.5, 5, 7.5, 10, 15, 20 ppb). Linear regression should be performed using a scientific calculator or statistical program.

Spiking procedures for recovery determination

To determine the recover, spike aflatoxin standard solution to initial weight of an aflatoxin-free material. The spiking level should be within the calibration range (preferably mid-range). So that solution can have an adequate concentration of aflatoxin, The correlation coefficient of calibration curve is an indicator of method quality. The correlation coefficient should be greater or equal to 0.995 to run the samples with it. After securing this relevant step it is possible to proceed to running samples.

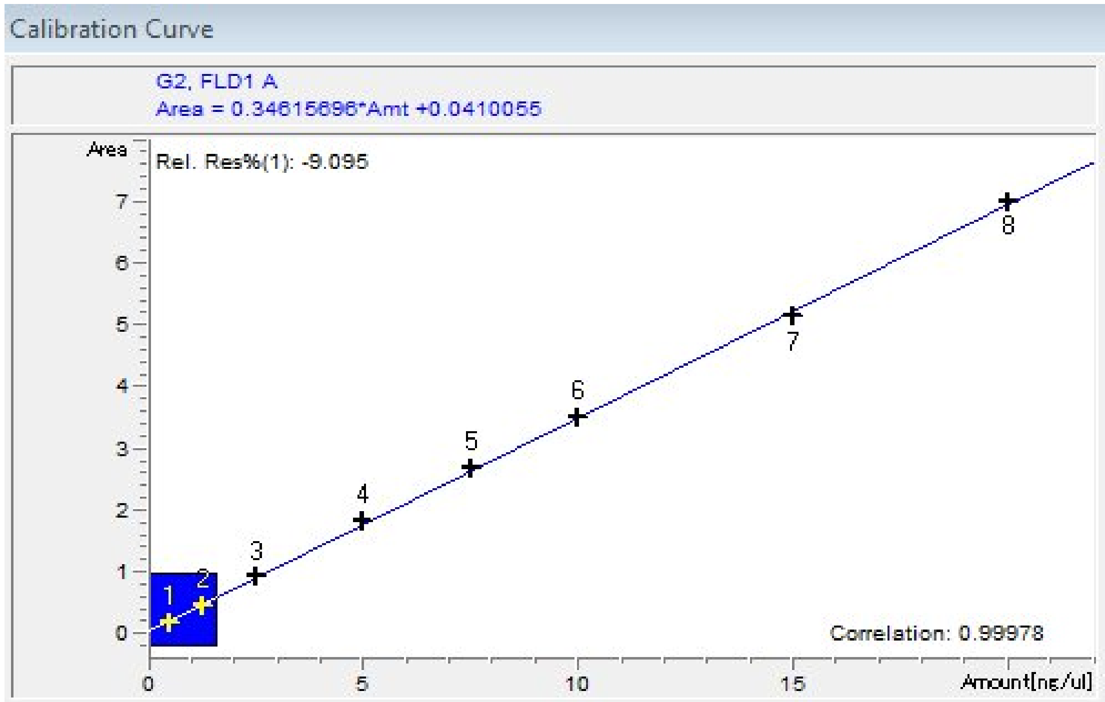


Figure 6: Calibration Curve

Calculation of Results

Plot the signal as x-axis (height or area) against the concentration of aflatoxins [ng/ml] as y-axis from the calibration solution. Draw the calibration curve and calculate slope (*a*) and intercept (*b*) using linear regression. y = area/height, x = concentration

$Y = ax + b$

Calculate concentrations using the equations (ã 2006 AOAC international)

Aflatoxin, ng/g = A x (T/I) x (1/W)

Where,

A = quantity (µg/l) (read from HPLC) x volume eluate injected in to HPLC (µL) / 1000= _________ (ng)

T = final test solution eluate volume = ________ (μL)

I = volume eluate injected into HPLC= _______ (μL)

W = mass (g) of commodity taken / volume of aflatoxin extraction solvent (ml) x volume of the purified extract taken (ml) = ___ (g)

3.6. Statistical Data Analysis

For data analysis, Excel Microsoft office 2007 and R Statistics software version 3.5.1 was used. The data obtained using questionnaires were subjected to statistical analysis using statistical package for social science (SPSS) computer programme. In the R 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 ($P < 0.05$) 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 management conditions affected for the possible mean aflatoxin level difference.

4. RESULTS

Field and postharvest practices can predispose crop produce to aflatoxin contamination. The risk of contamination is greater in developing countries where peasant farmers who constitute the majority face financial challenges and have little or no access to improved technology.

4.1. Aflatoxin Risk Factors Survey Result

Among dairy and beef producers about 41(76%) and 39(72%) didn't know the possible source and risk factors that influence aflatoxin production. Only 15(28%) know about risk factors that influence aflatoxin production of which 12(22%) of dairy and beef farmers respondents knows biological/biotic factors were the risk and the possible source of afalatoxin production and all of them said that the presence of other fungi or microbe and strain variation in the fungus was the types of risk factor that produce aflatoxin.

4.2. Demographic Information, Knowledge of Aflatoxin and Level of Education of Farmers

A semi-structured questionnaire result shows KAP (Knowledge, attitude and practice) assessments related to mold growth and formation of aflatoxins among dairy farmers and beef cattle producers. On this survey, a total of 54 participants (dairy farmers and beef finishers) were participated. Socio-demographic characteristics among dairy farmers and beef producers participated in this study were between the age of 30 -50 years, 80% were married. Sex distribution of the participants was seen to be male 80% (43 of 54) and only 20% were female.

The education level of the respondents varied from basic education to the highest education. Forty six percent (46%) of the dairy and beef farmers were secondary school leavers, 28% had higher education, and 22 %) of the dairy and beef farmers had primary/elementary education, while 4 % basic education level. When considering knowledge of based on farmers‟ education levels, about 56% of higher education level farmers had knowledge of aflatoxin. Farmers having higher education were significantly more aware of aflatoxin than those having primary education as shown in Table 4.

Table 4: Dairy farmers and beef cattle producers' education level and awareness of aflatoxin

Educational level of farmers		**Knowledge of afalatoxin**		
Educational level	N (%)	Know aflatoxin N (%)	Not know aflatoxin N (%)	Total N (%)
Basic	4 (7)	0 (0)	4 (100)	4 (100)
Elementary	13 (24)	3 (23)	10 (77)	13 (100)
Secondary	21(39)	6 (29)	12 (69)	21 (100)
Higher Education	16 (30)	9 (56)	7 (44)	16 (100)
Total	**54 (100)**	**18 (33)**	**36 (67)**	**54 (100)**

4.3. Dairy and Beef Farmer level of knowledge of aflatoxicosis and Factors influence Aflatoxin Production.

Most of the respondents 67% (37 of 54), 65% (35 of 54) did not know how animals get aflatoxicosis and how aflatoxin contaminated animal products respectively. Over all interviewed beef cattle producing farmers were not aware of animal could get aflatoxicosis and hence aflatoxin contaminate animal product on the other hand around eighteen dairy farmers have knowledge of aflatoxin contaminated animal product (milk) by aflatoxin.

Among dairy and beef producers about 23(43%) did not know about aflatoxin and 41(76%) and 39(72%) didn't know the possible source and factors that influence aflatoxin production. Only 15(28%) know about factors that influence aflatoxin production of which 12(22%) of dairy and beef farmers respondents knows biological/biotic factors were the risk and the possible source of afalatoxin production and all of them said that the presence of other fungi or microbe and strain variation in the fungus was the types of risk factor that produce aflatoxin.

As demonstrated below in table 5 a total of 54 participants (dairy farmers and beef cattle producers) were interviewed for their knowledge regarding aflatoxin contamination in dairy and beef feed. According to the result of knowledge assessment, awareness of mold growth and formation of mycotoxin is very low among the beef cattle producers. About eighteen dairy farmers (33%) respondents reported that they were aware of formation of mycotoxin (aflatoxin) the remaining 36 dairy farmers and beef cattle producers (67%) of respondents were not. On the other hand, 65 %(35 of 54) responded moisture and heat are the favorable conditions for mold growth (*shagata*) on animal feed.

