ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL SCIENCE

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Aflatoxins (B1, B2, G1 and G2) and Aflatoxigenic Fungi in Export Standard White and Red Sesame Seeds (*Sesamum indicum l.*) Grown in Humera and Wollega, Ethiopia

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A THESIS SUBMITTED TO ADDIS ABABA UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN FOOD SCIENCE AND NUTRITION

ADDIS ABABA, ETHIOPIA

June, 2017

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Abstract

The oilseed sector is one of the fastest growing sectors in Ethiopia, both in terms of its foreign exchange earnings and as source of income for millions of Ethiopians. Oilseed crops are recognized to be potentially suitable substrates for the production of aflatoxins by aflatoxigenic fungi. This study aimed to evaluate Knowledge attitude and practice (KAP) of sesame exporters about aflatoxin contamination, analyze thousand seed weight, moisture, crude fat and peroxide value of white and red sesame collected from Humera and Wollega varieties. Determining the level of aflatoxins of white and red Ethiopian sesame seeds. Compare the level of aflatoxins between white and red Humera and Wollega varieties. Isolate and identify aflatoxigenic fungi from sesame seeds. A total of 27 sesame samples were collected from Ethiopian Agricultural commodities warehouse service enterprise, located in Addis Ababa. Semi-structured questionnaires used for knowledge, attitude and practice survey of sesame exporters and Thousand seed weight, moisture, crude fat and peroxide value of sesame seeds have been done according to American oil chemists society (AOCS) method. Analysis of aflatoxins was done by using HPLC with solid phase extraction clean up method. Isolation and identification of aflatoxigenic fungi has been done by culturing sesame samples on Potato dextrose agar (PDA) media. Sixty percent of exporters doesn't have any idea about aflatoxins and all of them cannot make aflatoxins test for sesame seed before export. Thousand seed weight, moisture, crude fat and peroxide value were ranged 2.89-3.22g/1000 seed, 3.33-4.99 %, 46.75-57.75% and 2-8.2 meq/kg, respectively. Aflatoxins detected from 93 % of samples in the range of (0.44 - 48.28 ng/g). Average content of AFB1, AFB2, AFG1, AFG2 and total aflatoxins are 0.83 ng/g, 6.82 ng/g, 17.25 ng/g, 1.17 ng/g and 26.07 ng/g, respectively. AFB1 and AFG2 detected below European union (EU) maximum limit for individual aflatoxin (2 ng/g). AFB2, AFG1 and total aflatoxins content is above the permissible limit set by EU for both individual and total aflatoxins (15 ng/g). Red sesame variety indicates relatively higher level of aflatoxins contamination as compared with white Humera and white Wollega sesame. *Aspergillus flavus*, *A. parasiticus* and *A. niger* isolates identified. Based on the finding, the contamination level may affect national economy.

Key Words: ***Sesame, Aflatoxins, HPLC, Solid phase extraction, Oilseed***

Acknowledgement

First of all my heartfelt thanks goes to my advisor Dr. Ashagrie Zewdu for his full support, encouragement, availability when I need his support and guidance as an advisor, as father, elder brother as well as, as close friend. The words those come from him made me strong, standby and to use my full effort to deal with problems on my surrounding. He helped me to do things efficiently, timely, effectively and really I'm full of proud for being his student. Honestly, my successful accomplishment of this work is unthinkable without Dr. Ashagrie assistance. Thank you!

I proceed my long list of thanks for the whole staff of Addis Ababa university Center for food science and nutrition from the Head of the center Dr. Kaleab Baye to the staff clearer Aregash Beshir for the smile face that all of you showed me during my time in the center.

I would like to extend my appreciation to Ethiopian Agricultural Commodities Warehouse service enterprise (EACWSE), especially Yinebeb and Nuriye, Ethiopian Conformity Assessment enterprise, Ermi and Bless Agri Food laboratories, and Chemistry laboratory head Henok Ashagrie.

