ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

CENTER FOR FOOD SCIENCE AND NUTRITION

Level of aflatoxin in dairy feed, poultry feeds and feed ingredients produced by feed factories in Addis Ababa, Ethiopia

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Declaration

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

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Approval page

As thesis research advisors, we hereby certify that we have read and evaluated this thesis, prepared under our guidance and recommend that it be submitted as fulfilling the thesis requirement.

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TABLE OF CONTENT

ACKNOWLEDGEMENTS iii

LIST OF TABLES vi

LIST OF FIGURES vii

ABBREVIATIONS AND ACRONYMS viii

ABSTRACT ix

1. INTRODUCTION 1

1.1. Background 1

1.3. Significance of the study 3

1.4. Objectives 3

1.4.1. General Objective 3

1.4.2. Specific Objectives 3

2. LITERATURE REVIEW 4

2.1. Animal source of food 4

2.2. Animal feed ingredients 4

2.3. Common feed ingredients 5

2.3.2. Industrial by- products 6

2.5. Occurrence of aflatoxin in animal feeds 8

2.7. Prevalence of aflatoxin in animal feed and feed ingredient in Ethiopia 11

2.8. Effects of aflatoxin on food-producing animals 11

2.8.1. Effects of aflatoxin on dairy cattle 12

2.8.2. Effects of aflatoxin on poultry 13

2.9. Control measure of aflatoxin in feeds 14

2.9.1. Pre-harvest control method 15

2.9.2. Post-harvest control method 15

2.11. Animal feed factories in Ethiopia 18

3. **MATERIALS AND METHODS** 19

3.1. Description of the study area 19

3.2. Sampling and sample collection 19

3.3. Sample preparation 19
3.4. Study design for KAP (Knowledge Attitude and Practice) 20
3.5. Determination of nutritional analysis by NIR 21
3.6. Analysis of aflatoxins in the feeds 21
3.6.1. Chemicals and reagents 21
3.6.3. Extraction and clean up 23
3.6.4. HPLC determination and calculations 24
3.7. Statistical data analysis 24
4.1. Identification of aflatoxins 25
4.2. Preparation of calibration curve 26
4.3. Accuracy and precision 27
4.4. Level of aflatoxins in feed ingredients 29
4.6. Level of aflatoxins in the animal feeds 31
4.6.1. Aflatoxin levels in dairy feeds 31
4.6.2. Aflatoxin levels in poultry layer Feeds 33
4.8. Aflatoxin levels in poultry broiler feeds 34
4.9. Comparison of aflatoxin levels among the feeds 36
4.10. Aflatoxin knowledge assessment of feed producer and farmers 38
5. **CONCLUSIONS AND RECOMMENDATIONS** 41
5.1. Conclusions 41
5.2. Recommendations 42
6. **REFERENCES** 43
7. **ANNEXES** 51
Annexe-1. Interview form 51
Annex-2. NIR results of dairy, poultry and feed ingredients 52
Annex 3. Calibration Curve 54
Annex -4. A pictures of sample preparation and analysis 56
Annex-5. Chromatogram of individual aflatoxin in dairy, poultry feed and feed ingredient samples 57

LIST OF TABLES

Table 1. FDA maximum level of aflatoxin in animal feeds and food 17

Table 2. Structure of the commercial feed sub-sector in Ethiopia........ 18

Table 3. The retention time for mixed aflatoxin standard (20μg/L)........ 25

Table 4. Accuracy and recovery of data for aflatoxin analysis in feeds and ingredients 28

Table 5. Average levels of aflatoxin G2, G1, B2, B1 and total AF in feed ingredients........ 29

Table 6. Average levels of aflatoxin G2, G1, B2, and B1 in dairy cattle feed 32

Table 7. Average level of aflatoxin G2, G1, B2, and B1 in poultry layer feed........ 33

Table 8. Average level of aflatoxin G2, G1, B2, and B1 in poultry broiler feed 35

Table 9. Comparison of aflatoxin levels among the feeds........ 37

Table 10. Percentage of Aflatoxin B1 contamination in dairy, poultry feed and feed ingredients 38

Table 11. Summary of aflatoxin KAP among dairy, poultry feed producers and farmers........ 39

LIST OF FIGURES

Figure 1. Chemical structure of aflatoxin B1, B2, G1 and M1 (Rawal et al., 2010) _________ 9

Figure 2. Flow chart of study design _________ 20

Figure 3. HPLC Instruments at Bless Agri Food Laboratory Service Testing Laboratory, Addis Ababa. _________ 22

Figure 4. Chromatogram for blank (methanol) _________ 25

Figure 5. Chromatogram for 20μg/L mixed aflatoxin standard solution _________ 26

Figure 6. Overlaid chromatogram of the calibration series _________ 26

Figure 7. Chromatogram for sample _________ 27

Figure 8. Aflatoxin levels in feed ingredient collected from Addis Ababa _________ 30

Figure 9. Aflatoxin contamination of dairy feed in different feed factories _________ 32

Figure 10. Aflatoxin contamination of poultry layer feed in different feed factory _________ 34

Figure 11. Aflatoxin contamination of poultry broiler feed in different feed factories _________ 36

ABBREVIATIONS AND ACRONYMS

AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
AOAC	Association of Official Analytical Chemists
ANOVA	Analysis of Variance
BSG	Brewers Spent Grain
CEC	Commission of European Communities
CSA	Central Statistics Agency
EAFIA	Ethiopian Animal Feed Industry Association
EC	European Commission
EU	European Union
ESA	Ethiopian Standard Agency
μg/kg	Micro gram per kilogram
μg/L	Micro gram per liter
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FLD	Florescence Detector
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity Column
IARC	International Agency for Research on Cancer
KAP	Knowledge, Attitude and Practice
LOD	Limit of Detection
LOQ	Limit of Quantification
NIR	Near Infrared
ppb	parts per billion
RSD	Relative Standard Deviation
SPSS	Statistical Package for the Social Sciences
UK	United Kingdom
USA	United Sates of America
VDFCA	Veterinary Drug and Feed Administration Control Agency
WHO	World Health Organization

ABSTRACT

Aflatoxins are one of the major factors that affect the quality and safety of feeds that are transferred into livestock species and then to humans via animal sources of food (meat, egg and milk). The objective of this study was to detect and quantify the level of aflatoxins (B1, B2, G1, G2 and total aflatoxin) in dairy feed, poultry (layer and broiler) feed and feed ingredients produced in feed factories in Addis Ababa also to evaluate knowledge Attitude and Practice (KAP) of feed producers and farmers in regard to feed quality and safety. A total of 42 samples consisting of five dairy feeds, six poultry broiler feeds, six layer feeds and 25 feed ingredients were collected from seven dairy and poultry feed factories found in Addis Ababa and its surroundings. Samples were analyzed using High Performance Liquid Chromatography after cleaned up with Immunoaffinity columns. The evaluation of KAP was done using a structured questionnaire. In the feed samples analyzed, the aflatoxin B1 levels obtained ranged from (51.66 to 370.51) µg/kg in dairy cattle feed; from (1.45 to 139.51) µg/kg in poultry layer feed; and from (16.49 to 148.86) µg/kg in broiler feed. In feed ingredients on the other hand, aflatoxin B1 levels ranged from (2.64 to 46.74) µg/kg in maize grain; from Not Detected (ND) to 3.66 µg/kg in wheat bran; from ND to 12.77µg/kg in wheat middling; from ND to 3.43µg/kg in soybean; and from (110.93 to 438.86) µg/kg in niger seed cake. 100% of dairy feeds, 67% of poultry layer, 67% broiler feeds and 24% of ingredients contained aflatoxin in levels higher than the maximum tolerable limit set by the US Food and Drug Administration and Ethiopian Standard Agency. The result of KAP assessment revealed that only 63% of feed producers and farmers had awareness about the formation of aflatoxins in feeds. In conclusion, dairy feed from feed types and niger seed cake from feed ingredients were the most heavily contaminated with aflatoxins that need better feed management by the producers and strong regulation by the government. There should be regular monitoring of aflatoxin in animal feeds by the feed manufactures to prevent aflatoxin buildup in dairy feeds, poultry feeds, and feed ingredients to ensure the health of animals and safety of animal source of foods like milk, egg and meat.

Keywords: Aflatoxin, Feeds, Dairy, Poultry Layer, Poultry Broiler, Ingredients

1. INTRODUCTION

1.1. Background

Animal source foods such as milk, meat and eggs currently provide around 13% of the energy and 28% of the protein consumed globally; in developed countries, this rises to 20 and 48% respectively. Consumption of livestock products per capita has markedly outpaced growth in consumption of other major food commodity groups. Since the early 1960s, consumption of milk per capita in the developing countries has almost doubled, meat consumption more than tripled and egg consumption increased by a factor of five (Tilman, 2014).

Animal feeds have an important role in enabling economic production of animal-source foods. Feeds may be produced in industrial feed mills or simple on-farm mixers or by hand mixing (Afsah *et al.*, 2013). Poor quality and safety of animal feed directly affect the quality of meat, milk and milk products (Smith, 2015). Feed supply and feed safety are intimately linked because feeding stuffs origin, processing, handling, and storage, as well as many other factors related to the market, can affect at different levels both quality and safety of feed. One of the factors that affect animal feed quality and safety is fungal contamination of cereal grains, oil-seed meals, and forages which presents a major animal health risk throughout the world and particularly in the humid tropics (Pinotti, 2011).

Aflatoxins are capable of minimizing the nutritional value of animal feed by elaborating various mycotoxins; feeds contaminated with the toxins affect animal health and productivity (Akande *et al.*, 2006). The toxins are produced in cereal grains as well as forages before, during and after harvest, in various environmental conditions (Yiannikouris and Jonany, 2002). Aflatoxin is one of the different types of mycotoxins fungal metabolites produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin is produced at a temperature of 12-40°C and requires 3-18% moisture (Duncan and Hagler, 2008).

Several studies have been reported that high levels of aflatoxin in dairy cattle feeds, poultry layer and broiler feeds and feed ingredients were above the recommended FDA limit (Dawit *et al.*,2016; Rehrahie, 2018; Kotinagu, 2015; Greco, 2014). The presence of aflatoxins in feeds may decrease feed intake and affect animal performance. Besides, the possible presence of toxic residues in an edible animal product such as milk, meat, and eggs has detrimental effects on human health. Fungal contamination affects both the organoleptic characteristics and the alimentary value of feeds and entails a risk of toxicities (Akande *et al.*, 2006; Mkau, 2016: Dawit, 2016). Hence, this study will contribute to ensure the safety of animal-sourced products for human consumption as well as to safeguard the health of the consumers by increasing knowledge and awareness of aflatoxin contamination on animal feed.

1.2. Statement of the Problem

Inadequate supply of quality feed and the poor quality of ingredients used as feed are the major factors limiting livestock productivity in Ethiopia. Feed, usually grass based not available in sufficient quantity due to fluctuating weather conditions or when available are of poor nutritional quality. Small hold farms in Ethiopia have little chance of selecting feeds since farmers use what is available which is grain based and rarely find access to readymade feeds. This resulting in low milk and meat yields high mortality of young stock, longer parturition intervals, and low animal weights (Ahmed *et al.*, 2004).

The presence of aflatoxin contaminated milk in the market is becoming an alarming problem. A recent study conducted on dairy and feed samples collected from the greater Addis Ababa milk shed indicated that all collected milk and feed samples had detectable levels of aflatoxin. Most of the milk was contaminated with aflatoxin M1 and it was presumed that aflatoxin M1 contamination in milk must have originated from aflatoxin B1 contaminated cattle feed of lactating cows (Dawit *et al.,* 2016; Rehrahie, 2018).

Considering the serious health related issues associated with aflatoxin, it is important to study the contamination level of animal feeds thoroughly and look into possible factors for the contamination of animal source of food to reduce the health risk of consumers at risk for health problems and major economic setbacks. This study will investigate the occurrence and contamination level of aflatoxin in animal feeds and ingredients produced in Addis Ababa.

1.3. Significance of the Study

The study will help in creating awareness to feed suppliers on major animal feed ingredients that are susceptible to aflatoxin contamination. It will also assist regulatory bodies like the Veterinary Drug, Feed Administration and Control Authority (VDFACA) in implementing regulatory limits on aflatoxin levels in animal feed ingredients, and compound feed. Manufacturers will also have benefited by controlling the safety of ingredients used in the processing of animal feed.

1.4. Objectives

1.4.1. General Objective

To detect and quantify the level of aflatoxin in feeds and feed ingredients produced by animal feed producers in Addis Ababa.

1.4.2. Specific Objectives

- To determine the levels of aflatoxin in dairy, poultry (layer, broiler) feeds and feed ingredients in Addis Ababa.
- To compares the levels of aflatoxin in feeds and feed ingredients with the acceptable limit set by Ethiopia and international regulatory authorities.
- To assess (KAP) on aflatoxin among feed producers and farmers.

2. LITERATURE REVIEW

2.1. Animal source of food

Animal source of foods play an important role in human diet. Nutritionally they are important sources of protein of good quality and excellent sources of vitamins and minerals. In addition, animal foods are in general more distinctive in flavor and texture and often more palatable than foods of vegetable origin. Overcoming deficiencies in diet quantity and quality are major nutritional challenges globally, particularly in developing countries (Yang *et al.*, 2009)

Diet quantity is concerned with the availability and consumption of total food energy (kcal) and diet quality with the ability of the diet to supply protein of high biological value (presence of all essential amino acids) and adequate supplies of micronutrients (vitamins, minerals, and trace metals) to meet biological requirements under a wide range of physiologic and environmental conditions. From earlier emphasis on the protein gap, and then on the energy gap since the late 1980's to the present time, there has been an increasing awareness of "hidden" malnutrition or multiple micronutrient deficiencies, and an appreciation that diet quality is as important as diet quantity (Neumann *et al.*, 2002).

Animal source foods have a positive impact on the quality and micronutrient enhancement of the diet of women and children, and can prevent many micronutrient deficiencies. These deficiencies can impose a heavy individual and societal burden. The task remains to improve access to and utilization of animal source foods by poor families, "look to the farm and not to the pharmacy" (Siekmann *et al.*, 2003).

2.2. Animal Feed Ingredients

As livestock production and productivity increases, feed is the most important thing. Supplying adequate amount of feed for various livestock species involves the formulation of diet. Good quality feeds are produced from good quality feed ingredients (Pond *et al.*, 2004).