Table 5: Farmers' Knowledge level on storage condition, selection of feeds and Problem of storing feeds for long period of time

Storage condition favorable for mould growth		**Problem of storing feeds for long time**		**Feed selection criteria used**	
Factors	**N (%)**	**Factors**	**N (%)**	**Factors**	**N (%)**
Hot and Moist	35 (65%)	Mould	13 (24%)	Color	12 (22%)
Moist	7 (13%)	Insect	2 (4%)	Color and odor	17 (31%)
Hot	5 (9%)	Bad smell	1 (2%)	Quality	1 (2%)
Long term storage	3 (6%)	Insect and mould	1 (2%)	Color, odor, Moisture	7 (13%)
Don't know	4 (7%)	Don't know	37 (68%)	Other	4 (7%)

4.4. Dairy and Beef Farmers Level of Attitude

Majority of the dairy and beef cattle farmers about 67% do not prefer the lower quality with lower price to purchase dairy and beef cattle feeds they considered the quality of feeds rather than its price. About 35 dairy farmers (66%) of respondents were reported that the food that not use for human consumption due to mold growth (*shagata*), damage or expired had a problem used as animal feed. The majority of dairy farmers about 76% prefer compound feed, 11% cotton seed cake, 9% wheat bran, and 4% corn (Table 6).

Table 6: Summary of type of feed preferred by farmers

Factors	Frequency	Percent
Compound feed	41	76
Cotton seed cake	6	13
Wheat bran	5	9
Corn	2	4
Total	**54**	**100**

4.5. Sources of Farmers' Merchandise and Factors Considered During Purchase

All urban dairy farmers and beef cattle producers in the study site were used concentrate feed every day to feed milking cow and beef cattle to increase milk production and weight gain. The most common ingredients in concentrate feeds in the study site were wheat bran, wheat middling *noug* (*Guizotia abyssinicaor* Niger seed) cake linseed cake, corn, cotton seed cake and brewery grain.

Thirty nine percent (21 of 54), 28% (15 of 54) and 19 %(10 of 54) of dairy and beef cattle farmers responded that they have had purchased their animal feed from feed processors, traders and factories, respectively. However, only 3% dairy and beef cattle farmers purchased raw materials directly from farmers.

Table 7: Sources of compound feeds and feed ingredients

Source	Frequency (N=54)	Percentage (%)
Feed processor	21	39
Traders	15	28
Factories	10	19
Feed processors and traders	3	5
Farmers	3	5
Feed processors and factories	2	4
Total	**54**	**100**

Regarding to location of the feed storage system, about 59% of the dairy farmers and beef cattle producers store their dairy and beef feed in the house, 24% stored in the shade and 13 % stored in open space. Storage facilities at the dairy and beef cattle farmer's households were not ideal for keeping animal feeds because of most of the farmers around 80% (43 of 54) did not stored their feed for long time. Of those who stored on a raised and floor dry place storage accounts 11%, and 9%, respectively.

Most of the dairy and beef cattle producers were not aware of drying and ventilation use 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.

Table 8: Summary of aflatoxin practice among dairy farmers and beef producers

Place of storage	Frequency(N=54)	Percentage (%)
In the House	32	59
In the Shade	13	24
In open Space	9	13
Way of Storing Feeds		
Raise and Dry	6	11
On floor and Dry	5	9
Raised and Humid	0	0

4.6. Moisture Content of Feeds and Feed Ingredients

The moisture content of feeds was determined according to AOAC, 2000. Twenty four compound feeds were analyzed in the present study and 6.80- 10.98% range of moisture content was seen. The moisture content analyzed in individual feed component like in corn, linseed cake, *noug* seed cake, wheat middling and wheat bran showed 8.11-10.94%, 6.83-7.84%, 5.48-9.99, 9.02-9.49 and 9.10-9.83% moisture content respectively (Table-9).

Table 9: Sample of dairy and beef compound feeds and feed ingredient moisture content

Sample name	No. Obs	Mean	Std Dev	Minimum	Maximum
Compound Feed	24	8.852	0.914	6.80	10.98
Corn	9	9.739	1.137	8.11	10.94
Linseed Cake	4	7.138	0.471	6.83	7.84
Noug Seed Cake	5	8.042	1.753	5.48	9.99
Wheat Middling	9	9.247	0.130	9.02	9.49
Wheat bran	9	9.506	0.269	9.10	9.83

4.7. Contamination Level of Aflatoxin

A total of 60 animal feed samples were collected for analysis. The samples included all the commonly used dairy and beef cattle feeds such as mixed concentrate feed. All dairy and beef producing farmers used concentrate every day to feed their cattle. The most common ingredients in concentrate feeds were wheat bran, noug seed cake, linseed cake, corn, wheat bran, wheat middling and pea hulls. Dairy farmers also widely used agro industrial by-products, especially Brewer's dry yeast.

Among the 60 livestock compound feed and feed ingredients samples analyzed , 62% (37 of 60) were contaminated with aflatoxin B1,B2,G1 and G2 among them 100% (12 of 12) Dairy compound feed samples shown the mean concentration of 46.89 ppb which was higher than the FDA and EU standards (20 ppb). In Beef compound feed, 66.66% incidence was found (8 of 12) at mean concentration of 24.499 ppb which was below the FDA and EU (300 ppb). The contamination of AFB1, AFB2, AFG1 and AFG2 were found to be 83.3% (20 of 24), 60.8% (17 of 24), 62.5% (15 of 24) and 60.8% (17 of 24), respectively in livestock compound feed whereas, the contamination of aflatoxin AFB1,AFB2, AFG1 and AFG2 in feed ingredients were found to be 55.6%(20 of 36), 63.9%), (23 of 36), 58.3% (21 of 36) and 63.9% (23 of 36), respectively in the present study.

In comparison of the present investigation with different international standards the average level of aflatoxin G2, G1, B2, B1 and total aflatoxin in compound feeds of dairy cattle were 1.63 µg/kg, 12.69 µg/kg, 4.48 µg/kg, 27.83 µg/kg and 46.89 µg/kg, respectively, and only aflatoxin B1 and total aflatoxin were exceed the limit of FDA, food and agriculture organization (FAO) and World Health Organization (WHO) action level 20µg/kg. Levels of aflatoxin G1 in compound feeds were exceed the limit of EU 5µg/kg for dairy cattle feed. Among the beef compound feed analyzed for aflatoxin AFB 1,AFB2,AFG2,AFG1and total aflatoxin compound feeds of beef cattle were 9.39µg/kg,1. 37µg/kg 2.01µg/kg,11.47µg/kg and 24.49µg/kg all feeds samples were within the safe limit of FDA, FAO, EU and WHO standards (beef feed = 300 µg/kg).

The highest aflatoxin AFB1 level (196.59 µg/kg) was in a concentrate feed sample that contained a mixture of wheat bran, wheat middling noug *seed* cake and corn from Bishoftu.(Table 10).The contamination level of feeds belonging to dairy producers was significantly higher (46.89 µg/kg) than beef cattle producers (24.44 µg/kg). Regarding the study locations, the contamination level of feeds and feed ingredients in Bishoftu (132.85 µg/kg) was significantly higher than that of Adama (96.44 µg/kg).