Next, my closest friend Sisay Tolla I will never forget you, honestly two years with you seems only two days, you made me all the time laughing, and enjoying my time in the center. Laboratory assistances Debebe Hailu and Yemaneh H/mariam, Thank you both for your support. I will remember the lunch times that we had throughout the year with all of you.

My Lovely Dad thank you for covering the expense for my study and my dear brother Tilahun Iyasu for everything you did.

Finally I would like to thank almighty God for adding this day in my life.

Thank you all!

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Abbreviations

A: Adenine

AF: Aflatoxins

AF36: Aflatoxin 36, Non-aflatoxin producing strains of *Aspergillus flavus*

AFAR: Aflatoxin Aldehyde Reductase

AFB1 : Aflatoxin B1

AFB2 : Aflatoxin B2

AFG1 : Aflatoxin G1

AFG2 : Aflatoxin G2

C: Cytosine

CSA: Central Statistics Agency

CYP1A2: Cytochrome P-450 family 1, subfamily A, enzyme within subfamily 2

CYP3A4: Cytochrome P-450 family 3, subfamily A, enzyme within subfamily 4

DNA: Deoxyribonucleic Acid

DSW: Damaged, Shriveled, Weevil bored

EAC: East African Community

EACWSE- Ethiopian Agricultural Commodities Warehouse Service Enterprise

EC: European Commission

ECX: Ethiopian Commodity Exchange

EFMHCA: Ethiopian food, medicine and health care administration authority

EFSA: European Food Safety Authority

ELISA: Enzyme Linked Immunosorbent Assay

EU: European union

FAO: Food and Agricultural Organization

FSA: Food Standards Agency

G: Guanine

GSH: Reduced form of glutathione

GST: Glutathione *S*-transferase

HCC: Hepatic cell carcinoma

HPLC: High Pressure/Performance Liquid Chromatography

HP-TLC: High performance Thin-layer Chromatography

ICH: International Conference on Harmonization

JECFA: Joint Expert Committee for Food Additives

KAP: Knowledge Attitude Practice

LAB: Lactic Acid Bacteria

LD_{50}: Lethal Dose

LOD: Limit of Detection

LOQ: Limit of quantification

LSD: Least Significance Difference

NADPH: Nicotinamide adenine dinuclotide phosphate

PDA: Potato Dextrose Agar

RSD: Relative Standard Deviation

SNNPR: Southern Nations, Nationalities and Peoples Region

T: Thiamine

TLC: Thin-layer Chromatography

U: Uracil

UV: Ultra violate

WHO: World Health Organization

CHAPTER ONE

1. Introduction

1.1. Background

Oilseeds are generally, grown primarily for the production of edible or cooking oil. Oil seeds and their products are mainly consumed throughout the world as snack as well as part of the ingredients of certain dishes in human daily diet. Soybeans (*Glycine max*), sunflower seed (*Helianthus annuus*), canola (*Brassica napus*), rapeseed (*Brassica rapa*), safflower (*Carthamus tinctorium*), flaxseed (*Linum usitatissimum*), mustard seed (*Brassica carinata*), peanuts (*Arachis hypogae*), sesame seed (*Sesamum indicum*), linseed (*Lens culinaris*), castor bean (*Ricinus communis*) and cottonseed (*Gossypium spp.*) are used for the production of cooking oils, protein meals for livestock, and industrial uses (Princen, 1983).

Ethiopia is one of the major centers of origin and/or diversity for several oil crops. Rape seed (*Brassica carinata*), Niger (*Guizotia abyssinica*), Sesame (S*esamum indicum*) and Linseed (*Lens culinaris*) are the major indigenous oil crops having considerable diversity in the country (CSA, 2013). The oilseed sector in Ethiopia is one of the fastest growing sectors in the country, both in terms of its foreign exchange earnings and as source of income for millions of Ethiopians. Sesame is among the most important oilseed crops in the country, mainly as a commercial export commodity (CSA, 2010). In 2014/15, Ethiopian sesame exports were valued about $482 million from 292,298 metric tons sesame, which is the second largest export-revenue after coffee. Ninety five percent of exports are in the form of unprocessed seeds (FAS, 2016). The traditional importers of Ethiopian sesame seed were China, Israel, Turkey and other Middle Eastern countries. Currently, more European countries and Japan are also trying to enter the market (Gelalcha, 2009).