Among the various constraints limiting livestock productivity in Ethiopia, poor quality and quantity animal feed is the major one. Verifying to this background, critical feed scarcity for more than 9 months a year and average annual feed deficit of 35% of the requirement (maintenance plus production) has been noted in the status of feed resources in the central highlands of Ethiopia (Tangka, *et al.*, 2002).

Poor nutrition is a major barrier to market-oriented livestock production. It leads to a slow growth rate in growing animals and low production and reproduction performance. Poorly fed animals give a low output of meat and milk. Nutritional problems also lead to delayed age of onset of puberty, long parturition intervals, low conception rates, and low overall lifetime reproductive performance. Under poor feeding conditions, animals take too long to reach optimum slaughter weight and the meat produced by such animals may not satisfy the desired quality attributes to fulfill the demand of the consumers (Adugna *et al.*, 2008).

Feed resources are classified as natural pasture, crop residue, improved pasture and forage, agro-industrial by-products and other by-products like food and vegetable refusal (Gelayenew *et al.*, 2016). Out of these feed manufacturers utilize industrial by products and maize to fulfill the energy and maintenance requirement of the animals.

2.3. Common Feed Ingredients

Natural pasture and hay differ from place to place due to agro climatic conditions. Natural pasture for cattle is declining from time to time and crop residues are being utilized. The most common crop residues are cereal crop residues like barley/wheat straw, green maize fodder, sorghum and oat fodder (Adugna, 2008).

2.3.1 Maize Grain

Maize is the most common grain that is used for animal feed production. The corn plant is efficient at converting large amounts of sunlight into stable forms of chemical energy stored as starch, cellulose, and oil. Corn is the grain most routinely used in commercial poultry diets because it has good energy content and is easy to digest. The amino acid profile of the protein in corn complements the amino acid profile of the other ingredients, such as soybean meal, typically used in feed. Alternative grains are typically evaluated in relation to corn (Jacob, 2015).

2.3.2. Industrial By- products

Agro-industrial by-products produced in Ethiopia include by-products from flour milling, sugar factory, oil seed and brewery factories. These byproducts are mainly used for dairy, poultry and fattening animals. Agro-industrial by-products are rich in energy and/or protein contents or both. They have low fiber content, high digestibility, and energy values compared with the other class of feeds (Mengistu *et al.*, 2017).

Niger seed cake is one of the oilseed cakes commonly used as a protein supplement for livestock in Ethiopia. About 84802.34 tons of Niger seed is processed every year in the country from which about 50% niger seed cake is produced. The protein content of Niger seed cake varies from 28-38% with most values lying between 30-35%. The fat content varies from 2.1- 12.6% with an average of 8.4% and has high fiber (34.4%) and 8.4% lignin) content and low digestibility of 61.7% compared to most other oilseed cakes (Adugna, 2008).

Soybean meal is protein digestibility in poultry is approximately 85% ranging between 82% and 94% for individual amino acid digestibility. Among the vegetable protein sources, soybean meal is used to meet the animal's requirement for limiting amino acids in cereal-based (e.g. maize) diets, because it is usually the most cost-effective source of amino acids the carbohydrates in soybean meal are incompletely digested by colonic microbiota in mono-gastric (Kerley and Allee, 2003). Removal of raffinose and starchyose improve metabolizable energy content by 12%.

Wheat Bran is the wheat grain consists of about 82% endosperm, 15% bran, and 3% germ. Wheat bran is the major milling by-product used as livestock feed in Ethiopia. It is the outer fibrous layer separated from the rest of the grain and germ. It is the physically fibrous and flaky product. It is the outer kernel plus some flour with a protein content of 14-18%. It has high phosphorus (1%) but low calcium (0.1%) content. Wheat bran is quite palatable and is well known for its laxative characteristics because of its swelling and water holding capacity. This is due to its high fiber and non-starch carbohydrate content (Adugna, *et al.*, 2008).

Wheat middling is a by-product of wheat milling. During milling, 70 to 75 percent of the wheat grain becomes flour, and the remaining 25 to 30 percent results in by-products. Wheat middling consist of fine particles of wheat bran, wheat shorts, wheat germ, wheat flour, and some of the by-product from the tail of the mill. Wheat middling cannot contain more than 9.5 percent crude fiber and must have minimums of 14 percent protein and 3 percent fat (Jacob, 2015).

Molasses contains high levels of sugars which are readily digested in the rumen. It is also a good source of minerals such as calcium, potassium, sulfur and trace minerals but deficient in nitrogen and phosphorus. It can be a major or minor component of drought feed. It is a concentrated source of energy that can be stored for a long period. Since the protein content of molasses is negligible, it is usually fed with high-quality protein or urea. Molasses is often used as a carrier for urea because it is palatable and provides a wide range of minerals (Adugna *et al.*, 2008).

Brewers' spent grain (BSG) is the most abundant by-product generated from the beer-brewing process, representing 85% of the total by-products obtained. After the mashing process the insoluble part of the barley grain, the BSG, is in solution with the soluble (liquid) wort. The wort, which will be fermented into beer, is filtered through the BSG, which is a by-product and must be disposed of (Mussatto, 2014).

2.4. Feed Formulation from Feed Ingredients

Feed formulation is the process of quantifying the number of feed ingredients that need to be combined to form a single uniform mixture for livestock and animal industry. Nutritious feeds are formulated by using raw materials and ingredients such as oats, maize, soybean meal, etc. The formulated feeds provide various nutrients that are needed for the growth of livestock. A good animal yield is highly dependent on the quality and safety of feeds supplied therefore the formulated feed should provide a balanced diet for the animal with high-quality ingredients and adequate amount to provide healthier growth (Hardy *et al.*, 2003).

There are various methods of feed formulation calculation. They are amid to provide balanced nutrients for animals at the lowest possible cost using different mathematical techniques. Some of the methods are listed as follows: Pearson's square method, trial and error method, two by two matrix method, simultaneous equation method and linear programming method (Saxena *et al.*, 2012).

Feed formulation is the process of quantifying the amounts of feed ingredients that need to be combined to form a single uniform mixture (diet) for animals that supply all of their nutrient requirements. Feed formulation is done based on the class of the animal, feed ingredient types and constraints and cost and availability of ingredients. Formulation of animal ration is a complicated problem as; the requirement of animals varies with species, the stages of growth, body weight and physiological needs such as pregnancies, milk yield at certain level with different fat percentages (Hamre *et al*., 2013).

2.5. Occurrence of Aflatoxin in Animal Feeds

Aflatoxin is toxic metabolites produced by two species of the genus *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins started getting attention in 1960 when 100,000 turkeys and other poultry in the United Kingdom died in a single event. Cause of death was eventually traced to a toxic contaminant in groundnut meal used in the bird's feed which was later named aflatoxin (Sharma *et al*., 2017).

Eighteen different aflatoxins have been identified, but the major aflatoxins found in animal feedstuffs are aflatoxin B1 (AFB1), B2, G1 and G2. Lactating mammals that ingest AFB1 deposit the 4-hydroxylated metabolite, aflatoxin M1 (AFM1), in their milk. There is an agreement that around 0.3–6.2% of AFB1 in dairy cattle feeds is converted to AFM1 (Creppy, 2002). Aflatoxin M1 (AFM1) is the principal hydroxylated AFB1 metabolite present in the milk of cows fed with a diet contaminated with AFB1. And excreted within 12 hours of administration of contaminated feeds AFM1 in milk has been shown to decline as contaminated feed is withdrawn, with no traces of aflatoxin in milk being detected after 3–4 days of withdrawal (Melkamu, 2014).

Animals are exposed to aflatoxins through their feed. Aflatoxins can be found worldwide in a variety of food and feed commodities especially cereals; the contamination with aflatoxin-producing fungi and the production of the toxin in the products can occur in the field, during storage, transportation at almost all stages of the production chain. In finished animal feed, the contamination of an ingredient could cause the contamination of an entire feed batch (Gallo *et al.*, 2015).

Furthermore, in developing countries, a large proportion of food and feed are handled on the informal market, and animals are often fed crops that are considered unfit for human consumption because of mold, insect damage, or other problems. These crops are especially at risk for aflatoxin contamination.

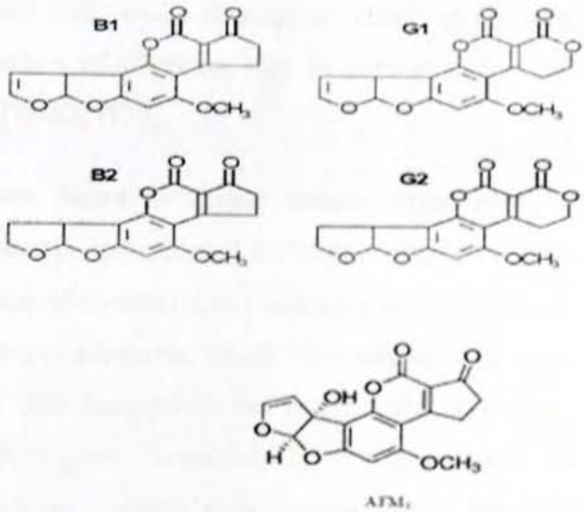


Figure 1. Chemical structure of aflatoxin B1, B2, G1 and M1 (Rawal *et al.*, 2010)

Aflatoxin is a global food safety concern as recognized by the World Health Organization with rural subsistence farming communities in developing countries being the populations most at risk of aflatoxin exposure. Staple foods that are susceptible to aflatoxin contamination, food insecurity, low aflatoxin awareness and lack of enforcement of regulatory limits are some of the contributors to the high level of aflatoxin exposure in these populations (WHO, 2015).

Molds commonly occurring in the natural environment produce mycotoxins in the process of secondary metabolism. *Aspergillus flavus* and *A. parasiticus* are species of molds, which are responsible for the production of aflatoxins and are crucial in the pathogenesis of human diseases. *Aspergillus* species present in decaying plants, the soil, and their spores are transferred via air currents and insects to crops and food storages. Aflatoxins B1, B2, G1, G2, M1 and M2 are the most common derivatives of aflatoxins (Geremew, 2016).

Aflatoxicosis in humans follows two pathways of the dietary exposure first by direct ingestion of aflatoxins (mainly B1) in contaminated foods of plant origin such as maize nuts and their products. Second by ingestion of aflatoxins carried over from feed into milk and milk products including cheese and powdered milk, where they appear mainly as aflatoxin M1. In addition to the carryover into milk, residues of aflatoxins may be present in the tissues of animals that consume contaminated feed (WHO, 1979).

Aflatoxin residues have been found in animal tissues, eggs, and poultry following the experimental ingestion of aflatoxin-contaminated feed Contamination of milk, egg, and meat can result from animal consumption of mycotoxin contaminated feed Ingestion of contaminated food is the main source of exposure to aflatoxins, which adversely affect the health of both humans and animals (Agage, 2004). The compounds can cause acute or chronic toxic effects of a teratogenic, mutagenic, carcinogenic, immunotoxic or hepatotoxic character. Molecular aflatoxins affect DNA mutations, postranslation peptids chains modification, proteins, and nucleic acids methylation and the formation of free radicals (Chu, 2006).

2.6. Physical Properties of Aflatoxins

Aflatoxins are crystalline odorless solids when isolated and the color range from pale white to yellow. The melting points range from 268 °C for B1 down to 190 °C for G2 optimal water activity for growth of *A. flavus*is high about 0.99. The maximum is at least 0.998 whereas the minimum water activity for growth has not been defined (Walier, 2003).

Production of toxins appears to be favored by high water activity. *Aspergillus flavus* is reported to grow within the temperature range 10-43 °C. The optimal growth rate occurs at a little above 30 °C, reaching as much as 25mm per day. The aflatoxins are produced by *A. Flavus* over the temperature range 12-40°C. It is not possible to specify an optimum temperature for the production of the toxins, although production between 20-30 °C is reported to be significantly greater than at higher and lower temperature (Ongoma, 2013).

2.7. Prevalence of Aflatoxin in Animal Feed and Feed Ingredient in Ethiopia

In the study conducted by Dawit (2016) on aflatoxin contamination of milk and dairy feeds in the Greater Addis Ababa milk shed, all the feed samples were contaminated with AFB1 ranging between 7µg/kg and 419µg/kg. Out of a total of 156 feed samples collected, only 16 (10.2%) contained AFB1 at a level less than or equal to 10µg/kg. At the same time, 41 (26.2%) of the feed samples contained AFB1 at a level exceeding 100µg/kg.

Rehrahie (2018) conducted a study on aflatoxins, heavy metals, and safety issues in dairy feeds, milk and water in some selected areas of Ethiopia; a total of 205 samples comprise of 115 concentrate feeds, 45 roughage feeds and 45 milk samples were analyzed using the ELISA technique. The outcomes showed that half of the feed samples (81) were free from aflatoxin, and the rest (79 samples) were within the EU standard of 5µg/kg and the USA standard of 20µg/kg. The pattern of aflatoxin contamination showed that concentrate feeds were more contaminated (7.67 ± 0.80µg/kg) than roughage feeds (0.41 ± 0.14µg/kg); hay (0.72 ± 0.25µg/kg) was more contaminated than straw (0.05 ± 0.05µg/kg) and oilseed cake based concentrate feeds were more contaminated (13.09 ± 1.12µg/kg) than concentrate feeds without oilseed cake (2.78 ± 0.66µg/kg).

A study conducted by Amare Ayalew in 2010, showed that Aflatoxins were detected in 88% of the samples of maize the mean value 27µg/kg. Additional monitoring of mycotoxins in maize from different regions of the country was verified in order to determine the actual risks from mycotoxins and possibly low mycotoxin risk maize production areas.

2.8. Effects of Aflatoxin on Food-Producing Animals

The presence of fungi in feed ingredients may cause general, unspecific problems for animal production, such as reduced feed intake or feed acceptability. However, diseases caused by mycotoxins, known as mycotoxicoses, are the most important health problems caused by fungi contamination. They are characterized by diffuse syndromes, but with a predominance of lesions in organs and tissues, such as liver, kidneys, epithelial and central nervous system, depending on the type of toxin. Thus, mycotoxicoses can cause significant losses to the animal industry worldwide (Rodrigues and Naehrer, 2012).