Table 10: Level of aflatoxin G2, G1, B2, B1 and total aflatoxin in compound feed

Sample Code	B1(μg/kg)	B2(μg/kg)	G2 (μg/kg)	G1 (μg/kg)	Total AFs(μg/kg)	Moisture (%)
BDCP 01	4.53	0.99	2.25	6.79	14.56	8.04
BDCP 02	1.31	0.37	1.41	3.56	6.66	8.79
BDCP 03	5.85	1.16	1.68	8.88	17.58	7.76
BDCP 04	7.82	1.39	1.83	12.53	23.58	8.44
BDCP 05	6.07	1.00	2.07	8.33	17.47	8.19
BDCP 06	5.19	0.79	0.94	4.81	11.73	8.31
BDCM 07	9.96	1.98	0	6.83	18.77	9.41
BDCM 08	29.62	4.11	1.93	8.49	44.16	9.64
BDCM 09	196.59	32.94	3.39	68.19	301.11	9.00
BDCM 10	21.43	3.81	1.64	6.85	33.73	9.19
BDCM 11	35.88	3.91	1.71	11.4	55.90	9.00
ABCM 12	9.73	1.36	0.76	5.59	17.43	9.46
ABCM 13	5.46	0.45	0	0.71	6.63	9.13
ABCM 14	0.26	0	0	0	0.26	7.98
ABCM 15	46.48	9.03	12.75	71.18	139.44	7.36
ABCM 16	6.94	1.21	2.05	10.24	20.45	9.54
ABCM 17	0	0	0	0	0	9.83
ABCM 18	0	0	0	0	0	10.98
ABCM 19	0.17	0	0	0	0.17	10.01
ABCM 20	0	0	0	0	0	6.80
ABCM 21	24.22	2.12	6.61	33.79	69.75	9.34
ABCM 22	0	0	0	0	0	8.98
ABCM 23	1.64	0	0	0	1.64	8.79
ABCM 24	27.59	3.66	2.72	21.68	55.65	8.47
Average	**18.61**	**2.93**	**1.82**	**12.08**	**35.69**	**8.85**

4.8. Detection of Aflatoxin in Individual Feed Samples

The common ingredients of compound feeds and feed ingredients were also tested individually using HPLC with immune affinity column clean up. For feed ingredients, 47.22% (17 of 36) show contamination of aflatoxin B1, B2, G1 and G2 in noug seed cake, lin-seed cake, corn, wheat bran and wheat middling. An incidence of 100% (5 of 5), with total mean concentration of 950.93 ppb of aflatoxin was seen in noug seed cake. In lin seed cake, an incidence of 75% (3 of 4) was contaminated with mean concentration of 326.85 ppb. An incidence of 66.66% (6 of 9) corn was contaminated with aflatoxin at mean concentration of 40.01ppb. In wheat bran and wheat middling an incidence of 11.11% (1 of 9),22.22 %(2 of 9) with a mean concentration 0.0146 µg/kg and 0.0266 µg/kg was found in dairy and beef feed ingredient, respectively which is lower than the FDA WHO and FAO (20 ppb) and EU standards for dairy feed (5 µg/kg).

According to the result of individual feed samples raveled that, all noug seed cake samples were contaminated with aflatoxin AFG2 with the range 1.09 to 114.49 µg/kg, AFG1: 6.34 to 735.98 µg/kg, AFB2: 2.69 to 151.71 µg/kg and AFB1: 16.85 to 980.28 µg/kg and total aflatoxin with range of 26.96 - 1953.14 µg/kg (Table 11). In- lin seed cake, 75% (3 of 4), samples was contaminated with aflatoxin AFG1with the range 0 to 483.176, AFG2 with the range 0 to 91.4673 µg/kg, AFG1: 6.34 to 735.98 µg/kg, AFB2: 0 to 59.24 µg/kg and AFB1: 0 to 315.49 µg/kg and total aflatoxin with range of 0 to 949.38 µg/kg was found in dairy and beef feed ingredient respectively both noug and lin seed cake is higher than the FDA and EU standards for dairy feed (20 µg/kg).

Table 11: Level of aflatoxin G2, G1, B2, B1 and total aflatoxin in noug cake (n=5)

Sample Code	B1(µg/kg)	B2(µg/kg)	G2 (µg/kg)	G1 (µg/kg)	Total AFs(µg/kg)	Moisture content (%)
BNC 05	16.85	2.6688	1.097	6.34	26.96	7.85
BNC 06	597.67	74.205	35.46	257.45	964.79	9.99
BNC 07	980.28	151.71	85.17	735.98	1953.14	9.33
BNC 08	115.39	20.08	9.36	33.25	178.08	5.48
BNC 09	670.23	116.88	114.49	730.09	1631.69	7.56
Average	**476.09**	**73.11**	**49.12**	**352.62**	**950.93**	**8.04**

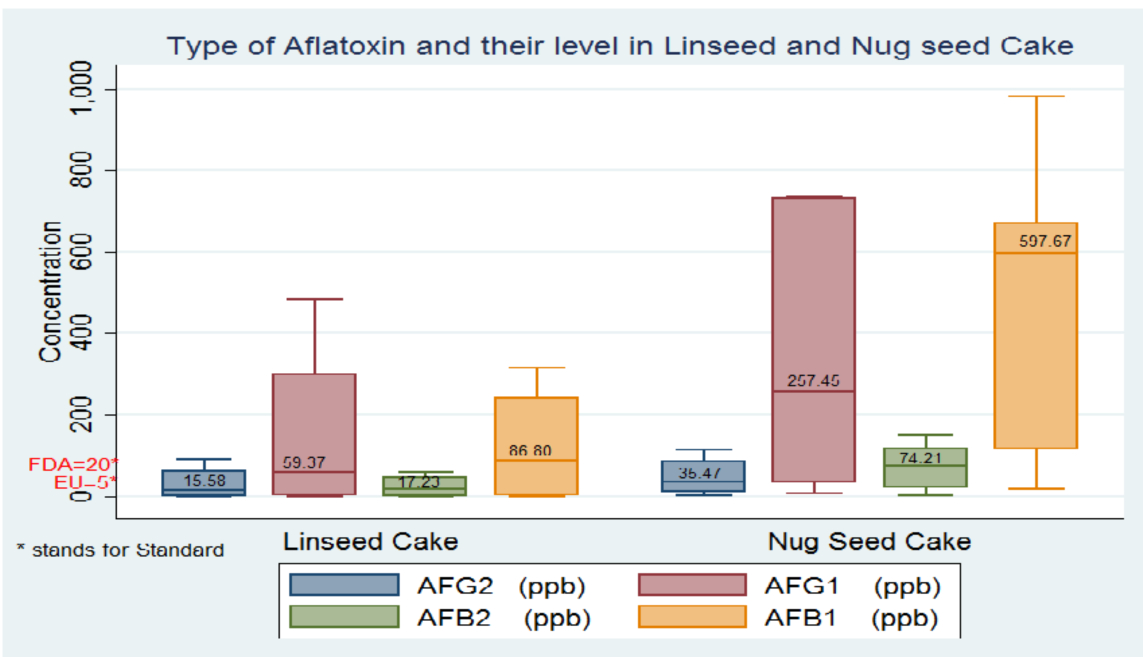


Figure 7: Type of aflatoxin and their level in linseed cake and Noug seed cake

Accordingly in 66.66 % (6 of 9) of corn were seen to have a significant level of aflatoxin and the reaming 33.33 %(3 of 9) were not contaminated with aflatoxin. Corn feeds tested were seen to have AFG2 with the range 0 to 11.74 µg/kg, AFG1: 0 to 95.68 µg/kg, AFB2: 20 to 15.48 µg/kg and AFB1: 0 to 98.97874 µg/kg and total aflatoxin with range of 0 - 221.8787 µg/kg (Table 12). The mean of aflatoxin G1, G2, B2, B1 and total aflatoxin level in meaze/corn were 17.78 µg/kg,2.16 µg/kg, 2.80 µg/kg, 17.26 µg/kg and 40 µg/kg, respectively. The mean aflatoxin AFB1, AFB2, AFG1 and G2 were not above the limit of FDA level 20µg/kg, FAO and WHO 20µg/Kg and but AFB1 and AFG1exceed the limit of EU which is 5µg/kg for dairy cattle feed.