Sesame *(Sesamum indicum L.)* is an important oil seed crops belonging to the family *Pedaliaceae*. It grows in tropical zones as well as in temperate zones between latitudes of 40°N and 40°S (Onsaard, 2012) (Ogbonna and Ukaan, 2013). Study reports indicate that Ethiopia is among the top six producers of sesame seed, in the world (Wijnands et al., 2009). Geographically, sesame is produced in different parts of Ethiopia at an elevation from sea level

of about 1500 meters. The dominant producers, who contribute over 83 percent to national production, are located in the regions of Tigray (West Tigray), Amhara (North Gonder), Wollega and most recently, in Benishangul-Gumuz Region (Metekel) (CSA, 2010).

Ethiopia has high quality sesame seed varieties suitable for wide range of uses, the two most widely known sesame varieties are Humera and Wollega derive their names from the areas where they are planted (FAS, 2016). Whitish Humera type has good demand in the world market & known for its top quality. Also it is used as a reference for grading in the international market and it is appreciated worldwide for its aroma and taste. Wollega type sesame is the other one which is mixed /brownish in color and used for oil extraction as it has high oil content (Yitbarek, 2015).

Sesame is nutritionally important and it is widely used for bakery products and as vegetable cooking oil. Sesame seeds are an important source of oil (44–58%), protein (18–25%), carbohydrate (13.5%) and ash (5%). The oil is an excellent vegetable oil because of its high content of antioxidants such as sesamin, sesamol and sesamolin and its fatty acid composition which shows remarkable stability to oxidation and have long shelf life (Elleuch et al., 2007).

However, oilseed crops are recognized to be potentially suitable substrates for the production of toxic secondary metabolites by molds, notably the production of aflatoxins by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Hesseltine et al., 1966). The fungi from the genus *Aspergillus*, especially *A. flavus* and *A. niger* are fungal species that often cause problems in oilseeds related to discoloration, rotting, seed shrinking, necrosis and germination, leading to a toxigenic potential mainly by aflatoxin production. Being hot and humid climate plant may also make sesame seed more prone to mycotoxin contamination (Chavan and Kakde, 2008).

The mycotoxin production in oilseed is dependent on the type of raw material and its composition. Nutritional factors such as carbon, nitrogen, amino acids and trace elements influence mycotoxin synthesis. Simple sugars such as glucose, maltose, lactose and sorbose also may assist in toxin production (Cuero et al., 2003) (Feng and Leornard, 1998) (Yu et al., 2003). Tryptophan inhibits formation of tyrosine assists in toxin generation by *A. flavus* (Yu et al., 2008). The production of aflatoxins is directly related to the number of double bonds in the molecules of free and esterified fatty acids: linoleic acid and trilinolein stimulate the production

of aflatoxins in greater proportion than oleic acid, triolein, and other saturated fatty acids and triacylglycerols. Lipid oxidation increases the biosynthesis of aflatoxins (Fanelli and Fabbri, 1989) (Bircan, 2006) (De Luca et al., 1995) (Jayashree and Subramanyam, 1999).

1.2. Statement of the problem

Studies on the occurrence of aflatoxins on sesame seeds were done in different countries of the world. For example, in Greek thirty samples of sesame products were examined for the presence of AFB1. After analysis, 77.6% of samples were found to be contaminated. Eight samples exceeded the European Union (EU) limit (2 µg/kg) (Eleni et al., 2016). In Nigeria analysis of thirty sesame samples showed that eight samples contained AFB1 within the range of 14.71-140.9 µg/kg (Makun et al., 2014). These results indicate that aflatoxin contamination in sesame were found to be beyond the tolerable limit set by the European Commission and require similar work in Ethiopia.