The effects of aflatoxin depend on genetic factors (species, breed strain); physiological factors (age, nutrition, exercise); and environmental factors (climatic, husbandry, housing). Developing fetuses are very susceptible to even low levels, and young and fast-growing animals are more affected than adults. Males are more susceptible than females (Grace, 2013).

The disease called aflatoxicosis causes acute and chronic presentation in animals. Acute aflatoxicosis causes death and chronic aflatoxicosis results in cancer, toxicity, and immune suppression. The liver is the primary target organ. AFB1 is a potent carcinogen by bioactivation of cytochrome P450 in the liver and AFB1-8,9-epoxide (AFBO) production. AFBO is needed for carcinogenic and toxic activity (Wang *et al.*, 2009).

2.8.1. Effects of Aflatoxin on Dairy Cattle

Ruminants are less susceptible to mycotoxins than monogastric, because of the rumen microbiota and the feed particles contained in the rumen compartment may be effective in the degradation, deactivation, and binding of these toxic molecules, hence protecting animals (Fink, 2008).

Acute aflatoxicosis is characterized by quick deterioration of general status, loss of appetite, low feed conversion, interference with reproductive capacity, immunosuppression, acute hepatitis, jaundice, hemorrhage and death (Abidin and Khatoon, 2012). The liver is the most importantly affected organ, with lesions caused by hemorrhagic necrosis, centrilobular congestion, a proliferation of cells of biliary ducts and fatty infiltration in hepatocytes (Oliveira *et al.*, 2014).

The effects of chronic toxicity are also characterized by hepatic lesions in a lower extent and include changes in the growth of rumen microorganisms and genetic changes. In cattle, the primary symptoms are reduced weight gain as well as liver and kidney damage; where milk production is also reduced. Different forms of the enzymes that metabolize aflatoxins (e.g. cytochrome P450s, glutathione S-transferases) are considered responsible for the different susceptibilities of different animals to the toxic effects of aflatoxins (Binder, 2007).

Aflatoxins affect milk quality. Cows metabolize AFB1 to form the monohydroxyderivative, aflatoxin M1 (AFM1), which is excreted into milk within 12 hours in the form of aflatoxin M1 with residues approximately equal to 1.7% of the dietary aflatoxin level. The FDA limits for aflatoxin M1 in milk is 0.5 ppb and for aflatoxin B1 should not be more than 20 ppb (Diaz *et al.*, 2004). AFM1 is a potential human carcinogen that is very resistant to thermal treatments such as pasteurization and freezing. The European Commission Regulation 1881/2006 sets a maximum limit of 0.05μg kg^{-1} for AFM1 in raw milk, heat-treated milk, and milk for the manufacture of milk-based products (EC, 2006).

2.8.2. Effects of Aflatoxin on Poultry

Poultry is highly susceptible to mycotoxicoses caused by aflatoxins and ochratoxins (Anjum*et al.*, 2012). In chicken, Total Aflatoxins impairs most of the important production parameters including weight gain, feed intake, feed conversion efficiency, pigmentation, processing yield, egg production, and male and female reproductive performance (Hussain *et al.*, 2010).

Aflatoxins are metabolic conversion by hepatic enzymes (the cytochrome P450 family) to the metabolically active metabolite Exo-AFB1-8, 9-epoxide to exert its toxicity. This metabolically active form of aflatoxin can bind with particular cellular compounds (proteins, DNA and RNA) to influence normal cellular activities, and is considered the active form responsible for the carcinogenicity and mutagenicity (Celik *et al*, 2000).

Aflatoxins are metabolized, bio transformed, and stored in poultry organs mainly in the liver (Gregory and Manley, 1982). but not all aflatoxins are assimilated and some traces are excreted in the litter. Aflatoxins can damage animal organs such as mucous membranes, digestive tract, and nervous and circulatory systems (Delbianchi *et al.*, 2005).

Aflatoxin toxicity in poultry is also associated with biochemical, hematological, reproductive and pathological changes (Ortatatli and Oguz, 2001). Aflatoxin intake in broilers is associated with liver damage, poor performance, immunosuppression, and mortality. Some of the metabolites formed during the metabolism of AFB1 are transmitted to edible animal products, liver, muscle, and eggs, which exert immunosuppressive, embryotoxic and teratogenic effects (Celik *et al.*, 2000).

Aflatoxin also can affect laying hens and lead to reduced egg production, poor egg quality and increased mortality of challenged hens. AFB1 adversely influences egg quality by decreasing shell thickness, egg weight, and egg energy deposition. The negative impacts of aflatoxin on laying hens can be induced when the feed contains 1-2 mg/kg (Verma *et al.*, 2007). Besides, aflatoxin in laying hen feed can result in an aflatoxin residue in the eggs, feed to egg AFB1 transmission ratio was approximately 5000:1 (Oliveira *et al.*, 2000).

2.9. Control measure of Aflatoxin in Feeds

Aspergillus flavus and *A. parasiticus*. Under favorable conditions typically found in tropical and subtropical regions, including high temperatures and high humidity, these molds, normally found on dead and decaying vegetation, can invade food crops. Drought stress, insect damage, and poor storage can also contribute to the higher occurrence of the molds including in more temperate regions (Wang, 2018).

Prevention measurements are focused on the minimization of crop contamination before harvesting (plant breeding and good agronomic practices) and during storage or postharvest (detoxification). Several methods of prevention and control are available to reduce the contamination with aflatoxins. However, mycotoxin contamination of food and feed is unavoidable (Kendra, 2007).

Food crops can become contaminated both before and after harvesting. Pre-harvest contamination with aflatoxins is mainly limited to maize, cottonseed, peanuts, and tree nuts. Post-harvest contamination can be found in a variety of other crops such as coffee, rice, and spices. Improper storage under conditions that favor mold growth (warm and humid storage environments) can typically lead to levels of contamination much higher than those found in the field (Wang, 2018).

2.9.1. Pre-Harvest Control Method

Biocontrol of aflatoxins refers to the use of organisms to reduce the incidence of Aspergilli in susceptible crops to reduce aflatoxin contamination. The most widely used biocontrol method employs atoxigenic strains of Aspergilli that can competitively exclude toxigenic strains from colonizing crops. Cultural practices including crop rotation, tillage, the timing of planting, and management of irrigation, fertilization can also help to prevent Aspergillus infection and subsequent aflatoxin accumulation by reducing plant stress (Munkvold, 2003).

2.9.2. Post-Harvest Control Method

Possible intervention strategies include good agricultural and storage practices including early harvesting, proper drying, sanitation, proper storage, insect management and appropriate use of pesticides during the manufacturing process could help in minimizing the fungal infection or insect infestations of crops (Wagacha and Muthomi, 2008).

Extrusion and heating: The greatest reduction in mycotoxin concentrations in extruded products seems to occur at temperatures greater than 160° C this may affect essential nutrients and compromise the nutritional quality of the food product Alonso *et al.*, (2000) reported that extrusion processing of kidney beans improved protein and starch digestibility.

Binding: The binders are meant to act like a 'chemical sponge' which will practically 'mop up' the mycotoxins from the gastrointestinal tract thereby preventing it from being available to other target organs in the body. The chemical structure of both the mycotoxin and the binder determines the effectiveness of adsorption. The important characteristics of the adsorbent to be taken into consideration include its physical structure, its charge, pore size and surface area (Kolossova *et al.*, 2012).

The best-known method for mycotoxin deactivation is with the use of adsorbents, which are known as mycotoxin binders, sequestering agents or enterosorbents Adsorption can be described as a process by which dissolved material either in liquid or gaseous state is bound to the surface of another and may be expressed in terms of adsorptive surface area per unit mass. These binders may be of microbial (organic) or inorganic nature which includes charcoal and clay minerals. The addition of binding agents to foods and feeds has received a lot of attention in the past years (Murugesan *et al.*, 2015).

Ammoniation: is a safe and effective way to decontaminate aflatoxins; it has been used with success in many countries but is not legal in others interventions aim to detoxify contaminated products. Treatment with gaseous ammonium can reduce aflatoxin levels dramatically, and can make feed safe and tolerated by animals (Peltonen *et al.*, 2001).

Blending: is one of the method of reducing moderate levels of aflatoxin contamination is to blend contaminated grain with a clean grain (blending 1 kilogram of grain with aflatoxin contamination five times above the limits with 9 kilograms of grain with no detectable aflatoxin would result in 10 kilograms of grain with aflatoxins at 50 percent of the permissible amount). The blending of contaminated crops has been practiced where highly contaminated crops are mixed with non-contaminated crops to produce a mix that has an average level below the legal limits. This is generally not allowed in the United States, since the feed would be considered adulterated, but has been allowed on exception during unusually contaminated harvests (Grace *et al.*, 2015).

2.10. Legislations Associated with Aflatoxin Levels in Feeds and Foods

Many aflatoxin regulatory levels are set depending on the particular agricultural commodity or compound feed/food, the type, and age of animal which will consume it and the intended use. Many countries base their regulations on the guidelines established by the European Union (EU) or by the United States Food and Drug Administration (FDA) Guidelines sometimes differ from each other; in most of the cases, the maximum allowed content of aflatoxins is lower in the regulations given by the EU than in those granted by the FDA. For example, USA Food and Drug Administration had established 20µg/kg for aflatoxin present in feed and 0.5µg/kg aflatoxin for milk (Khanafari, 2007).

European Union has introduced the maximum accepted residue levels for aflatoxin in animal feeds as 20µg/kg in all feed materials and in the most complete and complementary feedstuffs for cattle, sheep, goats, pigs and poultry, 5µg/kg in complete feeding stuffs for dairy animals and 10µg/kg for complete feeding stuffs for calves and lambs (CEC, 2003). And in Ethiopia it is 20µg/kg for both dairy and poultry feeds (ESA, 2019).

The action levels listed below were established by the Food and Drug Administration (FDA) for addressing aflatoxin levels in human food, animal feeds, and feed ingredients. Any feed, grain or ingredient moving in interstate commerce with aflatoxin levels those listed below are subject to restrictive action by the FDA depending on animal type as outlined below.

Table 1. FDA maximum level of aflatoxin in animal feeds and food

Species	Commodity	Maximum level(µg/kg)
Dairy animals	All feed and feed ingredients	20
Human	Milk	0.5
Human	Any food except milk	20
Poultry and dairy animal	Corn and other grain	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	Corn and other grain	200
Finishing beef cattle	Corn and other grain	300
Beef cattle, swine, poultry	Cottonseed meal	300

Source: (Carlson and Ensley, 2003)

2.11. Animal Feed Factories in Ethiopia

Commercial feed sector in Ethiopia had total number of 80 enterprises under four major categories (Table 2). The dominate feed processing plants were private enterprises and farmers' union engaged in production of compound feed followed by importers or manufactures of specialty ingredients (premixes, feed additives) and importers or manufactures of feed processing equipment (VDFACA, 2016).

Common feed ingredient used by feed processing plants included maize, wheat bran and wheat middling, rice bran, lentil bran and molasses as sources of energy while niger seed cake, soybean cakes and cottonseed were noted to be the major source of protein. Major mineral ingredients include limestone, and salt. Premixes of amino-acids trace minerals and vitamins and slaughter by-products such as meat and bone meal (EAFIA, 2017; Tolera, 2012).

Table 2. Structure of the commercial feed sub-sector in Ethiopia

Region	Private feed plants	Union feed plants	Specialty ingredient importers and manufacturers	Feed processing equipment	Total
Addis Ababa	10	1	10	4	25
Oromia	12	7	4	1	24
Amhara	44	6	0	0	10
SNNP	4	6	1	0	11
Tigray	2	8	0	0	10
Total	32	28	15	5	80

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in Addis Ababa, Ethiopia. It is a major trade center for animal feeds. In the city, there are 10 animal feed producers registered by VDFACA. But three of them are out of business. Thus, animal feed and feed ingredient samples were collected from seven dairy and poultry feed factories in Addis Ababa. These factories use five common ingredients namely maize, wheat bran, wheat middling, soybean cake and niger seed cake for producing dairy cattle, poultry layer and broiler feed in different rations.

3.2. Sampling and Sample Collection

A total of 42 samples of dairy and poultry feed and feed ingredients were collected from 7 dairy and poultry feed factories in Addis Ababa. The samples included 25 feed ingredients (five maize, five wheat middling, five wheat bran, five soybean meal, and five Niger seed cake) and 17 feeds (five dairy cattle, six poultry layer and six broiler feed). To achieve reasonably representative samples, primary large samples of approximately 10 kg were collected from different parts of the store, sack or unit of feed. The samples were homogenized and quartered to obtain a 1 kg of laboratory sample (ES 1029:2019). About one kilogram of feed samples were collected and transported to the laboratory for analysis in sealed plastic sample bags. The samples were stored in the refrigerator at 4 ^{o}C until the time of analysis.

3.3. Sample Preparation

Sample preparation was conducted using the method of AOAC Official method 950.02 for animal feed. The distribution of aflatoxin is extremely non-homogeneous, laboratory samples were homogenized by grinding the entire laboratory sample. The laboratory sample was finely ground and mixed thoroughly using a process that approaches complete homogenization as possible. After and before grinding, the grinder was cleaned to prevent cross-contamination.

The study design followed in this study is demonstrated in Figure 2.