Table 12: Level of aflatoxin G2, G1, B2, B1 and total aflatoxin in corn (N=9)

Sample Code	B1(µg/kg)	B2(µg/kg)	G2 (µg/kg)	G1 (µg/kg)	Total AFs(µg/kg)	Moisture content (%)
BCO 28	0	0	0	0	0	10.93
BCO 29	0	0	0	0	0	8.67
BCO 30	46.6	46.6	6.43	48.78	110.43	9.75
BCO 31	1.63	1.63	0	0.63	2.53	10.94
BCO 32	0	0	0	0	0	9.82
BCO 33	6.66	6.67	0.27	8.72	16.08	10.65
ACO 34	98.98	98.98	11.74	95.68	221.88	10.53
ACO 35	0.84	0.84	0.61	2.44	4.32	8.11
ACO 36	0.66	0.66	0.42	3.75	4.84	8.25
Average	**17.26**	**2.80**	**2.16**	**17.77**	**4o**	**9.73**

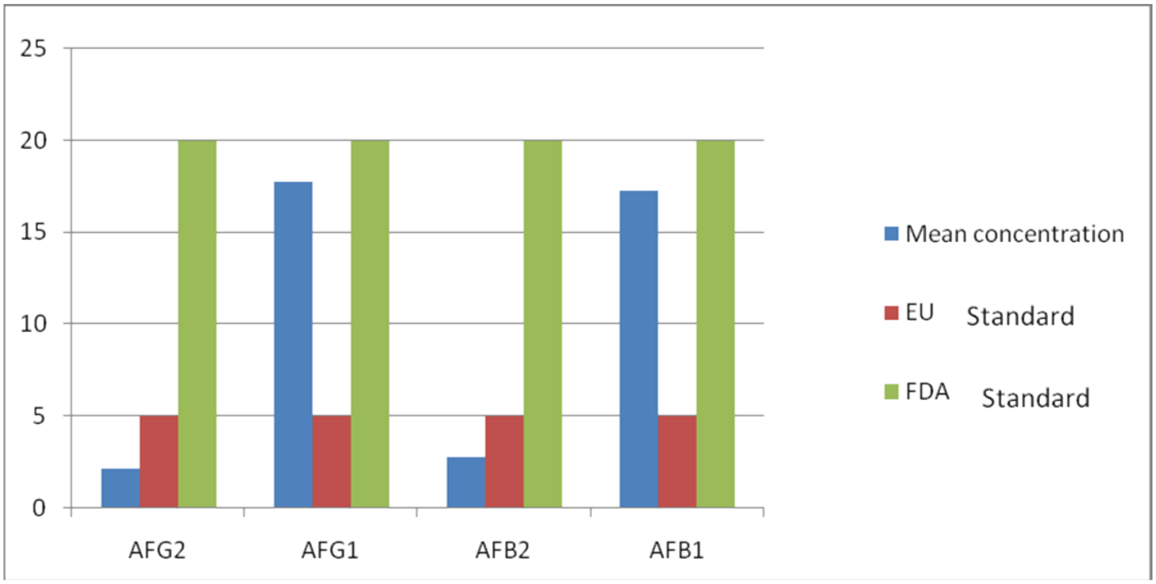


Figure 8: Type of aflatoxin and their mean concentration with EU and FDA Standard in corn

Nine Wheat bran and nine wheat middling feeds samples were analyzed and found that only 11.11% (1 of 9) and 22.22% (2 of 9) of the samples were contaminated with aflatoxin G1, B2 and B1 respectively. The AFB1, AFB2, AFG1 and total aflatoxin seen in these feed type were not above the limit of FDA action level 20µg/kg, food and agriculture organization (FAO)/World Health Organization (WHO) 20µg/Kg and EU 5µg/kg for dairy cattle feed.

4.9. Correlation Between Moisture Content and Aflatoxin in Feed

There was a negative correlation between aflatoxin type in feed and moisture content in feeds collected from the corresponding dairy and beef farm, with a correlation coefficient of -0.0141.The ANOVA results revealed that, there was a significant mean differences in aflatoxin contamination of aflatoxin between and within group of feed type among the two study sites (Bishoftu and Adama) at a significance Probability- value of (Sig) = 0.00 for aflatoxin within and between group of feed type (compound feed and ingredient feed). Taking alpha (α) level of significance 0.05. $P = 0.00 < 0.05$ for B1, B2, G1 and G2.

In conclusion there was a significant mean difference between and within a group of feed in the two study sites.

5. DISCUSION

5.1. Survey Result

Field and postharvest practices can predispose crop produce to aflatoxin contamination. The risk of contamination is greater in developing countries where peasant farmers who constitute the majority face financial challenges and have little or no access to improved technology.

Among dairy and beef producers about 41(76%) and 39(72%) didn't know the possible source and risk factors that influence aflatoxin production. Only 15(28%) know about risk factors that influence aflatoxin production of which 12(22%) of dairy and beef farmers respondents knows biological/biotic factors were the risk and the possible source of afalatoxin production and all of them said that the presence of other fungi or microbe and strain variation in the fungus was the types of risk factor that produce aflatoxin.

The results of this study is in contrary with the factors that influence mycotoxin production are either biological (biotic), environmental (abiotic) or nutritional (Diener and Davis, 1966; Okelloetal., 2010). Some of the biotic factors include cultivar susceptibility and growth stage, insect and bird damage and presence of other fungi or microbes and strain variation in the fungus while abiotic factors include mechanical damage, moisture, temperature, pH and other crop stresses such as drought, soil type, suitability of substrate, excessive rainfall, gaseous exchange andgaseous environment and preservatives and crowding of plants (CAST, 1989; CRA, 2011; Suttajit, 1989; Robens, 1990; William *et al*., 2004). Nitrogen stress is another biotic factor which can also predispose crops to aflatoxin contamination. This is consistent with the findings by Strosnider *et a*l., (2006) who reported that it is advisable to keep the grains from contact with the earth, raising them on wooden pallets or on concrete floor and ensuring adequate ventilation in a storage facility helps to prevent an increase in moisture content, insect and rodent infestation during storage, and is a critical measure against aflatoxin contamination.

Most of the dairy and beef cattle producers were not aware of drying and ventilation use 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.The findings by Hawkins *et al*., (2005) confirms that high levels of humidity, temperature and poor aeration during storage are important factors that may contribute to aflatoxin contamination. In another study, Smith et al. (1985) further emphasizes that aflatoxin contamination can occur when food commodities are stored under high moisture and temperature conditions.

5.2. Laboratory Result

The detection and level of aflatoxin in this study showed that 23 feed samples (38%), did not contain any level of the toxins; whereas 37 of the feed samples (62%) were found to be contaminated with aflatoxins. Out of this 23feeds and feed ingredientsamples (38.33%) were found to haveaflatoxins within the EU permitted level of 5µg/kg, and the FDA permitted level of 20 µg/kg AFB1, respectively (Table 12). This result was similar to the AFB1 level of feeds reported in India almost all (83%) of the feed samples fulfilled the permissible limits of the E.U. and the FDA. (Senerwa, 2016). Another study by (Kang‟ethe *et al*. 2007) who reported that out of 70 feeds obtained from farmers, 98.6% had traces of aflatoxins B1 while 83% (58) had aflatoxin levels equal or above 0.01mg Kg-1.

The results of this study is in contrary with the AFB1 level of feeds reported in India, Kenya and previous authors in Ethiopia in which 17% and 15%, 46% and 45% of the feed samples did not fulfill the U.S.A AFB1 level recommended for feeds (Table 1). The reason for the difference of AFB1 in feeds between Ethiopia and Kenya; as well as Ethiopia and India could be due to the difference in feed type used in Ethiopia (wheat by-products based feeds) and other African countries and India (by-products of maize and groundnut based feeds) which are the most susceptible crops to AFB1 contamination as compared to wheat-based feeds (lewis l. *et al*., 2005).

Table 13: Aflatoxin AFB1, AFB2, AFG1 and AFG2 (µg/kg) in feeds and feed ingredients in this study and other countries in comparison with EU and USA permissible limits

Country	N	Range (µg/kg)	0 level	EU Standard	USA Standard	> USA Standard	Reference
Ethiopia	60	0-1953	38.3	20	18.33	23.33	This study
Portugal	312	5.1-74	0	84	14	2	Dawit G. (2016)
Indian	40	1.8-245	28	13	55	17	Senerwa D.M.(2016)
Kenya	144	1-9961	0	27	54	46	Makau C.M.,(2016)
Indian	356	-	46	NA	39	15	Rehrahie M. (2001)
Ethiopia	156	7-419	0	5	50	45	Idris Y. M. (2010)

In the present study analysis of individual feeds revealed that noug cake, was highly contaminated with aflatoxin AFG2, AFG1, AFB2, AFB1 and total aflatoxin and corn had relatively low levels of aflatoxin contamination as compared to noug cake, while aflatoxin contamination in wheat bran and wheat middling too much lower than noug seed cake and corn. Previous study conducted in Ethiopia by Dawit *et al*., 2016 reported that level of aflatoxin B1 in noug seed cake were ranging from 290 to 397µg/kg, where as in the present investigation aflatoxin B1 in noug seed cake was range from 26.96 to1953.14 µg/kg which means much higher than the previous findings. Our finding was comparable with the finding of Barbara Szonyi, (2015) in Addis Ababa using ELISA and Yohannes *et al*. (2018) in Gurage zone using HPL(C,Muluken Fikere, 2017) in around Addis Ababa, Kang, (2009) in Kenya reported the presence of aflatoxin in noug seed cake which exceeded the limit of FDA action level and European Union.