There are numbers of studies conducted in Ethiopia by different authors on Aflatoxins contamination on different agricultural commodities, food and feeds like groundnut, maize, sorghum, milk, shiro and ground red pepper (Ayalew, 2010) (Eshetu, 2010) (Ephrem et al., 2014) (Mohammed and Chala, 2014) (Ayalew, et al., 2006) (Fufa and Urga, 1996). A study by Amare *et. al.,(*1995*)* reported aflatoxin levels of 5-250 µg/kg in groundnut seed from eastern Ethiopia (Ayalew et al., 1995). Alemayehu *et. al.,* (2012) reported that total aflatoxin levels in *Aspergillus flavus* positive samples of groundnut seed varied between 15 and 11865 µg/kg (Alemayehu et al., 2012). These results indicated aflatoxins contamination of groundnut samples from Ethiopia, at levels much higher than any international acceptable standards, which is far beyond the Food and Agricultural Organization of the United Nations (FAO), World Health Organization (WHO) standard (15 µg/kg) and the European Union (EU) limit i.e. 4-15 µg/kg.

The quality parameters for export standard sesame seed are thousand seed weight greater than 3g, 40-50 % of oil content, pearly white seed color and 99 % seed purity is required for export (Wijnands et al., 2007). In Ethiopia, Ethiopian Commodity exchange authority (ECX) has set the following quality parameters for export standard sesame seeds in accordance with minimum internationally agreed standards. The minimum international standard of oil content is 52%, 48%, and 45% for first, second and third grades respectively. Minimum acceptable moisture

content is 6-8% for all grades, the percentage of foreign bodies such as dirt, branches, stones etc. internationally agreed standard is 2% for first grade, 4% for second, and 6% for third grade sesame (ECX., 2015). Test for aflatoxins is not part of the ECX standard for sesame seed to be export standard.

The occurrence of aflatoxins in sesame seed may reduce its quality and quantity, which is capable of affecting the health of people who consume the sesame seed and the country's foreign exchange as well as it will interrupt the wellbeing of Ethiopia's national economy, since Sesame seed is the second largest export revenue generator after coffee. So, it is expected to be free from any potential food safety hazards including aflatoxin.

Even though, various studies have been conducted in Ethiopia on aflatoxin occurrence in different agricultural commodities, food and feed by different authors, there is no single study conducted on aflatoxin contamination of sesame seed so far. So, this study was conducted to investigate Aflatoxins and Aflatoxigenic fungi in Export Standard White and Red Sesame Seeds of Humera and Wollega varieties, Ethiopia.

1.3. Significance of the study

- ✓ Determining the level of aflatoxin of the sesame may make Ethiopian sesame export ready and increase its destination to different parts of the world.
- ✓ Maintaining the wellbeing of Ethiopian economy by preventing loss of export revenue.
- ✓ For government bodies like EFMHCA (Ethiopian food, medicine and health care administration authority) and Ethiopian standards Authority to set the limit for regulation.
- ✓ The study may serve as baseline for further studies that can be done on aflatoxin contamination of sesame seed.

1.4. Research questions

- What is the knowledge, attitude and practice (KAP) of sesame seed exporters about Aflatoxins?
- Is the aflatoxin content of Ethiopian export standard red and white sesame seed above the permissible limit of East African standard or not?

- Is there significant difference between white and red Humera and Wollega sesame seed varieties in the aflatoxins content?
- What are the most prevalent aflatoxigenic fungi in sesame?

1.5. Objectives

1.5.1. General objective

To investigate Aflatoxins and Aflatoxigenic fungi in export standard White and Red sesame seed of Humera and Wollega varieties, Ethiopia.

1.5.2. Specific objectives

- To evaluate KAP of sesame exporters about aflatoxin contamination.
- To determine thousand seed weight, moisture, crude fat and peroxide value of white and red sesame collected from Humera and Wollega varieties.
- To determine the level of Aflatoxins (B1, B2, G1 and G2) of white and red Ethiopian Sesame seeds.
- To compare the level of Aflatoxins between white and red Humera and Wollega varieties.
- To isolate and identify aflatoxigenic fungi from sesame seeds.