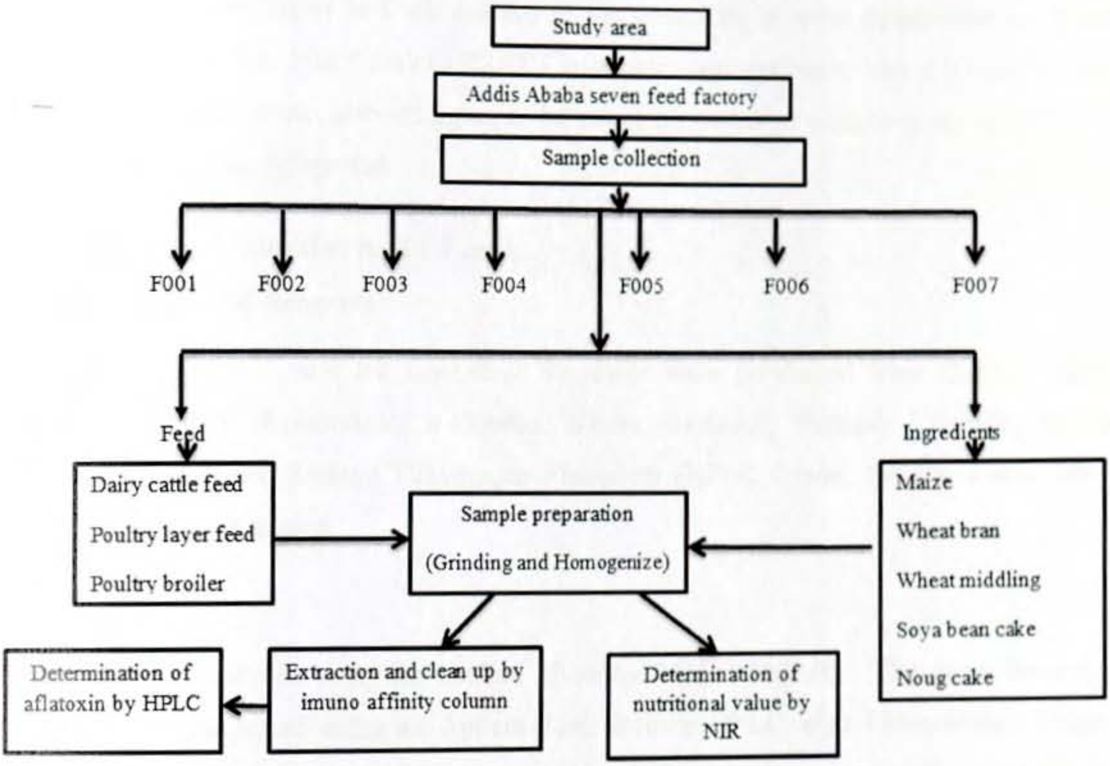


Figure 2. Flow chart of study design

3.4. Study Design for KAP (Knowledge Attitude and Practice)

A structured questionnaire was also designed for collecting information from feed producer, dairy and poultry farmers to capture their knowledge, attitude, and practice (KAP) on aflatoxin contamination. The questionnaire was focused on their storage practices; awareness of mold growth and the formation of aflatoxin on animal feed. A total of 30 participants were engaged in the survey. The response was classified in three parts; the first part was general information about the formation of fungi, the second part contained selection criteria for quality dairy and poultry feeds and the final part was KAP related to the transfer of mycotoxin in the animal source of food milk, egg, and meat.

3.5. Determination of Nutritional Analysis by NIR

Moisture, fat, protein, fiber and ash content of the feed sample were determined using NIR analysis technique. The NIR (model DS2500) instrument was calibrated using (Grain, vegetal-protein, by-product) so that infrared light can be detected number of organic compounds present in the samples will be interpreted.

3.6. Analysis of Aflatoxins in the Feeds

3.6.1. Chemicals and Reagents

Aflatoxin G2, G1, B2, and B1 Analytical Standards were purchased from (Sigma Aldrich GMBH, Germany), Acetonitrile, n-Hexane Water, Methanol, Sodium Chloride, Sodium Phosphate Dibasic and Sodium Dihydrogen Phosphate (HPLC Grade, ≥99.9% assay, Merck KGaA, Germany) were used.

3.6.2. Materials

Aflatoxins were analyzed using the method given by AOAC 2003.02. The quantification of aflatoxins was performed using an Agilent 1260 Infinity HPLC with Fluorescence detector setting (Excitation at 365nm and Emission at 440nm). Column oven temperature was set at a Temperature of (390C); injection volume was set at (20μl); a quaternary pump delivering a flow rate of 1.2 ml/min and a mobile phase composition: Acetonitrile, Water, Methanol (15: 60: 25 v/v). The data was processed by Agilent Open Lab CDS software.

Extraction and cleanup was done using Immunoaffinity Column, (Afla-clean Select from LC-Tech GMBH, Germany). Derivatization was achieved by hydroxylation from the water of the mobile phase under UV light at 265nm wavelength so that Aflatoxins B2 and G2 will be formed and flourish well for easy detection, unlike the parent B1 and G1. A reversed-phase C-18 HPLC column with 5 μm particle size, 10 cm length, and 4.6 mm internal diameter was used for separation.

Figure 3. HPLC Instruments at Bless Agri Food Laboratory Service Testing Laboratory, Addis Ababa.

3.6.3. Analytical Quality Assurance

3.6.3.1. Limit of Detection

The limit of detection was defined as the minimum quantity of aflatoxins substance giving a signal-to-noise ratio of at least 3 ($S/N \geq 3$). The Limit of Detection was measured for all 4 aflatoxins by injecting standard solutions of different concentrations and measuring for each substance the amount injected on the column and the S/N values.

3.6.3.2. Limit of Quantification

The limit of quantification was defined as the lowest concentration of aflatoxin that can be determined with acceptable accuracy and precision. LOQ was determined based on the amount of analyte which can be reproducibly quantitated above the baseline noise, that gives $S/N > 10$ (FDA, 2010). This also corresponds to the lowest concentration of the calibration curve 0.5µg/kg.

3.6.2.3. Accuracy

The accuracy of the method was determined by analysis of samples containing known aflatoxin amounts. The closeness of test results to the "true" or accepted value. The accuracy of the method was determined by spiking sample with aflatoxin standard analyzing in replicates and evaluating the recovery against the AOAC guideline for the respective concentration value (Horwitz, 2002).

3.6.2.4. Precision

The precision of the method was evaluated through the repeatability of the method by assaying ten replicate injections of aflatoxin mixed standard at the same concentration (20μg/kg) during the same day under the same experimental conditions to obtain an acceptable %RSD which is less than 11% as the specification required by AOAC (Horwitz, 2002).

3.6.2.5. Linearity

Linearity was determined by injecting a series of concentration of (0.2, 0.5, 2, 5, 10, 20, 30, 50, and 100) ppb total aflatoxin standard. The concentration range (0.2-100) ppb and regression equation were found by plotting the peak area (Y) versus the aflatoxin concentration (X) expressed in ppb.

3.6.2.6. Recovery

The recovery of standards must be processed with every batch of the sample. The mixture of aflatoxin standards G2, G1, B2, and B1 was spiked to 20g sample whose aflatoxin content is previously determined these spiked samples were then loaded onto the Immunoaffinity column and analyzed in duplicate by the HPLC. The method was evaluated as the percent of recovered concentration recommended value of codex Aliment Arius (1 – 15μg/kg is between 70 to 110 % and for > 15μg/kg the recommended percent of recovery is between 80 – 110 %).

The %Recovery was calculated using the following formula;

$$\%Recovery = \frac{Cs - Co}{Ca} \times 100 \ldots\ldots\ldots\ldots Eq1$$

Where; Cs is the concentration of aflatoxin in the spiked sample in μg/kg,

Co is the concentration of aflatoxin in the original sample in μg/kg, and

Ca is the amount of aflatoxin added to the original sample in μg/kg.

3.6.3. Extraction and Clean up

20g of feed sample and 2 g of salt (NaCl) was weighed and transferred into a blender jar and 100 mL of 80% methanol/20% water plus 50 mL of n-hexane were added. The mix was blended at high-speed blender jar for 45 minutes and the blended contents were filtered through whatman No.4 filter paper into a 500-mL filtration flask. From the filtrate, in the case of phase separation, the lower phase was used for the next steps. 7mL was taken and diluted with 43mL of phosphate buffer solution (pH 7.2). The diluted filtrate (equivalent to 1.4gm of the sample) was passed through the column at the flow rate 2mL per min. The column was washed by passing 10mL of water and the toxin was eluted by 1mL of 100% pure methanol two times and collected with a vial for HPLC injection.

The affinity column contains antibodies raised against aflatoxin B1, B2, G1, and G2. The detection for aflatoxin was 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 in addition to this remove the substances, which may interfere with the detection of the analyte.

3.6.4. HPLC Determination and Calculations

The aflatoxins in the methanol eluate are injected into the HPLC for the detection and quantification of aflatoxins in the sample. The aflatoxin content of in the feed samples was calculated using the following formula.

$$\text{Aflatoxin } (\mu g/kg) = n \ x \left(\frac{V_e}{V_I}\right) x \left(\frac{1}{W_e}\right) \times DF \quad \text{.......Eq2}$$

Where;

V_e is final volume collected after elution from immunoaffinity column (μl)

V_I is volume eluate injected into HPLC (μl)

$W_e$is weight of matrix represented by final extract (gm.)

DF is a dilution factor

3.7. Statistical Data Analysis

For data analysis, Microsoft Excel 2013 and IBM SPSS Statistics version 20 software were used. In the SPSS method, one-way analysis of variance (ANOVA) was performed to evaluate the levels of total aflatoxin mean comparison between the study sites. A p -value of less than 0.05 was considered to show statistical significance. Assumptions of ANOVA were checked. As there was one dependent variable one-way ANOVA was used. The dependent variable level was aflatoxin concentration and the independent variable in the study area.

4. RESULTS AND DISCUSSION

4.1. Identification of Aflatoxins

The retention time of mixed aflatoxin standard and peak area as shown in Table 3, gone a good precision having retention time range between 1.76 –2.23 % RSD and peak area range between 3.76 - 4.28 %RSD which had values within FDA standard % RSD <5% for peak area and retention time and < 3.0% for retention time. The elution order of individual aflatoxin was in the order of AFG2, AFG1, AGB2, and AFB1 with 5.36, 6.25, 6.96 and 8.48 retention times respectively.

Table 3. The retention time for mixed aflatoxin standard (20µg/L)

Aflatoxin type	Retention time (minutes)	N	% RSD of Peak area	% RSD of Retention time
G2	5.36 ± 0.12	6	4.28	2.23
G1	6.25 ± 0.11	6	3.96	1.76
B2	6.96 ± 0.13	6	3.76	1.87
B1	8.48 ± 0.15	6	4.13	1.79

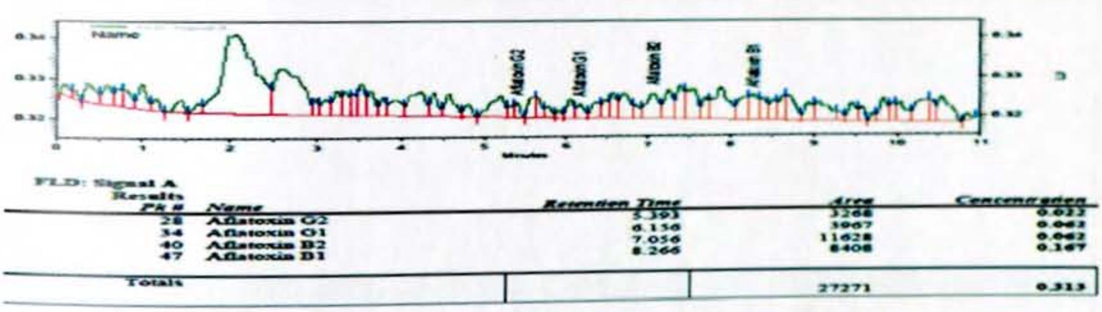
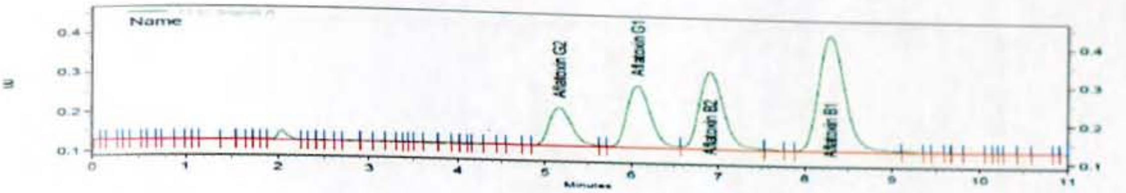


Figure 4. Chromatogram for blank (methanol)



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentn
44	Aflatoxin G2	5.162	307424	2.000
46	Aflatoxin G1	6.070	524762	8.000
47	Aflatoxin B2	6.905	663997	2.000
50	Aflatoxin B1	8.287	1064133	8.000
Totals			2560316	20.000

Figure 5. Chromatogram for 20μg/L mixed aflatoxin standard solution

4.2. Preparation of calibration Curve

Calibration curves were prepared for all four aflatoxins by using the standard solutions covering the concentration ranges from 0.5 to 10μg/L for Aflatoxin G2; 2 to 40μg/L for Aflatoxin G1; 0.5 to 10μg/L for Aflatoxin B2 and 2 to 40μg/L for Aflatoxin B1. Peak areas of the different aflatoxins were plotted against the concentrations and linear regression analysis was used to calculate the equation and the correlation coefficient of the standard curves. The acceptance criteria for correlation are > 0.99 on the FDA standard. All correlation curves were ≥ 0.9985, demonstrating the linearity over the concentration ranges studied.

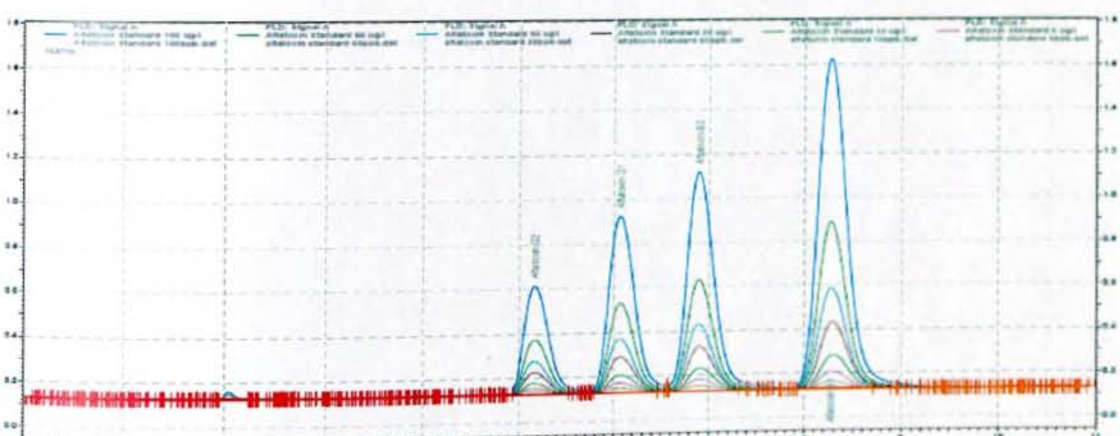
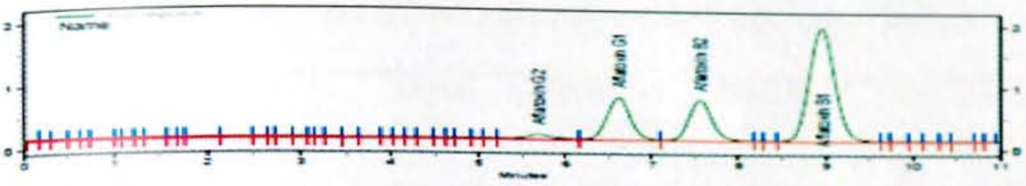


Figure 6. Overlaid chromatogram of the calibration series



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
33	Aflatoxin G2	5.688	224006	1.502
34	Aflatoxin G1	6.624	2082917	32.479
35	Aflatoxin B2	7.560	1960180	6.977
38	Aflatoxin B1	8.964	6098537	46.312
Totals			10365640	86.270

Figure 7. Chromatogram for sample

4.3. Accuracy and Precision

The accuracy and the precision of the method were determined by analysis of samples containing known aflatoxin amounts. The recovery acceptance criteria for this concentration range is between 70% and 120%and the %RSD was less than 11% as the specification required by AOAC. All the feed sample different matrices recovery calculated ranged from 78.75% to 112.0% which is within the acceptable range showing the accuracy of the method (Table 4).