In comparison of the present investigation with different international standards the average level of aflatoxin AFG2, AFG1, AFB2, AFB1 and total aflatoxin in noug seed cake were 49.12 µg/kg, 352.62 µg/kg, 73.11 µg/kg, 476.09 µg/kg and 950.93 µg/kg, respectively was exceeding the acceptable limit of US Food and Drug Administration (FDA) 20µg/Kg, FAO, WHO 20µg/Kg and EU 5µg/Kg for dairy feed. The reasons for high Aflatoxins content in noug seed 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 Noug seed has fewer diseases than other oil seeds, molds such as *Aspergillus* Noug*, A.flavus, Penicilliumsp, Alternaria alternata, Rhizoctoniasolani and R. bataticolaare* 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 noug seed cake may be support mold growth and formation of aflatoxin.

According to the result of individual samples in meaze/corn/ about 33.33% (3 of 9) of the samples was not contaminated and the reaming 66.66 % (6 of 9) contaminated with aflatoxin..The mean aflatoxin AFB1, AFB2, AFG1 and G2 were not exceed the limit of FDA action level 20µg/kg, food and agriculture organization (FAO)/World Health Organization (WHO) 20µg/Kg and but the mean aflatoxin AFB1 and AFG1exceed the limit of EU 5µg/kg for dairy cattle feed.

The study conducted by Amare, 2010. In corn sample from Adama, Ambo and Diredawa showed that 88% of the samples contain 27µg kg-. Another study by Muture *et al*., (2005) in Makueni County of Kenya had aflatoxins levels greater than the Kenya regulatory limit (20 µg/kg). Findings by Lewis *et al*., 2005 where 55% of maize products sampled during 2004 aflatoxicosis outbreak had aflatoxins levels greater than Kenya regulatory limit of 20 µg/kg. (Lunyasunya *et al*., 2005).

The result of this study in wheat bran and wheat middling samples showed that from the total eighteen samples (each 9 wheat bran and wheat middling) of only 1 (11.11%) and 2%(22.22) of the samples were contaminated with aflatoxin G1, B2 and B1, respectively. In comparison the level of aflatoxin B1 in wheat bran with the recent study conducted in Addis Ababa and its surroundings by Dawit, (2016), who reported that the level of aflatoxin B1 in wheat bran ranges from 9- to 31 μg/kg and in the present investigation found from 0 to 0.13μg/kg which is much lower than the previous findings. In comparison to the present investigation with different international standards the average level of aflatoxin G1, B2, B1 and total aflatoxin in wheat bran were .015ng/g, 0ng/g, 0 ng/g and 0.13ng/g respectively, Aflatoxin B1, B2, G1 and total aflatoxin were not above the limit of FDA action level 20μg/kg, FAO, WHO 20μg/Kg and EU 5μg/kg for dairy cattle feed.

The lowest AFBI was recorded from wheat bran and wheat middling (concentrate feed) with AFBI of 0.13μg/kg and 0.015μg/kg, respectively. The pattern of AFB1 contamination in roughage feeds and concentrate feeds was similar to the report of Makau, (2016) who showed that the concentrate feeds (47.841μg/kg) contained almost 10 times more AFBI than the roughage feeds (5.14 g/kg) indicating concentrate feeds are more susceptible to AFB1 contamination than roughage feeds. This might be due to the fact that concentrate feeds are rich in nutritional composition than roughage feeds which can provide adequate substrate for growth and reproduction of fungi. This is justified by the fact that compared to grass hay which contains 59 gm CP/kg feed, noug and noug based

concentrate feeds contained CP contents of 312 g/kg and 251.3g/kg, respectively (Rehrah ia and Ledin (2001), Ephrem (2015) also reported that food stuffs with high fat content are good substrates for aflatoxin producing fungi and are more susceptible toAFB1contamination as compared to other dairy feeds Makun 2012).

In general, the highest AFB1 content in feeds observed in Bishoftu might be due to the relatively optimal temperature which could be aggravated by the relatively longer storage duration.This is comparable to the report of Dawit, 2016).

6. CONCULUSION AND RECOMMENDATION

The result of knowledge, attitude and practice (KAP) assessment in this study revealed that, awareness of factors that influence aflatoxin production, knowledge about possible source of aflatoxin and formation of mycotoxin is very low among the dairy and beef cattle farmers. In this study, the majority feeds and feed ingredients samples were contaminated with AFG1, AFG2, AFB1, AFB2 exceeding the FDA and EU limit action levels. Whereas few feed samples were found to be within the stringent EU and the more permissible USA standards. The pattern of aflatoxin distribution amongst the different feed sources showed that concentrate feeds such as *noug* seed cakes were more contaminated, followed by lin seed cake, corn, cereal by product (wheat bran and wheat middling), respectively. The aflatoxin contamination level was higher in dairy than beef cattle producers. All variations in aflatoxin contents amongst feeds and sampling sites were due to different nutritional contents of feeds, environmental condition of the site and the storage situations by the value chains actors in different areas.Therefor; based on the above conclusion the following recommendations were forwarded:

- 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 contaminati on of the feed.
- There is a need to educate and train dairy and beef farmers on good agricultural practices and good storage practices to produce grains and feeds with low levels aflatoxin contamination
- Regulatory body should be provide regulatory limit of aflatoxin in dairy and beef cattle feeds and also conduct post marketing surveillance related to aflatoxin contamination of feeds.
- Further research should focus on risk mitigation targeted at mixed feed containing noug seed cakes as the primary source of aflatoxin contamination in dairy production.

- Further studies are required considering season of the year and different storage conditions such as use of palate, ventilation, and duration of feed storage on aflatoxin and nutrient contents of feeds.

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8. APPENDICIES

Appendix I: Questionnaire used to assess Knowledge, Attitude and Practices (KAP) of aflatoxin Contamination in Compound feed and feed ingredient of Dairy and beef producers

PART I SOCIO-DEMOGRAPHIC CHARACTERSICS

Statement	Response		
	Frequency		Percentage
Age	20-30	7	13
	30-50	37	69
	>50	10	18
Sex	Male	43	80
	Female	11	20
Marital status	Single	13	24
	Married	39	72
	Windowed	0	0
	Diverse	2	4
Educational	Illiterate	0	0
	Basic	2	4
	Primary/Elementary	12	22
	Secondary	25	46
	Higher Education	15	28
Family income	2000-4000	30	56
	4000-6000	11	20
	6000-10,000	4	7
	>10,000	9	17

PART II: KAP Questioner for dairy and Beef producers

Summary of aflatoxin Knowledge among dairy farmers and beef producers

Statement	**Response**		
Do you know aflatoxin?		Frequency	Percentage
	Yes	18	33
	No	13	24
	Don’t know	23	43
Do you have knowledge about possible source of aflatoxins?	Yes	13	24
	No	41	76
Do you know factors that influence aflatoxin production?	Yes	15	28
	No	39	72
Do you know that animal can get aflatoxicosis?	Yes	11	20
	No	6	11
	Don’t know	37	69
Do you think it contaminate animal product?	Yes	18	33
	No	1	2
	Don’t know	35	65
If yes, which product?	Milk	15	28
	Meat	2	4
	Both meat and milk	1	2
What is mould? can you describe it	Yes	49	91
	No	5	9
	Don’t know	0	0
Do you have information about how mould can develop?	Yes	49	91
	No	5	9
	Don’t know	0	0
Do you have selection criteria for quality of animal feed?	Yes	49	91
	No	5	5
If your answer is yes, what are your selection criteria?	Color	12	22%
	Odor	13	24%
	Color, odor	17	31%
	Color, Oder, Moisture	7	13%
Which storage conditions are favorable for mould growth on animal feed	Moist	7	13%
	Hot and Moist	35	65%
	Hot	5	9%
	Don’t know	4	7%