CHAPTER TWO

2. Literature Review

2.1. Oilseeds

Oilseeds are generally, grown primarily for the production of edible or cooking oil. Oil seeds and their products are mainly consumed throughout the world as snack as well as part of the ingredients of certain dishes in human daily diet (Princen, 1983). They have been part of the diet for a long time and their production has shown a remarkable increase in the last few decades. This development is due to increased use of edible fats as vegetable oils. Today's dietary trend is gradually replace fat from animal origin with those from vegetable, including countries where people traditionally consume fat predominantly from animals. The change is connected with the notion of healthier lifestyle and the need to eat food with a positive influence on health, increasing the use of foods rich in proven beneficial components (Tuberoso et al., 2007).

Soybeans (*Glycine max*), sunflower seed (*Helianthus annuus*), canola (*Brassica napus*), rapeseed (*Brassica rapa*), safflower (*Carthamus tinctorium*), flaxseed (*Linum usitatissimum*), mustard seed (*Brassica carinata*), peanuts (*Arachis hypogae*), sesame seed (*Sesamum indicum*), Linseed (*Lens culinaris*), castor bean (*Ricinus communis*) and cottonseed (*Gossypium spp.*), used for the production of cooking oils, protein meals for livestock, and industrial uses (Princen, 1983). Sesame seeds are commonly used as a raw material for oil extraction. They have oil content between 48 - 58%; as a result, they have become one of the main sources of edible oil (Elleuch et al., 2007) (Cano-Medina et al., 2011).

2.2. Sesame

Sesame (*Sesamum indicum* L.) is an important oilseed crop belonging to family *Pedaliaceae.* It is an ancient oil-yielding crop cultivated for its flavorsome. It is also an edible seed and has high quality oil (Pathak et al., 2014). Sesame is nutritionally important and it is widely used for bakery products and as vegetable cooking oil. The seed is good source of oil (44–58%), protein (18–25%), carbohydrate (13.5%) and ash (5%) depending on the variety. The oil is an excellent

vegetable oil because of its high content of antioxidants such as sesamin, sesamol and sesamolin and its fatty acid composition which shows remarkable stability to oxidation and have long shelf life (Elleuch et al., 2007) (Cano-Medina et al., 2011). Sesame seeds are also very useful for body as they are anti-aging and rich in vitamins E, A and B complex and minerals such as calcium, phosphorus, iron, copper, magnesium, zinc and potassium (Bukya and Vijayakumar, 2013).

2.2.1. Growth Conditions for sesame

Sesame grows in hot and humid climate with temperature around 27 °C and annual precipitation of 625-1100 mm. The crop is intolerant to water logging or poor drainage and excessive rain fall (Ayana, 2015) (MARD, 2008). Sesame is annual crop which is primarily adapted to areas with long growing seasons and it prefers slightly acid to alkaline soils (pH 5-8) with moderate fertility. Clay soils are not suitable for cultivation of sesame seed, which is more prone to water logging. Sesame will not withstand water over the stem because it limits oxygen presence to the roots and suffocates the plants. Even if the plants do not die, they will be more susceptible to root rots and will yield less. Sesame will perform best on fertile and well-drained soils such as silt loams and it is adapted to sandy loam soils that provides them adequate moisture during seedling establishment and it has been grown satisfactorily on silty clay loam soils (Langham, 2008).
Planting sesame is management of adequate moisture and temperature to establish a strong stand advantage for sesame to compete against weeds for light, moisture, and nutrients before residual herbicides wear off. Successful establishment of sesame requires careful seedbed preparation and close attention to soil moisture. Sesame will not emerge from soils that are even slightly crusted (Langham, 2008). Maturity depends on the weather condition and it usually varies from 90-105 days. Mid October to November is the usual harvesting time. Harvesting begins when two third of the plant and seed pods turn yellow (Yitbarek, 2015).