Table 4. Accuracy and recovery of data for aflatoxin analysis in feeds and ingredients

Matrices		Aflatoxin (µg/kg)	Aflatoxin Spiked (µg/kg)	Aflatoxin obtained (µg/kg)	%Recovery	%RSD (n = 6)
Maize	G2	0.32	1	1.28 ± 0.02	96.0	1.56
	G1	7.36	4	10.96 ± 0.30	90.0	2.73
	B2	0.41	1	1.35 ± 0.01	94.0	0.7
	B1	7.29	4	11.01 ± 0.26	93.0	2.36
Wheat bran	G2	0.26	1	1.30 ± 0.03	104.0	2.30
	G1	1.61	4	5.76 ± 0.16	103.75	2.78
	B2	0.32	1	1.25 ± 0.00	93.0	0.1
	B1	0.91	4	4.85 ±0.13	98.50	2.68
Wheat middling	G2	0.19	1	1.26 ± 0.03	96.84	2.38
	G1	2.29	4	6.36 ± 0.16	101.75	2.52
	B2	0.23	1	1.26 ± 0.04	103.0	3.17
	B1	1.30	4	4.45 ± 0.14	78.75	3.12
Soybean cake	G2	0.24	1	1.36 ± 0.05	112.0	3.67
	G1	2.36	4	6.43 ± 0.15	101.75	2.33
	B2	0.45	1	1.35 ± 0.03	90.0	2.22
	B1	2.50	4	6.43 ± 0.17	98.25	2.64
Niger seed cake	G2	4.30	2	6.35 ± 0.15	102.5	2.36
	G1	125.51	8	133.61 ± 1.99	101.25	1.49
	B2	10.69	2	12.75± 0.24	103.0	1.88
	B1	76.64	8	84.56 ± 1.22	99.0	1.44
Dairy feed	G2	2.23	2	4.17 ± 0.16	97.00	3.84
	G1	56.51	8	64.25 ± 0.99	94.37	1.54
	B2	10.46	2	12.73 ± 0.26	96.75	2.04
	B1	76.51	8	84.56 ± 1.23	100.62	1.45
Poultry layer feed	G2	2.90	3	5.96 ± 0.06	102.00	1.06
	G1	58.67	12	70.86 ± 0.16	101.58	0.22
	B2	12.26	3	15.18 ± 0.23	97.33	1.51
	B1	87.16	12	99.16 ± 1.03	100.58	1.03
Poultry broiler feed	G2	1.16	3	4.21 ± 0.15	101.66	3.56
	G1	17.23	12	29.35 ± 0.98	101.00	3.34
	B2	1.55	3	4.58 ± 0.08	101.00	1.75
	B1	16.35	12	28.26 ± 0.12	98.08	0.24

4.4. Level of Aflatoxins in Feed Ingredients

The total aflatoxin content of maize collected ranged between 2.64μg/kg and 106.72μg/kg with a mean value of 26.92μg/kg and the aflatoxin B1 content ranged between 2.64μg/kg and 46.74μg/kg with a mean value of 12.71μg/kg. The total aflatoxin content of wheat bran collected ranged between "Non-Detected" (ND) and 5.82μg/kg with a mean value of 3.7μg/kg and the aflatoxin B1 content ranged between ND and 3.66μg/kg with a mean value of 2.29μg/kg. The total aflatoxin content of wheat middling ranged between ND and 4.75μg/kg with a mean value of 2.2μg/kg and the aflatoxin B1 content ranged between ND and 12.77μg/kg with a mean value of 1.35μg/kg. The total aflatoxin content of soybean cake ranged between ND and 7.39μg/kg with a mean value of 3.36μg/kg and the aflatoxin B1 content ranged between ND and 3.43μg/kg with a mean value of 1.67μg/kg. The total aflatoxin content of niger seed cake ranged between 251.94μg/kg and 491.66μg/kg with a mean value of 385.45μg/kg and the aflatoxin B1 content ranged between 110.93μg/kg and 438.86μg/kg with a mean value of 288.34μg/kg (Table 5).

Table 5. Average levels of aflatoxin G2, G1, B2, B1 and total AF in feed ingredients

Sample	N	Level of aflatoxin in μg/kg						
		G2	G1	B2	B1		Total AF	
		Mean	Mean	Mean	Mean	range	mean	range
Maize	5	1.35	11.54	1.32	12.71	2.64-46.74	26.92	2.64-106.72
Wheat bran	5	ND	1.5	ND	2.29	ND-3.66	3.7	ND-5.82
Wheat middling	5	ND	0.85	ND	1.35	ND-12.77	2.2	ND-4.75
Soybean cake	5	ND	1.69	ND	1.67	ND-3.43	3.36	ND-7.39
Niger seed cake	5	3.28	82.9	10.91	288.34	110.93-438.86	385.45	251.94-491.66

ND= Not Detected (<0.5μg/kg)
a, b-Means with different superscripts in a column differ significantly ($P < 0.05$)

Different studies on aflatoxin levels in feed ingredient have been conducted in different parts of the world. In Iran, the mean value of AFBs in maize, wheat grain, and wheat bran and soybean meal was 2.35, 1.54, 3.05 and 6.0μg/kg respectively. Nemati *et al*., (2014) which is similar to the reports of this study. In India, the mean value of AFB1 by using high-performance thin-layer chromatography scanner in maize, wheat bran, and soybean cake was 62, ND and 50 μg/kg (Kotinague *et al*., 2015).

A study by Dawit *et al.*, (2016) conducted in Ethiopia wheat bran and niger seed cake samples showed of AFB1 levels ranging between 9 and 31µg/kg in wheat bran and 290 and 397µg/kg in niger seed cake respectively, which is similar to the reports of this study.

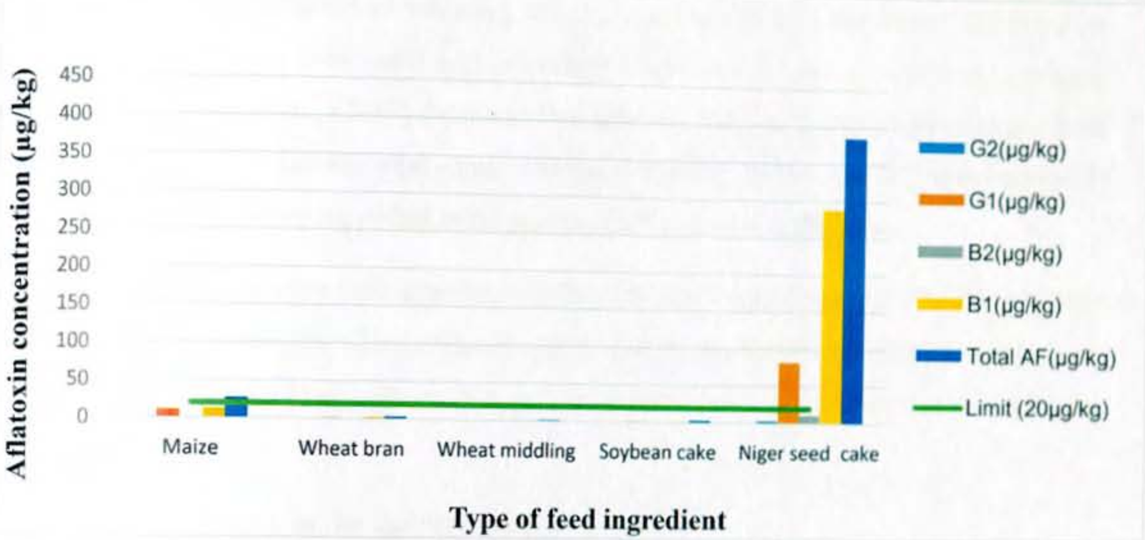


Figure 8. Aflatoxin levels in feed ingredient collected from Addis Ababa

Out of a total of 25 dairy and poultry feed ingredient samples collected from the seven feed factories in Addis Ababa, 24% (6 of 25) showed contamination of aflatoxin B1 and total aflatoxin level higher than the recommended 20µg/Kg limit set by the US Food and Drug Administration (FDA) and (FAO)/World Health Organization (WHO), Ethiopia has a similar standard of 20µg/Kg for aflatoxin B1 and 40µg/Kg for total aflatoxin. 20% (1 of 5) contamination in maize grain and 100% (5 of 5) had aflatoxin B1 and total aflatoxin in niger seed cake higher than the tolerable limit set by the US Food and Drug Administration (FDA) and (FAO)/World Health Organization (WHO), Ethiopia has a similar standard of 20µg/Kg for aflatoxin B1 and 40µg/Kg for total aflatoxin. The other feed ingredients; wheat bran, wheat middling and soybean cake samples were tasted below the tolerable limit.

The possible reason for high aflatoxin content in niger seed cake samples could be due to improper storage management and moisture content which could increase the risk of mycotoxin formation and aflatoxin contamination. 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 encourages mold growth due to moisture and heat. According to Schmidt *et al*., (2010) the interaction between water activity and temperature have a prominent effect on aflatoxin production. The high nutrient content and moisture content in Niger seed cake might have supported mold growth and formation of aflatoxin.

Among the dairy and poultry feed ingredients in this study, aflatoxin content of niger seed cake showed significant difference when compared to the rest of the ingredients (maize, wheat bran, wheat middling and soya bean cake) with a p value of less than 0.05 for aflatoxin B1 and Total aflatoxin.

4.6. Level of aflatoxins in the animal feeds

4.6.1. Aflatoxin Levels in Dairy feeds

The total aflatoxin content of dairy cattle feed collected from Addis Ababa feed factories ranged between 114.23μg/kg and 557.12μg/kg with a mean value of 313.03μg/kg and the aflatoxin B1 content ranged between 51.66μg/kg and 370.51μg/kg with a mean value of 192.80μg/kg.

Dawit *et al*, (2016) reported that analysis for AFB1in dairy feed samples in the Greater Addis Ababa milk shed was conducted by a commercial enzyme-linked immunosorbent assay (ELISA). AFB1 results ranged between 7μg/kg and 419μg/kg relatively similar to this study because the same ingredient niger seed cake was used. In Kenya 74 dairy feed samples showed aflatoxin concentration ranging between 0 and 147.86μg/kg (Makau *et al*., 2016). This is lower than this study. 112 dairy feed samples analyzed in Costa Rica showed an aflatoxin concentration 290.04μg/kg (Granados *et al.,* 2017). The reasons for difference of AFB1 in feeds in Ethiopia and other country could be due to the difference in feed types commonly utilized.

Table 6. Average levels of aflatoxin G2, G1, B2, and B1 in dairy cattle feed

Sample	G2(μg/kg)	G1(μg/kg)	B2(μg/kg)	B1(μg/kg)	TotalAF(μg/kg)
Dairy feed F001	2.43	93.99	12.64	153.61	262.67
Dairy feed F002	4.39	162.40	19.82	370.51	557.12
Dairy feed F003	5.14	156.67	11.48	311.57	484.86
Dairy feed F004	3.52	52.00	7.05	51.66	114.23
Dairy feed F006	2.26	56.70	10.69	76.64	146.29
Average	3.55	104.35	12.34	192.80	313.03

All five dairy feed samples in this study contained aflatoxin B1higher than the regulatory limits set by FDA, and ESA Standards (20μg/kg). Aflatoxin B1 levels in dairy feed and feedstuff are important to human health since approximately 1-2% of the aflatoxin B1 in the animal feed is transformed to aflatoxin M1 in milk. Therefore, aflatoxin B1 concentration in feed above standards may result in milk containing a higher aflatoxin M1 (ORUC *et al.*, 2007).

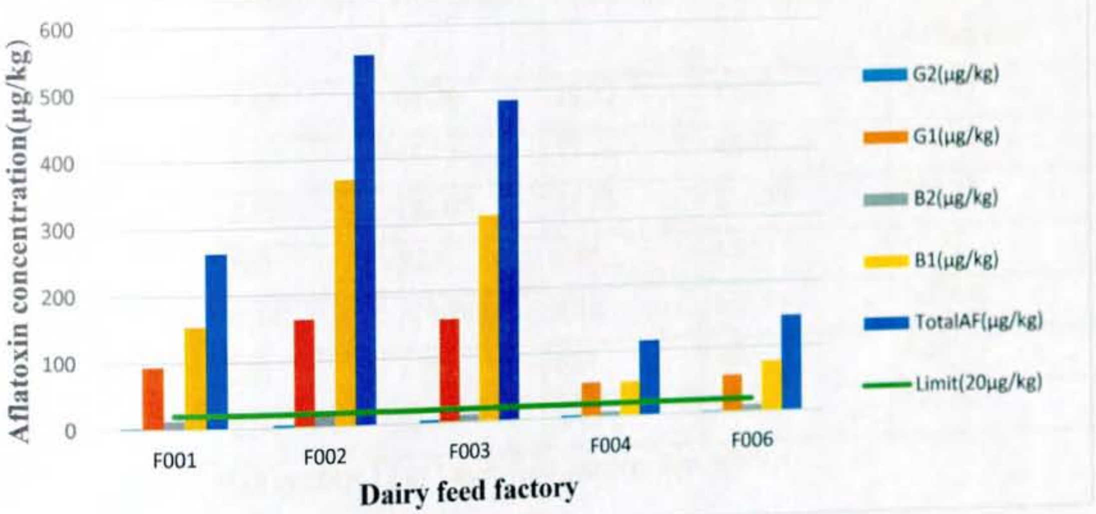


Figure 9. Aflatoxin contamination of dairy feed in different feed factories

4.6.2. Aflatoxin Levels in Poultry Layer Feeds

The total aflatoxin content of poultry layer feed collected from Addis Ababa feed factories ranged between 3.5μg/kg and 260.60μg/kg with a mean value of 143.04μg/kg and the aflatoxin B1 content ranged 1.45μg/kg and 139.51μg/kg with a mean value of 74.80μg/k (Table 7).