Do you get a problem when you store animal feed for a longtime	Yes	17	31%
	No	37	69%
If your answer is yes, what type of problem?	Insect	2	4%
	Mould	14	26%
	Both insect and mould	1	2%
Do you think animal feed can be contaminated with aflatoxin?	Yes	18	33%
	No	0	0%
	Don't know	36	67%
If your answer is yes which animal feed are the most contaminated?	Noug seed cake	8	15%
	Wheat bran	2	4%
	Brewery grain	1	2%
	Wheat bran and brewery grain	2	4%
	Compound feed	3	6%
	Corn	2	4%
	Poultry waste	1	2%
Do you think moldy feed can affect animal health?	Yes	43	80%
	No	0	0%
	Don't know	11	20%
Do you think mould growth can create on animal feed?	Yes	45	83%
	No	0	0%
	Don't know	9	17%

Summary of aflatoxin Attitude among dairy farmers and beef producers			
Statement	**Response**		
		Frequency	Percentage
Type of feed you prefer for Dairy/Beef cattle	Noug seed cake	0	0
	Wheat bran	5	9
	Corn	2	4
	Compound feed	41	76
	Cotton seed cake	6	11
Do you prefer the lower quality with lower price to purchase dairy feed?	yes	18	33
	no	36	67

Is there a problem the food that not uses for human consumption, due to mold growth, damage or expire used as animal feed?	Yes	35	66
	No	10	18
	Don't know	9	16

Summary of aflatoxin Practices among dairy farmers and beef producers

Statement	Response		
		Frequency	**Percentage**
Where do you get animal feed?	Feed processor	21	39
	Farmers	3	5
	Factories	10	18
	Processors and Traders	3	5
	Processors and Factories	2	4
	Traders	15	28
Do you store your animal feed for long time?	Yes	7	13
	No	47	87
If your answer is yes, how would you store your feed?	Raise and Dry	6	11
	Raised and Humid	0	0
	On floor and Dry	5	9
Where do you store animal feed for a long time?	Open space	9	13
	In the shade	13	24
	In the house	32	59

Do you have the habit to clean the store before storing of animal feed	Yes	43	83
	No	11	20
Do you dry animal feed before storage?	Yes	14	26
	No	40	74
Have you used aflatoxin/mould mitigation methods?	Yes	34	63
	No	20	37

Appendix 2: Flow Chart of AFLATEST® High Performance Liquid Chromatogra-phy (HPLC)

AFLATEST®HPLC OVERVIEW

SAMPLE EXTRACTION
Grind and weigh sample
Blend sample with salt and methanol/water mixture
Filter through filter paper

↓

DILUTION AND FILTRATION
Dilute aliquot of extracted sample with PBS
Filter through syringe filter

↓

AFFINITY CHROMATOGRAPHY
Pass filtrate over affinity column
Pass water over column, Elute with methanol;
derivatize using TFA and collect eluate in a cuvette.

↓

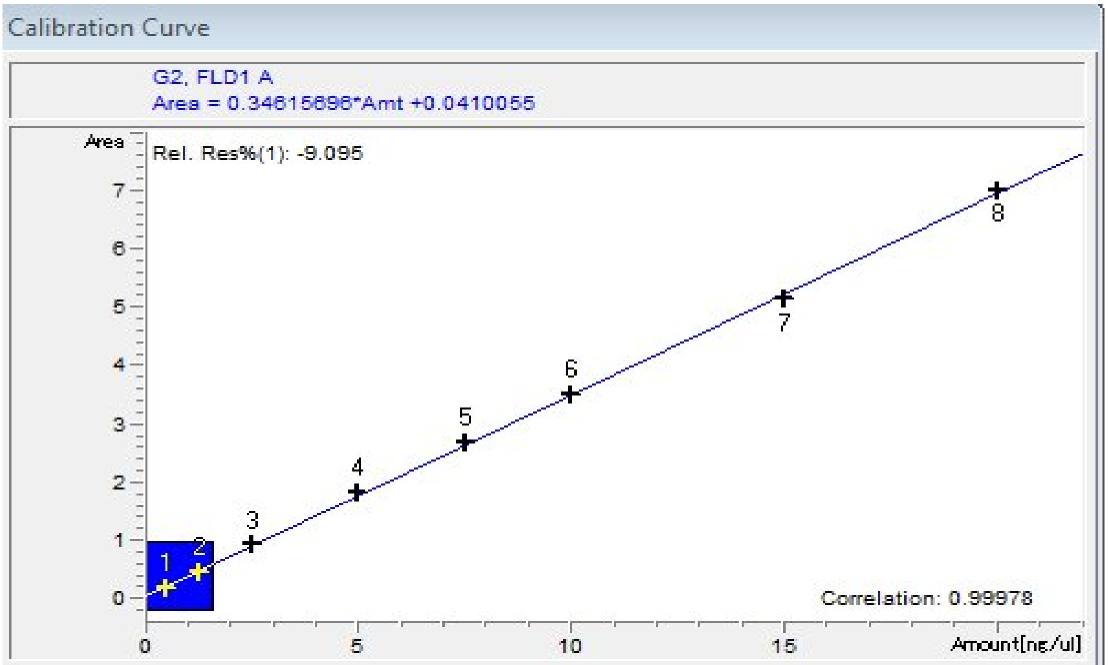
MEASURE AFLATOXIN
Dilute with mobile solvent
Inject onto HPLC

Appendix 3: Method Parameters

The wavelength conditions of the FLD for Aflatoxin was set to be 360 nm for the excitation wavelength, 450 nm for the emission wavelength; Mobile phase A consisted of water, B is acetonitrile, and C is methanol (60:25:15, v/v), respectively. Isocratic conditions were used for quaternary pump system. The flow rate was 1ml/minute and the injection volume was 20 µl and it was standard injection with needle wash. Stop time for quaternary pump was 10 min. The temperature of the column was 35°C on both sides.

CONDITIONS			
Column	ZORBAX SB-C18, 3.5µm, 150 x 4.6 mm with precolumn		
Eluent A	Water		
Eluent B	Acetonitrile		
Eluent C	Methanol		
Isocratic condition	%A	% B	%C
	60	25	15
Flow rate	1 ml/min		
Injection volume	20 µl		
Column temperature	35°C both sides		
UV detection	220 nm (20 Hz) Ex 360 nm Em 440 nm		
FLD detection	(5 Hz, 0.1s, Sensitivity: High, Gain 16)		
Post column derivatization with UVE photochemical reactor at 100 µA			
Standard	sample Mixture of aflatoxin B1, B2, G1 and G2 (0.5 – 20 ppb each)		