2.2.2. Sesame production in Ethiopia

According to FAO, (2008) the top ten sesame producing countries in the world are China, India, Myanmar, Sudan, Uganda, Nigeria, Pakistan, Ethiopia, Bangladesh, and Central African Republic (FAOSTAT, 2008). Ethiopia's share covers 8.18% of the total world production (FAO, 2015). In Ethiopia there are regions which satisfy the above requirements for the cultivation of

sesame. The major sesame growing areas are located in the Northwest; in Humera area in Tigray near the border with Sudan and Eritrea; in Metema in North Gondar and in Wollo area of Amhara region, Chanka area in Wollega of Oromia, in Pawi area in Benshangul Gumuz region, and a small amount of sesame is currently produced in SNNPR (Dawit and Meijerink, 2010) (CSA, 2008). The map below shows the major areas that well suit for the production of sesame in Ethiopia.

Figure 2.1 Main sesame growing regions in Ethiopia, underlined (Dawit and Meijerink, 2010).

As average production statistics from 2005-2012 obtained from CSA (2013) depict, almost 37 % of the country's total sesame seed production comes from the Amhara regional state, with 30 % coming from Tigray and 16 percent from Oromia. However, for the stated period, the highest average productivity for Tigray was about 9 quintal/hectare, followed by Amhara region about 8 quintal/hectare (CSA, 2013) (FAO, 2015). The total area, production and productivity during 2013 were 0.299 million ha, 0.220 million tonnes and 0.735 t /ha, respectively; and the total area and production were increased by 61.23 % and 17.91 %, respectively, while the total productivity was decreased by 27.23 % when compared with 2008 (CSA, 2013).

Table 2. 1 Area cultivated, production and productivity of sesame from 2009-2013 (CSA)

Year	Areas in Hectares	Production in tons	Production in ton/hectares
2009/10	315.85	260,534	0.83
2010/11	384.68	327,741	0.85
2011/12	328.32	247,783	0.75
2012/13	239.53	181,376	0.76

2.2.3. The quality parameters for Ethiopian export standard sesame

Regarding quality of sesame seeds, white seeds with a white to golden color, are mainly used in raw form because of their aesthetic value and are mostly priced higher than mixed seeds (yellow to dark brown seeds) which are generally crushed into oil (Wijnands et al., 2007). The major quality requirements for sesame seed export are thousand seed weight should be greater than 3g, 40-50% oil content, pearly-white seed color and 99% seed purity is required for export standard. Sesame being sold as plain seed, while quality characteristics such as oil content, percentage of admixture, fatty acid profile are not commonly analyzed due to lack of capacity to accurately measure the quality standards of sesame (Gelalcha, 2009).

Currently in Ethiopia grading of sesame seeds can be done by ECX (Ethiopian Commodity Exchange). Previously it was done by Ethiopian Quality and standards Authority until ECX established in 2008. According to ECX the quality parameters for Ethiopian export sesame seed types have to fulfill the standards set by the authority as well as the minimum agreed international standards. The minimum international standard of oil content is 52%, 48%, and 45% for first, second and third grades respectively. On the other hand, the minimum acceptable moisture content is 6-8% for all grades, the percentage of foreign bodies such as dirt, branches, stones etc. internationally agreed standard is 2% for first grade, 4% for second, and 6% for third grade sesame (Wijnands et al., 2007). Ethiopian sesame is mostly identified and graded as Humera, Gondar, and Wollega and to some extent mixed types and its oil content is 43-56%. According to the study on the Physico-Chemical properties of sesame varieties grown in Northern area of Ethiopia by Zebib et al., (2015), determined average fat content of 50.88% - 52.67%, Carbohydrate 8.3% - 11.69%, crude protein 22.58% - 24.27%, moisture (wb) 3.17% - 3.96%, average values of thousand seed weight were ranged from 2.74 - 3.16 g. After comparing

with sesame varieties that grown in other part of the world they concluded that the Ethiopian sesame varieties were good source of nutrients and functional foods for human nutrition and utilization (Zebib et al., 2015).