Different studies on aflatoxin levels in poultry layer feed were conducted in different parts of the world. In Jordan, 52 poultry feed samples showed a maximum aflatoxin B1 concentration level of 12.7 μg/kg (Alshawabkeh *et al.,* 2015) which is significantly lower than what is observed in this study. In North Asia, 622 poultry feed samples showed an aflatoxin B1 level ranging between 8.7 μg/kg and 225 μg/kg (Rodrigues *et al.,* 2012) where both lower and higher values were relatively similar to the outputs of this study. The significant variation in aflatoxin level between the samples may be associated with storage conditions including factors like warmer temperature and humid environmental condition.

Table 7. Average level of aflatoxin G2, G1, B2, and B1 in poultry layer feed

Sample code	G2(μg/kg)	G1(μg/kg)	B2(μg/kg)	B1 (μg/kg)	Total AF(μg/kg)
Layer feed F001, LF1	3.08	66.28	12.22	95.19	176.77
Layer feed F001, LF2	2.85	58.75	12.22	87.09	160.91
Layer feed F002	2.95	102.65	18.85	121.50	245.95
Layer feed F004	ND	5.84	0.63	4.05	10.52
Layer feed F005	6.14	110.79	4.16	139.51	260.60
Layer feed F006	ND	2.06	ND	1.45	3.51
Average	2.50	57.73	8.01	74.80	143.04

LF1- Layer feed sample, high quality; LF2- Layer feed sample, low quality

Aflatoxin B1 and Total Aflatoxin levels of the poultry feeds collected from four factories were higher than the regulatory limits set by both FDA and WHO. The remaing two had AFB1 contamination level of 4.05µg/kg and 1.45µg/kg respectively which were below the limit. The low amount of aflatoxin contamination in poultry layer feed in F004 could be due to low amount of niger seed cake content in the feed since the feed has low nutritional quality as the NIR analysis (Annexes) shows but feed F006 has good nutritional quality and its low aflatoxin contamination might have been reduced by using protein sources other than niger seed cake such as soya bean cake.

Aflatoxin B1 adversely influences egg quality by decreasing shell thickness, egg weight, and egg energy deposition and lead to reduced egg production, poor egg quality and increased mortality of challenged hens. Besides, aflatoxin in laying hen feed can result in an aflatoxin residue in the eggs; therefore, it is very important to control aflatoxin concentrations in feeds for laying hens (Oliveira *et al.*, 2000).

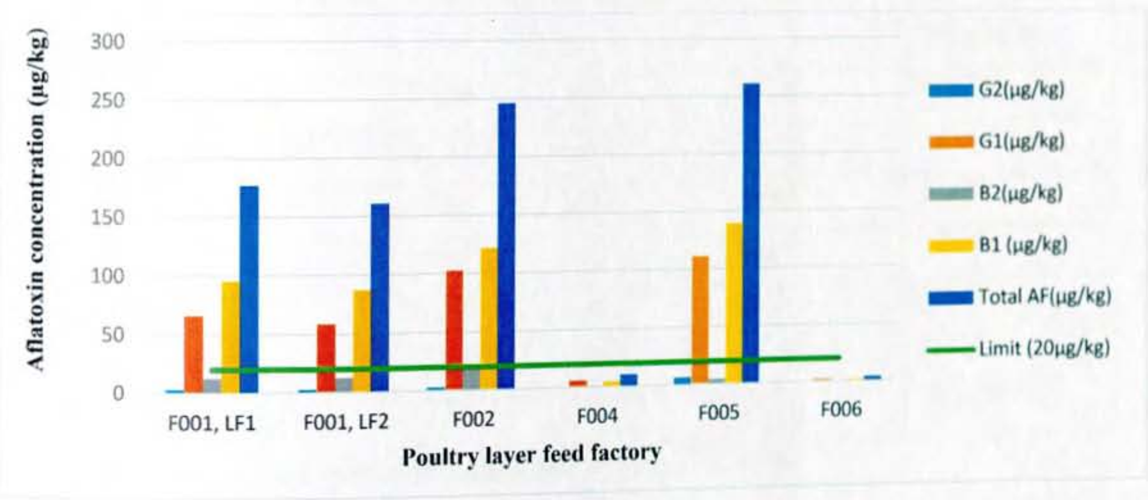


Figure 10. Aflatoxin contamination of poultry layer feed in different feed factory

4.8. Aflatoxin Levels in Poultry Broiler Feeds

The total aflatoxin content of poultry broiler feed collected from Addis Ababa feed factories ranged between 36.51µg/kg and 287.44µg/kg with a mean value of 151.07µg/kg and the aflatoxin B1 content ranged between 16.49µg/kg and 148.86µg/kg with a mean value of 71.22µg/kg (Table 8).

In a study on finisher broiler feeds conducted in Iran, aflatoxin B1 content ranged between 1.28 and 40 μg/kg and mean of AFBs 20.72μg/kg (Anjum *et al.,* 2012). In Pakistan range of 10 to166 μg/kg and mean aflatoxin content of 47.64 μg/kg were reported in poultry broiler finisher feed (Nemati *et al.*, 2014). The values reported by both studies above were significantly lower than the findings of this study.

The significant variation in aflatoxin level for broiler feed samples could be due to the utilization of different feed ingredients than that is used in Ethiopia. In finished animal feed, the contamination of an ingredient could cause the contamination of an entire feed batch (Gallo et al., 2015). The introduction of a feedstuff contaminated with aflatoxin-producing fungi could lead to the spoilage of other feed shipments and serves as a fungi source in the feed industry environment difficult to eliminate (Kovalsky *et al.*, 2016).

Table 8. Average level of aflatoxin G2, G1, B2, and B1 in poultry broiler feed

Sample	G2(μg/kg)	G1(μg/kg)	B2(μg/kg)	B1(μg/kg)	Total AF(μg/kg)
Finisher F001	3.15	78.96	13.24	99.16	194.51
Finisher F002	3.56	91.31	5.03	72.59	172.49
Finisher F004	1.18	17.18	1.66	16.49	36.51
Finisher F005, BF1	7.11	125.62	5.85	148.86	287.44
Finisher F005, BF2	4.56	91.60	6.03	72.59	174.78
Finisher F006	1.26	19.61	2.20	17.61	40.68
Average	3.47	70.71	5.67	71.22	151.07

BF1- Broiler feed sample, high quality; BF2-Broiler feed sample low quality

The limit for aflatoxin in poultry broiler feed set by the European Commission is 5 μg/kg and FAO/WHO level of 20μg/kg. Six poultry broiler feed samples collected from the feed factories in Addis Ababa. Four samples (67%) were aflatoxin B1 levels higher than the tolerable 20 μg/kg limit set by the Food and Drug Administration and European Commission (Figure 13).

Two of them (F004 and F006) lower than the tolerable limit the reason is as similar to the other feed. In Ethiopia limit set for poultry broiler feed aflatoxin B1, 20 µg/kg and total aflatoxin 40µg/kg similar to FDA. The high contamination of aflatoxin in broiler feed could be as a result poor storage condition and might be manufactured using an ingredient that is stored in bulk and for long times under condition that is a favorite for development of fungi

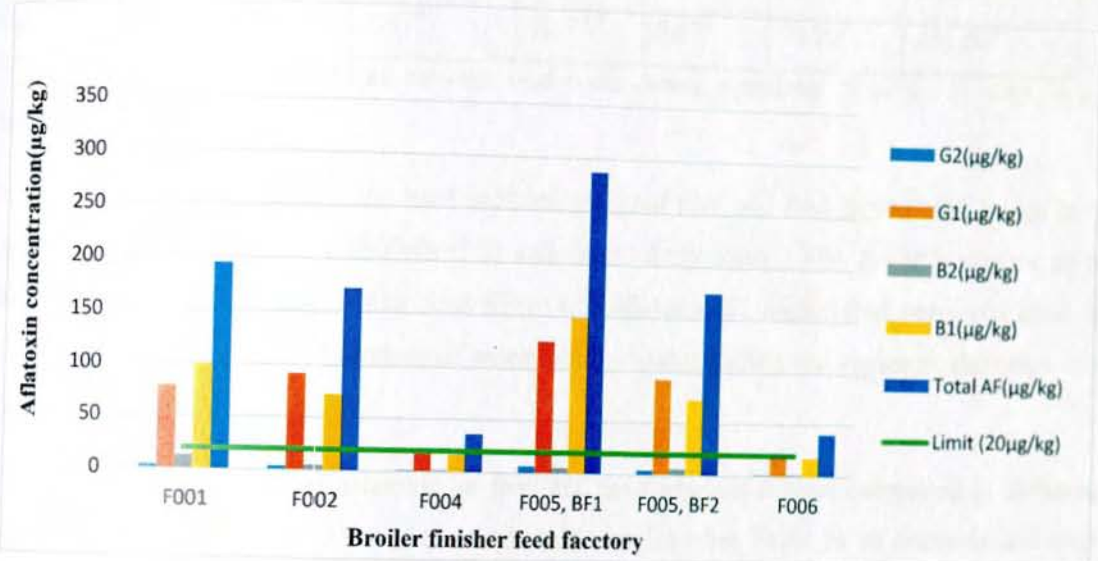


Figure 11. Aflatoxin contamination of poultry broiler feed in different feed factories

4.9. Comparison of Aflatoxin Levels among the Feeds

The ANOVA result of aflatoxin in different feed types collected from feed factories in Addis Ababa is shown in Table 9. There was no significant difference (P>0.05) between poultry layer and poultry broiler feed but in dairy feed, there was significant variation of Aflatoxin B1 and total aflatoxin. The reason for the higher level of aflatoxin contamination in dairy feeds could be due to the higher amount of niger seed cake used in the preparation of dairy feed. The contamination of an ingredient could cause the contamination of an entire feed batch (Gallo *et al.*, 2015). The high aflatoxin contamination of dairy feed shows a higher risk of aflatoxin contamination which leads to contaminate milk unless diluted with or replaced by other feeds.

Table 9. Comparison of aflatoxin levels among the feeds

Types of feeds	N	Mean aflatoxin level(µg/kg)				
		G2	G1	B2	B1	Total AF
Dairy feed	5	3.55[a]	104.35[b]	12.34[a]	192.80[b]	313.03[b]
Poultry layer feed	6	2.50[a]	57.73[a]	8.01[a]	74.80[a]	143.04[a]
Poultry broiler feed	6	3.47[a]	70.71 [a]	5.67[a]	71.22[a]	151.07[a]

[a-b] Different alphabate superscripts between food types denote significant difference ($p<0.05$) using duncan multiple range test

In summary (Table 10), from the total samples analyzed feed and feed ingredients, maize 20% (n=1/5) and niger seed cake 100%(n=5/5) and in the dairy feeds 100% (n=5/5), poultry layer feeds 67%(4/6) and poultry broiler feed 67% (4/6) aflatoxin B1 higher than permitted level of FDA and ESA (20µg/kg). The values of wheat bran, wheat middling and soybean cake below the tolerable limit.

Various studies on levels of aflatoxin in feed and feed ingredient were conducted in different parts of the world. Iran, 43.36% of the feed stuff samples were found to be contaminated with aflatoxin B1, which exceed the permissible concentration of AFB1 (Ersali *et al*, 2008). Turkey by Sahin *et al*, (2016) found that from n = 76 cattle feed samples, 26.3% and in India feed ingredients n=49, 24.5% (kotinagu *et al* 2015). The study conducted in Ethiopia (Dawit *et al.*, 2016) also similarly observed that a total of 156 dairy feed samples were analyzed for aflatoxin B1 using competitive enzyme immunoassay and 100% of samples exceeded the FAO level of 20µ g/Kg. The result of which is similar to the reports of this studies used the same type of raw material for dairy feeds

Table 10. Percentage of Aflatoxin B1 contamination in dairy, poultry feed and feed ingredients

Feed/feed ingredients	No. of samples	Positive samples higher than FDA limit (20 µg/kg)	Percent (higher than FDA limit)	Mean in µg/kg	Range in µg/kg
Dairy cattle feed	5	5	100%	192.80	51.66-370.51
Poultry layer feed	6	4	67%	74.80	1.45-139.51
Poultry broiler feed	6	4	67%	71.22	16.49-148.86
Feed ingredients					
Maize grain	5	1	20%	26.92	2.64-46.74
Wheat bran	5	0	-	3.70	ND-3.66
Wheat middling	5	0	-	2.20	ND-12.77
Soybean cake	5	0	-	3.36	ND-3.43
Niger seed cake	5	5	100%	385.45	110.93-438.86

Note: - ND= <LQ(<0.5µg/kg) ND= Not detected

LQ= Limit of quantification

4.10. Aflatoxin Knowledge Assessment of Feed Producer and Farmers

Knowledge, attitude, and practice (KAP) related to food quality and safety issues of dairy and poultry feed factories and farmers were assessed. A total of 30 participants; 7 dairy and poultry feed producers and 23 dairy and poultry farmers, were interviewed for their knowledge, attitude and practice (KAP) regarding aflatoxin contamination in dairy and poultry feed.

According to the results summarized in table 11 below, awareness of mold growth formation of mycotoxin is very low in dairy and poultry farmers. Knowledge about aflatoxins and its associated health impacts is very low as the farmers perceive that eating moldy food may be harmful but they considered milk, egg, and meat from animals fed moldy feeds to be safe. This could be supported by previous studies that showed lower levels of awareness about aflatoxins; 25% in the greater Addis Ababa milk shed of Ethiopia (Dawit, 2016), 6% in Vietnam (Lee *et al.*, 2017), 12% in Zimbabwe (Bankole, 2003), and 20% in Tanzania (Magembe *et al.*, 2016).