Appendix 4: Calibration Curve

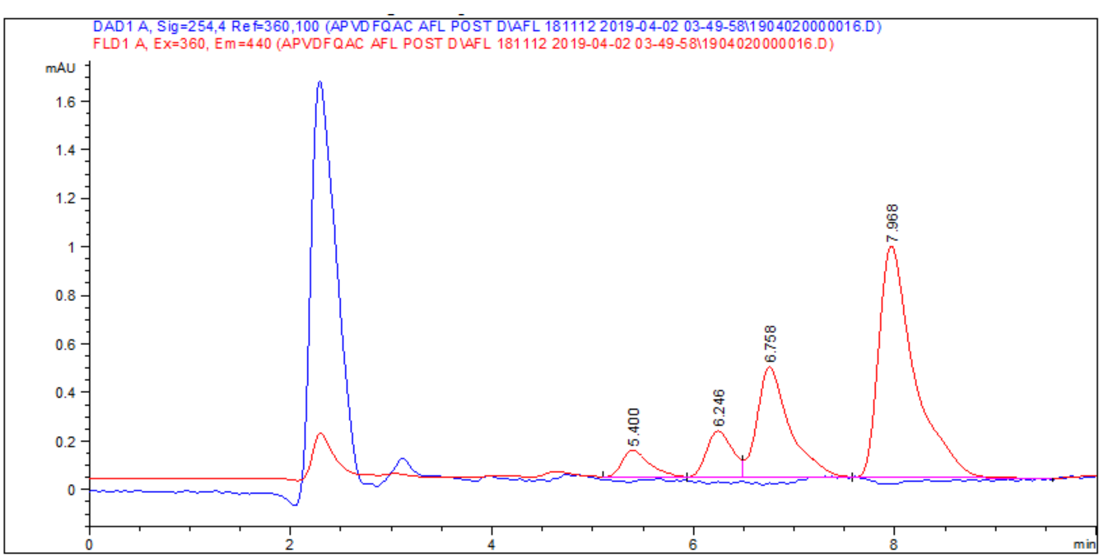


#	RT	Signal	Compound	Lvl	Amt[ng/ul]	Area	Rsp.Factor	Ref	ISTD
1	5.398	FLD1 A	G2	1	0.500	1.9461e-1	2.569	No	No
				2	1.250	4.7078e-1	2.655		
				3	2.500	9.1999e-1	2.717		
				4	5.000	1.824	2.740		
				5	7.500	2.691	2.787		
				6	10.000	3.510	2.849		
				7	15.000	5.130	2.924		
				8	20.000	7.004	2.856		
2	6.250	FLD1 A	G1	1	0.500	9.1543e-2	5.462	No	No
				2	1.250	1.9611e-1	6.374		
				3	2.500	3.9042e-1	6.403		
				4	5.000	7.7678e-1	6.437		
				5	7.500	1.146	6.545		
				6	10.000	1.485	6.732		
				7	15.000	2.167	6.921		
				8	20.000	2.912	6.868		

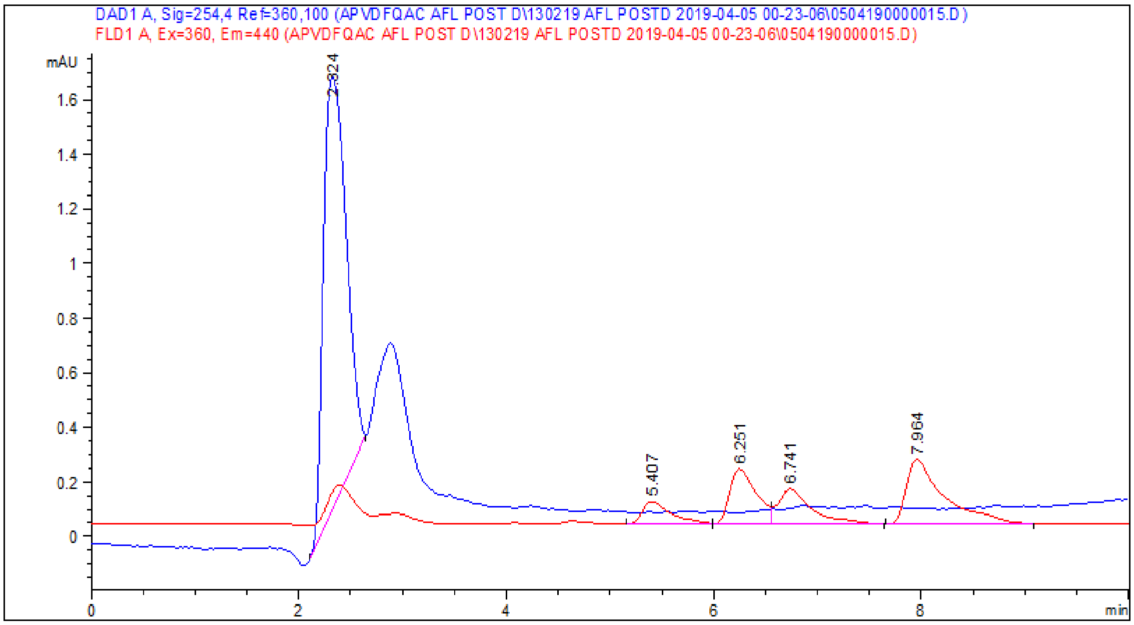
3	6.758	FLD1 A	B2	1	0.500	4.9952e-1	1.001	No	No
				2	1.250	1.174	1.065		
				3	2.500	2.328	1.074		
				4	5.000	4.606	1.086		
				5	7.500	6.736	1.113		
				6	10.000	8.917	1.121		
				7	15.000	13.115	1.144		
				8	20.000	18.018	1.110		
4	7.970	FLD1 A	B1	1	0.500	2.0940e-1	2.388	No	No
				2	1.250	4.9687e-1	2.516		
				3	2.500	9.5024e-1	2.631		
				4	5.000	1.878	2.663		
				5	7.500	2.744	2.733		
				6	10.000	3.665	2.729		
				7	15.000	5.415	2.770		
				8	20.000	7.467	2.679		

Appendix 5: Graphs of Samples Results Reading through HPLC INSTRUMETS

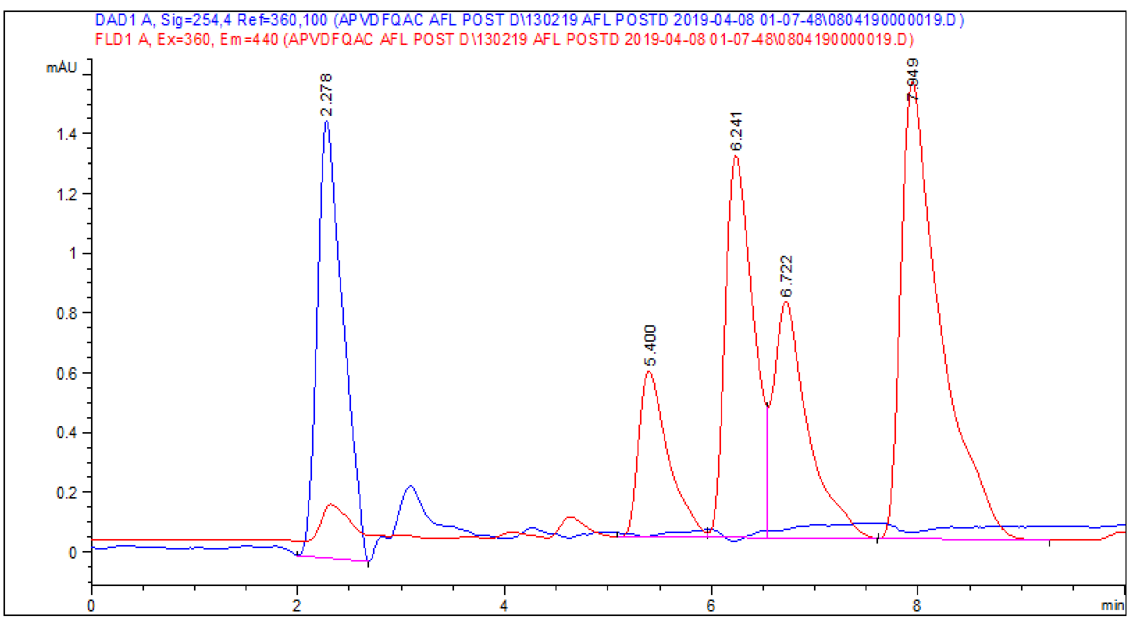
Sample: compound feed (code BDCP-09)



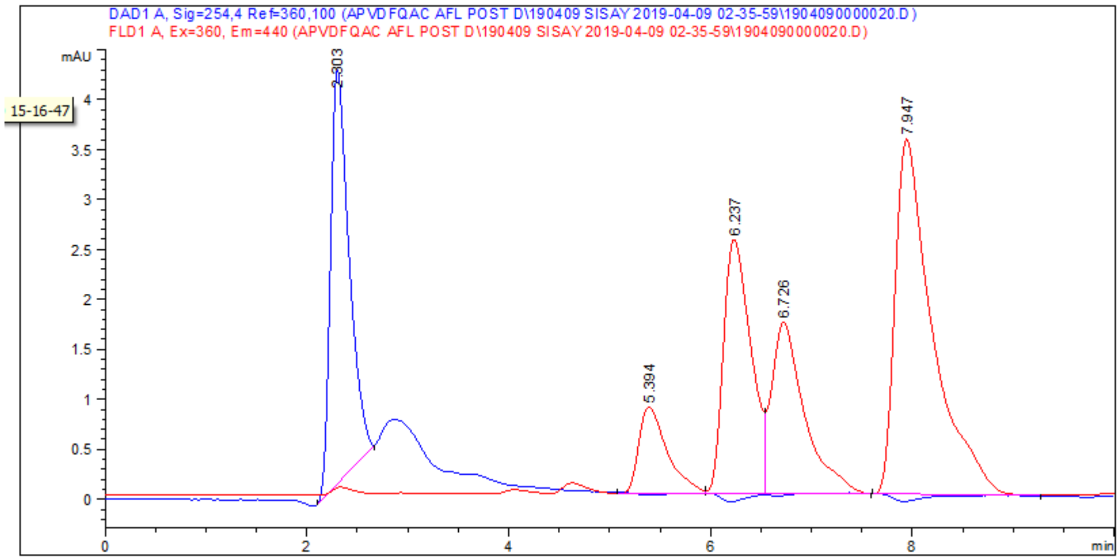
Sample: Compound feed (Code ABCM 15)