2.2.4. Sesame seed grading parameters of ECX

Sesame Seeds shall have a good natural color, free of objectionable odor, free of oilseeds other than sesame seeds, free of non-edible seed such as castor seed, contain no live or dead insects, not contain more than 10% moisture by weight and shall comply with the following requirements:

Table 2. 2 Grading parameters for whitish Humera and reddish sesame seed [32].

Parameters	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**UG**
Total Impurity (Foreign Matter and DSW) Max % by weight	1	3	5	7	15
Contrasting Colour, max % by weight	1	2	4	6	7

Table 2.3 Grading parameters for whitish Wollega sesame seed [32].

Parameters	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**	**UG**
Total Impurity (Foreign Matter and DSW) Max % by weight	1	3	5	7	10	20
Contrasting Colour, max % by weight	1	2	4	7	9	10

Table 2. 4 Grading parameters for mixed Humera and Mixed Reddish Sesame Seed [32]

Parameters	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**UG**
Total impurity (Foreign Matter and DSW) max % by weight	1	3	5	7	15
Contrasting Colour, % by weight	> 7				

Table 2.5 Grading parameters Mixed Wollega Sesame Seed [32]

Parameters	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	UG
Total Impurity (Foreign Matter and DSW) max % by weight	1	3	5	7	10	20
Contrasting Colour, % by weight	> 10					

2.3. Mycotoxins

Mycotoxins are naturally occurring toxins, produced by filamentous fungi in many agricultural crops, especially in cereals and oilseeds both in the field, after harvest, during storage, and later when processed into food, animal feed, and feed concentrates (Smith and Henderson, 1991). There are wide variety of mycotoxins but the most common and prevalent types of mycotoxins in different agricultural products are Aflatoxins, Fumonisin, Ochratoxins, Patulin, and Zearalenone. Aflatoxins (AFs), are mycotoxins, of some Aspergillus molds such as *Aspergillus flavus*, *Aspergillus parasiticus* and rarely *Aspergillus nomius* (Ali and Yoshizawa, 2005). They occur particularly in regions or countries with climates of high temperature and humidity or where there are poor crop harvesting and storage conditions, which encourage mould growth and mycotoxin development. Aflatoxins occur on crops in the field prior to harvest. Post harvest contamination can occur if crop drying is delayed and during storage of the crop if water is allowed to exceed critical values for the mould growth. Insect or rodent infestations facilitate mould invasion of some stored commodities. Aflatoxins are found occasionally in milk, cheese, peanuts, cottonseed, corn, pasta, condiments, nuts, almonds, figs, spices, and a variety of other foods and feeds. Milk, eggs, and meat products are sometimes contaminated because of the animal consumption of aflatoxin contaminated feed (JECFA, 1997).

According to WHO, (1979) 17 aflatoxins have been isolated, but only 4 of them are well known and studied extensively from toxicological point of view. These are B1, B2, G1 and G2 (WHO, 1979). Their name given based up on the light they fluoresce on ultraviolet light. B1 and B2 fluoresces blue, while Gl and G2 fluoresces green. Numbers 1 and 2 indicate major and minor compounds, respectively. In some animal species in dairy cattle, aflatoxin B1 and B2 are partially metabolized to the hydroxylated derivates namely M1 and M2, respectively. In monkey

B1 metabolized into P1. Toxigenic *A. flavus* isolates generally produce only aflatoxins B1 and B2, whereas *A. parasiticus* isolates produce aflatoxins B1, B2, G1 and G2. Generally AFB2, AFG1 and AFG2 do not occur in the absence of AFB1. In most cases AFG1 is found in higher concentrations than AFB2 and AFG2 (Weidenbörner, 2001).

In 1993, World Health Organization (WHO) for cancer research institutions designated aflatoxins as a Class 1 carcinogen, and as highly poisonous toxic substances (IARC, 2002). Aflatoxins are harmful to human and animal liver tissue, it can lead to liver cancer or even death. In the natural food contaminated with aflatoxin B1 is most common and most acutely toxic to various species. Among the known AFs, AFB1 is most commonly encountered and considered the most toxic and classified as a human carcinogen (Yunus et al, 2011). The median LD_{50} (lethal dose) of AFBl is 0.36 mg/kg body weight. Their order of toxicity is B1 > G1 > B2 > G2 (WHO, 1979).