From the seven feed factories, 86% of the respondents reported that they were aware of mold growth and formation of mycotoxin while from the 23 dairy and poultry farmers, 79% of the respondents were not familiar about mold growth and aflatoxin formation. On the other hand, more than 50% of the respondents have answered that they don't know about aflatoxin in feed transfer to animal source of food.

Out of the 30 dairy and poultry feed producer and farmers were interviewed 25(80%) are not aware of cleaning, drying, ventilation, pest control, and visual inspection prior and after storage of the feed. This practice is critical in feed handling since in the storage fungi can grow easily and results in the formation of aflatoxin. According to the survey result, over 50% of the dairy and poultry feed producers and 40% of farmers did not know that milk, egg, and meat could be contaminated with aflatoxin as a result of contaminated dairy and poultry feed. Feed processors were more aware of aflatoxin than the dairy and poultry farmers yet they do not control the quality of the raw materials and finished products in fear of risking their business.

Table 11. Summary of aflatoxin KAP among dairy, poultry feed producers and farmers

Questions	**Response**	**Respondents**	
Knowledge (K)		producers	Farmers
Do you know what aflatoxin is?	Yes	6 (86%)	5(21%)
	No	1 (14%)	18(79%)
Can it cause adverse health effects even when found in small amount?	Yes	4 (57%)	2(9%)
	No	3 (33%)	21(91%)
Do molds cause harm to human and animal health?	Yes	6 (86%)	5(22%)
	No	1 (14%)	18(78%)
Do poor storage conditions pose a risk of aflatoxin contamination in feeds?	Yes	5 (71%)	4(17%)
	No	2 (29%)	19(83%)

Does feeding cows moldy feeds make milk unsafe for human consumption?	Yes No	4 (57%) 3 (43%)	5(22%) 18(78%)
Is meat from animals fed moldy feed unsafe for human consumption?	Yes No	2 (29%) 5 (71%)	3(13%) 20(87%)
Attitude(A)			
Do you think that animal feeds should be free from molds like that of human foods?		4(57%) 3(43%)	2(9%) 21(91%)
Practice (P)			
Do you store animal feeds for a long time?	Yes No	3(43%) 4(57%)	22(96%) 1(4%)
Do you clean the storehouse before storage?	Yes No	5(71%) 1(19%)	7(30%) 16(70%)
Do you dry animal feed before storage?	Yes No	3(43%) 5(57%)	4(17%) 19(83%)
Do you perform post storage activities like visual inspection, moisture and pest control ventilation	Yes No	2(28%) 5(72%)	3 (13%) 20(87%)
Do you carry out a regular aflatoxin analysis to assure consumers of the safety of feeds?	Yes No	1(14%) 6(76%)	0 (0%) 23 (100%)

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Aflatoxins were detected in all dairy feeds, poultry feeds and feed ingredients investigated in this study. According to the result of this study, 100% of the dairy feed contained aflatoxin B1 higher than the maximum tolerable limit set by FDA and ESA (20 μg/kg). The regulatory limit should be implemented properly with regular analysis of animal feed and feed ingredients. Employment of proper aflatoxin decontamination and deactivation strategy will help reduce aflatoxin exposure of human and animal.

For poultry layer and broiler feed analyzed in this study showed 67% of aflatoxin B1 levels higher than the maximum tolerable limit set by the FDA and ESA (20 μg/kg). The results indicated that these feed products can be major sources of the aflatoxin transferred in to source of food (eggs and meat). Therefore, the need for surveillance and constant monitoring programs could be crucial.

From the five feed ingredient types evaluated in this study 100% of the niger seed cake and 20% of the maize samples showed aflatoxin B1 above the tolerable limit. Wheat bran, wheat middling and soybean cake on the other hand contained aflatoxin in levels below the tolerable limit set by FDA and national standards.

The KAP assessment outcome about mycotoxin specifically aflatoxin, in the feed factories and dairy and poultry farmers are not satisfactory with awareness value of 63% of the respondents. Attitudinally, most don't expect and seem to worry about the presence of aflatoxin in the feed. Almost all dairy and poultry feed factories (76%) do not determine the level of aflatoxin in the incoming raw materials and final products in their laboratories.

5.2. Recommendations

- Since the major objective of the study was quantification of aflatoxin in dairy feeds, poultry feeds and ingredients, with a finding of most results exceeding legal limits, it is recommended that there should be control of mold growth and aflatoxin production by ensuring that animal feeds are dried adequately. The dried feeds should then be stored in well-aerated storage structures and at low moisture level to discourage mold growth and mycotoxin.
- Since the KAP done in the study shows knowledge gap in feed producers and farmers, Policies should be designed to build awareness programs about the health risks associated with spoilage molds in feed and food for stakeholders of animal feed production. Knowledge transfer on how habits affect health is recognized as an effective mechanism especially those involved in inspection and regulatory control.
- There should be regular monitoring of AFB1 in animal feeds as an essential pre-requisite to prevent aflatoxin buildup in dairy feed, poultry feeds, and ingredients to reduce mold growth.
- Regulations related to alternative uses of contaminated commodities as feed, such as the use of binders, blending, and decontamination technologies need to be included in the standards for feed development process based on scientific evidence.
- Based on the result of this study it is recommended for feed producers and dairy and poultry farmers to use another protein source of feed like soybean cake rather than feeding niger seed cake due to its high level of aflatoxin contamination.

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7. ANNEXES

Annexe-1. Interview form

I am Genet Mengesha, a postgraduate student of Addis Ababa University, Center for Food Science and Nutrition, conducting a study on Occurrence of Aflatoxins in Animal feeds and ingredients used for product of animal source of food in Addis Ababa. The purpose of my visit is to know the knowledge, Attitude and practice (KAP) on mold growth and formation of aflatoxin in animal feeds. If you are willing to participate in the study, I will ask you few questions for a few minutes. Your honest answers to these questions will help me for a better understanding of the topic, and will eventually help in designing and implementing appropriate interventions to alleviate related problems.

I really appreciate your input.

Name of the interviewer: Genet Mengesha

Signature: --

Date of the interview: ---------------------------------

KAP Questioners for animal feed producer, dairy and poultry farmers

Knowledge (K)	YES	NO
1. Do you know what aflatoxins are?		
2. Can aflatoxins cause adverse health effects even when found in		
3. Do molds cause harm to human and animal health?		
4. Do poor storage conditions pose a risk of aflatoxin contamination in		
5. Does feeding cows moldy feeds make milk unsafe for human		
6. Is meat from animals fed moldy feed unsafe for human consumption?		

Attitude (A)		
7. Do you think that animal feeds should be free from molds like that of human foods?		
8. Do you think animal feeds could be contaminated with mycotoxins such as aflatoxins?		
9. Do you think that the health of animal can be affected by its feed?		
10. Do you think that expired and or damaged items can be suitable for animal feed?		
Practice (P)		
11. Do you store animal feeds for a long time?		
12. Do you clean the storehouse before storage?		
13. Do you dry animal feed before storage?		
14. Do you perform post storage activities like visual inspection, moisture and pest control ventilation?		
15. Do you carry out a regular aflatoxin analysis to assure consumers of the safety of feeds?		

Annex-2. NIR results of dairy, poultry and feed ingredients

NIR Results of Ingerident (g/100g)

Samples	Moisture	Fat	Protein	Fiber	Ash
Maize	12.50	3.60	7.42	2.38	1.42
Wheat bran	11.78	3.36	12.57	9.48	3.42
Wheat middling	11.78	12.54	3.52	8.31	2.39
soybean cake	7.68	5.37	43.45	5.49	5.25
Niger seed cake	8.28	10.14	38.50	18.97	7.73

NIR Results of Dairy Feed (g/100g)

Sample	Moisture	Fat	Protein	Fiber	Ash
Dairy F001	9.94	3.61	20.00	10.69	6.01
Dairy F002	9.56	5.28	14.08	8.57	5.92
Dairy F003	9.72	3.24	20.21	9.35	6.03
Dairy F004	8.72	4.63	12.69	11.58	6.52
Dairy F 006	5.63	4.46	21.80	12.71	8.28

NIR Results of Layer Feed (g/100g)

Samples	Moisture	Fat	Protein	Fiber	Ash
Layer 1 F001	8.99	9.4	25.39	8.60	7.01
Layer 2 F001	9.35	5.28	22.83	7.06	7.06
Layer F002	9.75	5.96	23.88	4.87	6.83
Lyer F004	9.05	4.38	14.91	6.27	4.18
Layer F005	9.10	3.38	19.16	3.96	5.74
Layer F006	9.08	5.23	20.27	10.50	7.12

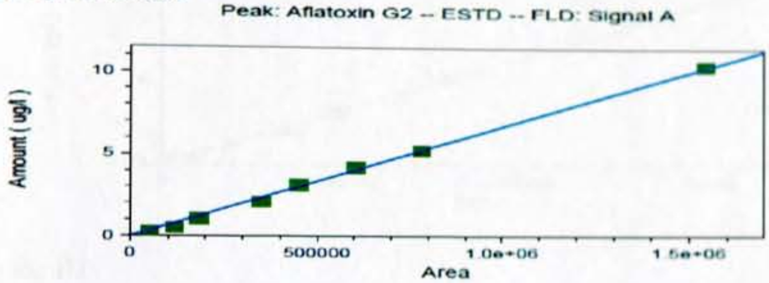
NIR Results of Broiler Feed (g/100g)

Samples	Moisture	Fat	Protein	Fiber	Ash
Finsher F001	8.36	4.11	19.30	8.02	5.55
Finsher F002	8.29	4.60	20.92	7.41	5.31
Finsher F004	8.69	4.78	15.64	8.26	4.76
Finsher1 F005	7.65	5.29	24.86	7.25	5.72
Finsher2 F005	8.03	4.58	21.16	7.19	5.07
Finsher F006	8.41	4.35	22.57	6.28	4.90

Annex 3. Calibration Curve

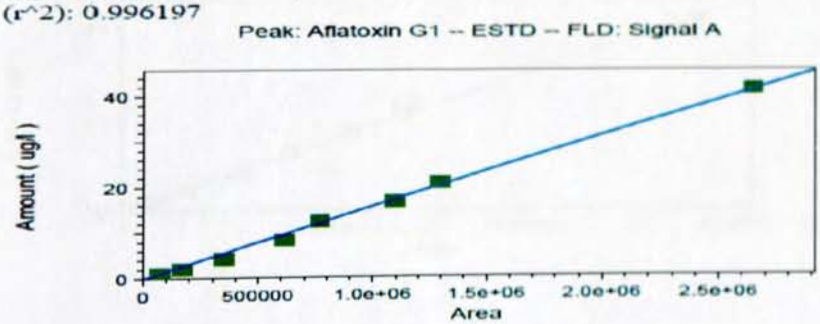
Calibration curve for G2

Aflatoxin G2 (FLD: Signal A)
Average RF: 5.91021e-006 RF StDev: 1.19187e-006 RF %RSD: 20.1663
Scaling: None LSQ Weighting: None Force Through Zero: On
Replicate Mode: Replace
Fit Type: Linear
y = 6.73640e-006x + 0.000000
Goodness of fit (r^2): 0.997016



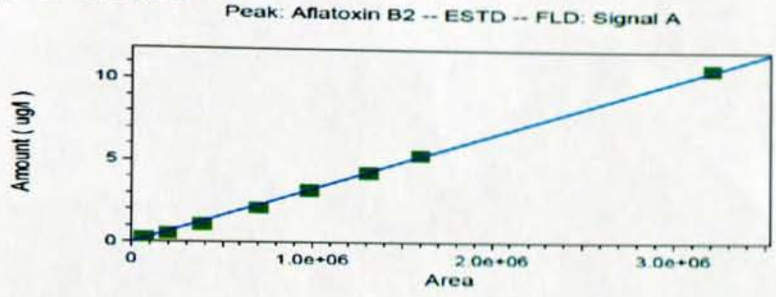
Calibration curve for G1

Aflatoxin G1 (FLD: Signal A)
Average RF: 1.39912e-005 RF StDev: 2.03677e-006 RF %RSD: 14.5575
Scaling: None LSQ Weighting: None Force Through Zero: On
Replicate Mode: Replace
Fit Type: Linear
y = 1.55910e-005x + 0.000000
Goodness of fit (r^2): 0.996197



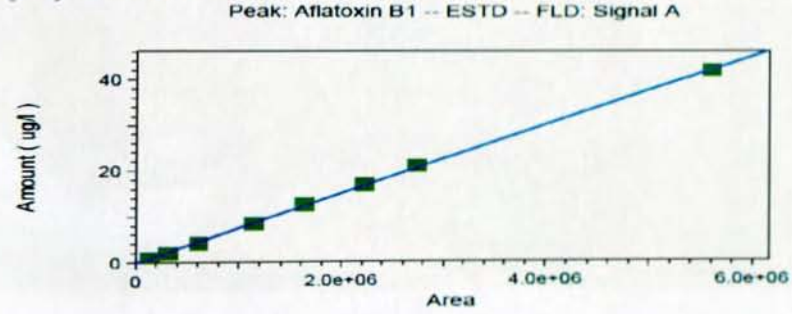
Calibration curve for B2

Aflatoxin B2 (FLD: Signal A)
Average RF: 3.08175e-006 RF StDev: 2.73879e-007 RF %RSD: 8.88711
Scaling: None LSQ Weighting: None Force Through Zero: On
Replicate Mode: Replace
Fit Type: Linear
y = 3.31315e-006x + 0.000000
Goodness of fit (r^2): 0.998638



Calibration curve for B1

Aflatoxin B1 (FLD: Signal A)
Average RF: 7.19243e-006 RF StDev: 5.32443e-007 RF %RSD: 7.40283
Scaling: None LSQ Weighting: None Force Through Zero: On
Replicate Mode: Replace
Fit Type: Linear
y = 7.52622e-006x + 0.000000
Goodness of fit (r^2): 0.999559



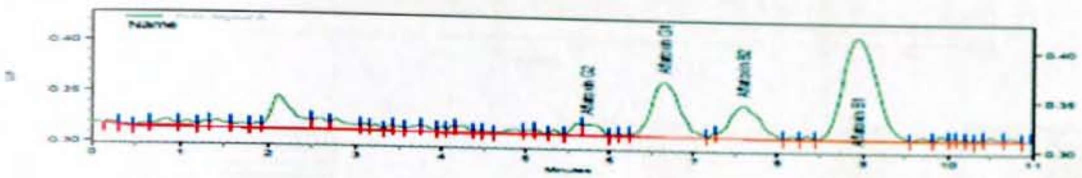
Annex -4. A Pictures of sample preparation and analysis