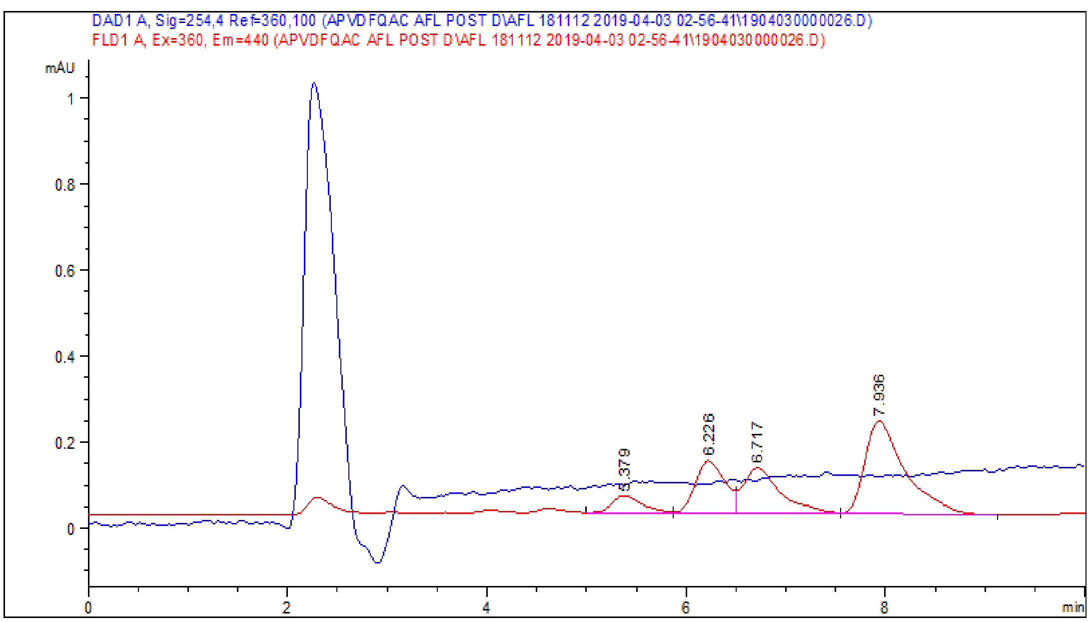
Sample: Lin seed Cake (code: ALC 03)



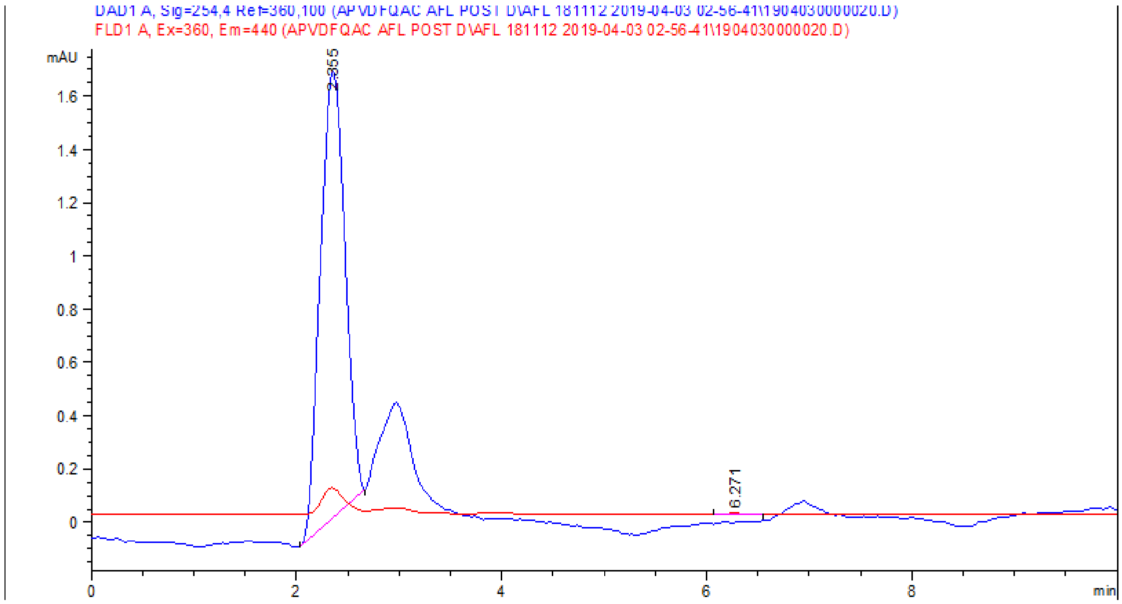
Sample: Noug seed cake (Code BNC 09)



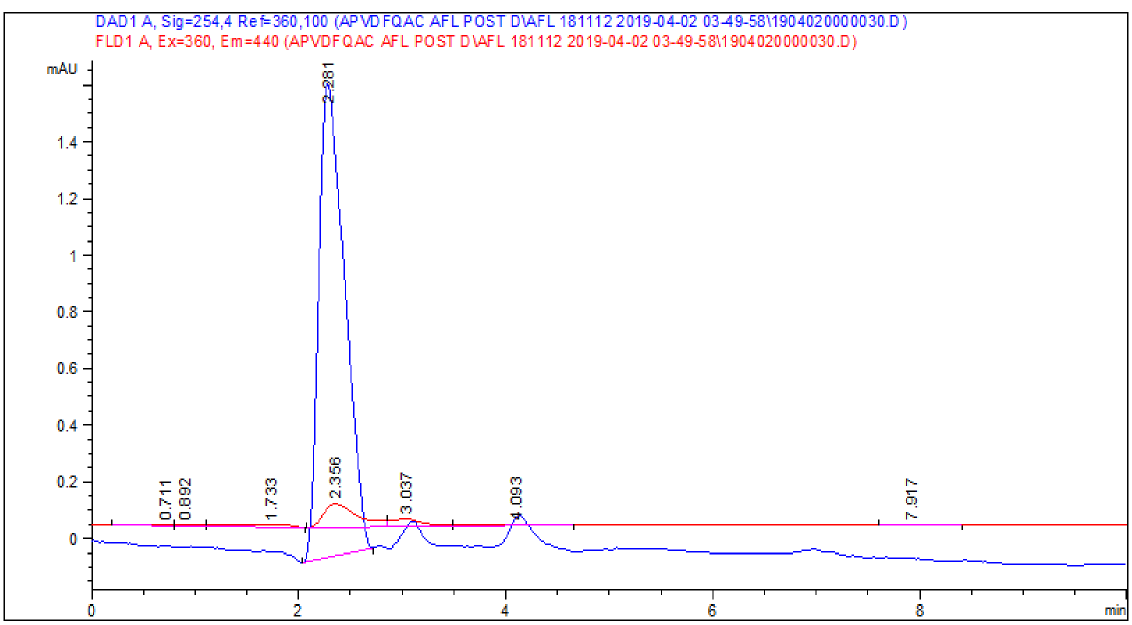
Sample: Corn (Code; BCO 30)



Sample: Wheat Bran (Code BWB 26)



Sample Wheat Middling (Code BWM 13)



Appendix 6: Storage of Compound feeds and Feed Ingredients in Beef Cattle producers

Appendix 7: Storage of Compound feeds and Feed Ingredients in Dairy farmers