Sample preparation

Extraction and clean up

Annex-5. Chromatogram of individual aflatoxin in dairy, poultry feed and feed ingredient samples

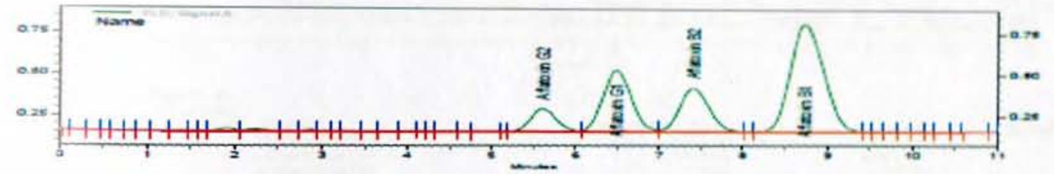
Maize F002



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
29	Aflatoxin G2	5.724	27502	0.184
32	Aflatoxin G1	6.638	219288	3.419
34	Aflatoxin B2	7.589	136417	0.441
37	Aflatoxin B1	8.942	473882	3.694
Totals			857089	7.739

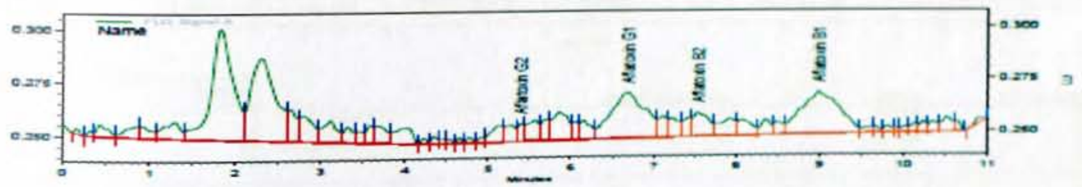
Maize F003



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
28	Aflatoxin G2	5.609	496383	3.329
29	Aflatoxin G1	6.487	1458118	22.736
30	Aflatoxin B2	7.409	1082940	3.314
32	Aflatoxin B1	8.734	2986040	22.729
Totals			6023481	52.108

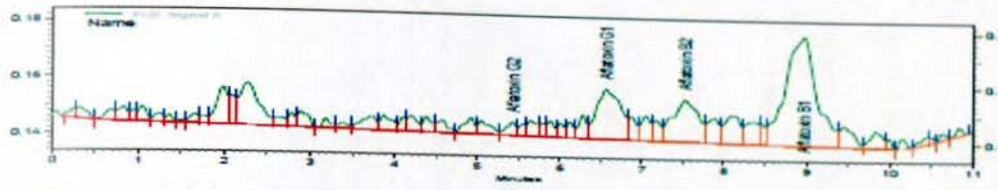
Wheat bran F005



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
26	Aflatoxin G2	5.393	7585	0.061
32	Aflatoxin G1	6.696	103001	1.606
36	Aflatoxin B2	7.546	24495	0.101
41	Aflatoxin B1	8.993	106476	0.910
Totals			241557	2.669

Wheat middling F003

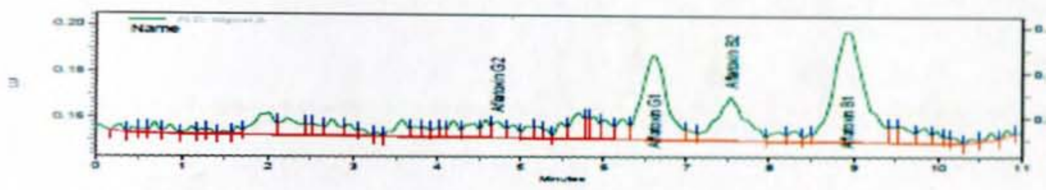


FLD: Signal A

Results

Pk #	Name	Retention Time	Area	Concentration
28	Aflatoxin G2	5.429	6697	0.045
36	Aflatoxin G1	6.566	61636	0.961
40	Aflatoxin B2	7.524	55337	0.196
45	Aflatoxin B1	8.971	173847	1.421
Totals			297517	2.622

Soybean cake F001

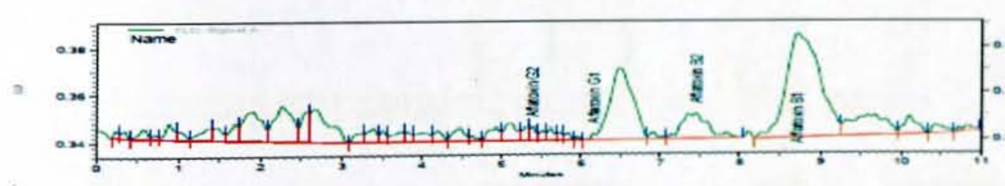


FLD: Signal A

Results

Pk #	Name	Retention Time	Area	Concentration
27	Aflatoxin G2	4.723	11485	0.077
38	Aflatoxin G1	6.602	129118	2.013
40	Aflatoxin B2	7.538	77448	0.262
44	Aflatoxin B1	8.928	206042	1.666
Totals			424093	4.017

Soybean cake F007

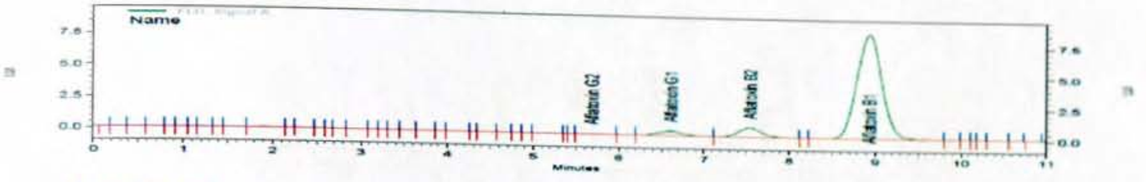


FLD: Signal A

Results

Pk #	Name	Retention Time	Area	Concentration
26	Aflatoxin G2	5.386	5141	0.034
32	Aflatoxin G1	6.156	105926	1.682
34	Aflatoxin B2	7.452	40239	0.149
35	Aflatoxin B1	8.712	217421	1.781
Totals			368727	3.686

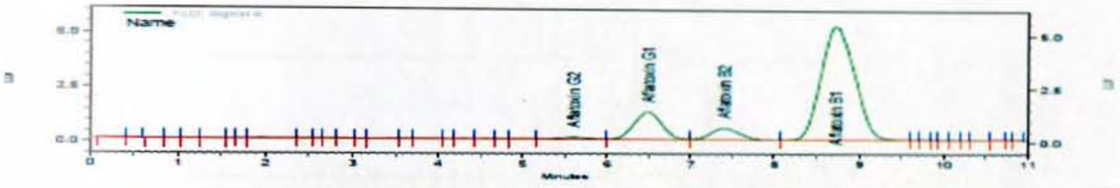
Niger seed cake F001



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
33	Aflatoxin G2	5.681	42619	0.286
35	Aflatoxin G1	6.610	1173878	18.304
36	Aflatoxin B2	7.546	2345925	7.148
38	Aflatoxin B1	8.935	28388892	215.207
Totals			31951314	240.944

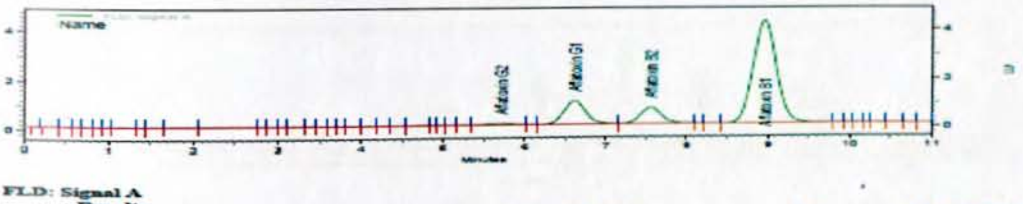
Niger seed cake F003



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
23	Aflatoxin G2	5.602	370692	2.486
24	Aflatoxin G1	6.487	4976519	77.698
25	Aflatoxin B2	7.394	2185574	6.661
26	Aflatoxin B1	8.726	24334534	184.487
Totals			31867319	271.232

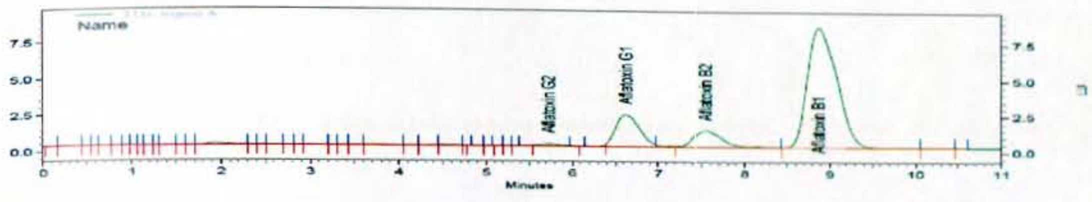
Dairy feed F001



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
30	Aflatoxin G2	5.717	173954	1.167
32	Aflatoxin G1	6.624	2960618	46.164
33	Aflatoxin B2	7.560	2019844	6.158
36	Aflatoxin B1	8.964	14139726	107.240
Totals			19294142	160.729

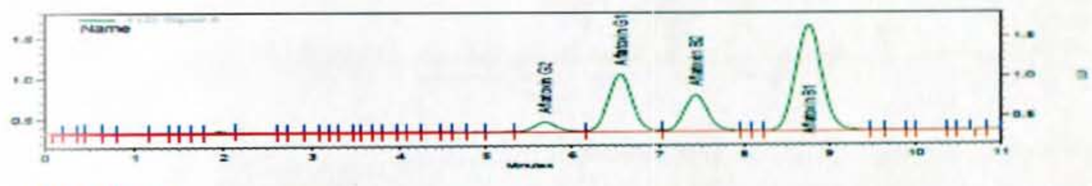
Dairy feed F002



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
35	Aflatoxin G2	5.724	454249	3.046
37	Aflatoxin G1	6.624	7230287	112.741
38	Aflatoxin B2	7.538	4481330	13.650
39	Aflatoxin B1	8.870	33903328	256.990
Totals			46069194	386.406

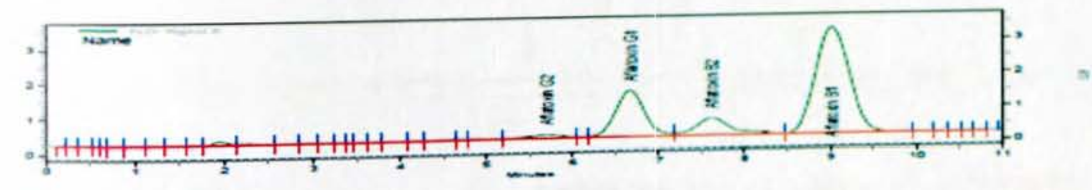
Dairy feed F004



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
30	Aflatoxin G2	5.681	374147	2.620
31	Aflatoxin G1	6.566	2327228	36.284
32	Aflatoxin B2	7.438	1463760	4.850
35	Aflatoxin B1	8.762	4746683	35.725
Totals			8911818	79.378

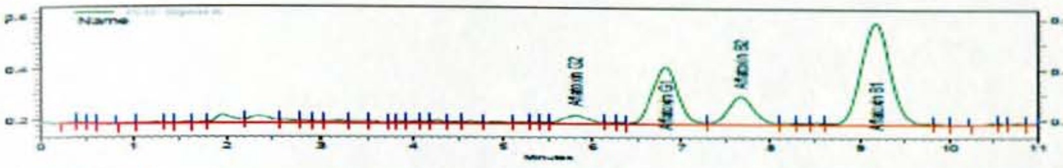
Broiler finisher F005



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
25	Aflatoxin G2	5.702	515772	3.489
27	Aflatoxin G1	6.674	5336200	83.206
28	Aflatoxin B2	7.618	2392289	7.289
29	Aflatoxin B1	9.014	14227410	107.905
Totals			22471671	201.889

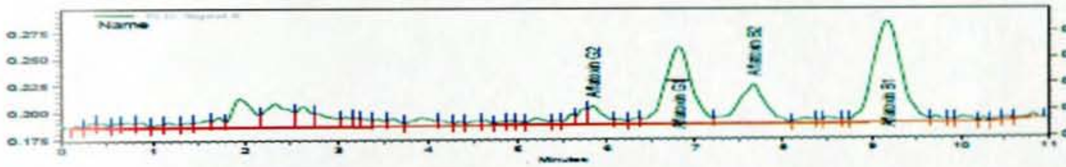
Broiler finsher F004



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
29	Aflatoxin G2	5.810	127484	0.866
32	Aflatoxin G1	6.826	783190	12.212
33	Aflatoxin B2	7.668	387258	1.202
37	Aflatoxin B1	9.180	1523999	11.651
Totals			2821931	26.920

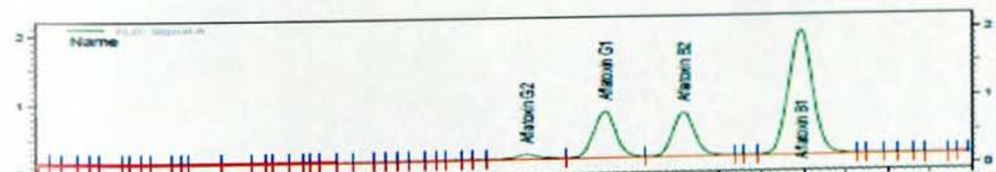
Layer F004



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
33	Aflatoxin G2	5.846	34574	0.232
36	Aflatoxin G1	6.811	262383	4.091
37	Aflatoxin B2	7.668	137286	0.444
42	Aflatoxin B1	9.180	360040	2.832
Totals			794283	7.598

Layer 1 F001



FLD: Signal A
Results

Pk #	Name	Retention Time	Area	Concentration
33	Aflatoxin G2	5.688	224006	1.602
34	Aflatoxin G1	6.624	2082917	32.479
35	Aflatoxin B2	7.560	1960180	5.977
38	Aflatoxin B1	8.964	6098537	46.312
Totals			10365640	86.270