Aflatoxins have been given considerable attention because of their demonstrated carcinogenic potential and hepatotoxic effects in both humans and animals. The disease caused by the consumption of substances or foods contaminated with aflatoxins is called aflatoxicosis. Humans are exposed to aflatoxins primarily through the consumption of contaminated agricultural or animal products. Other modes of exposure include the inhalation of toxins through occupational exposure (Wu et al, 2011). Human exposure to aflatoxins have a negative impact on health, it can lead to acute or chronic aflatoxicosis, based on the duration and amount of exposure, and compound existing health issues or the risk of disease transmission. Acute toxicity of aflatoxins in human characterized by high fever, high colored urine, vomiting, and edema of feet, Jaundice, rapidly developing ascitis, portal hypertension and a high mortality rate. Chronic (long term) exposure to aflatoxins in the diet increases risk with a synergistic effect from increased alcohol consumption. Aflatoxin B1 has been implicated as a cause of human hepatic cell carcinoma (HCC). Aflatoxin B1 also chemically binds to DNA and caused structural DNA alterations with the result of genomic mutation (Groopman et al., 1985).

2.3.1. Characteristics of Aflatoxins

Aflatoxins are crystalline substances, freely soluble in moderately polar solvents such as chloroform, methanol and dimethyl sulfoxide, and dissolve in water to the extent of 10-20 mg/liter. Crystalline aflatoxins are extremely stable in the absence of light and particularly UV radiation, even at temperatures in excess of 100ºC. A solution prepared in chloroform or benzene is stable for years if kept cold and in the dark. They fluoresce under UV radiation. Aflatoxin B1 has also been reported to react additively with a hydroxyl group under the catalytic influence of a strong acid (Andrellos et al., 1964). Treatment with formic acidthionyl chloride, acetic acid thionyl chloride or trifluroacetic acid results in addition products of greatly altered chromatographic properties, but relatively unchanged fluorescence characteristics. Ozonolysis results in fragmentation of aflatoxin B1 and the products of this reaction include levulinic, succinic, malonic and glutaric acids (Van dorp et al., 1963). The presence of the lactone ring makes the compound labile to alkaline hydrolysis, and partial recyclization after acidification of the hydrolysis product has been reported (De Iongh et al., 1962). Although few systematic studies have been carried out on the stability of the aflatoxins, the general experience would seem to indicate that some degradation takes place under several conditions. The compounds appear partially to decompose, for example, upon standing in methanolic solution, and this process is greatly accelerated in the presence of light or heat. Substantial degradation also occurs on chromatograms exposed to air and ultraviolet or visible light. These processes may give rise to some of the non aflatoxin fluorescent compounds typically seen in chromatograms of culture extracts. The nature of the decomposition products is still unknown, and the chemical reactions involved in their formation remain to be established (Wogan, 1966).

Table 2.6 Physical Properties of Aflatoxins (Hartley et al., 1963)

Aflatoxin	Molecular Weight	Molecular Formula	Melting Point (M.P)
B1	312	$C_{17}H_{12}O_6$	268-269*
B2	314	$C_{17}H_{14}O_6$	286-289*
G1	328	$C_{17}H_{12}O_7$	244-246*
G2	330	$C_{17}H_{14}O_7$	237-240*
M1	328	$C_{17}H_{12}O_7$	299
M2	330	$C_{17}H_{14}O_7$	293

* Decompose

2.3.2. Chemical Structure of Aflatoxins

Their structure consists of a bifuran ring fused to a coumarin nucleus with a pentenone ring (in B and M aflatoxins) or a six membered lactone ring in G aflatoxins. The four compounds (B1, B2, G1, and G2) are separated by the color of their fluorescence under long wave ultraviolet illumination (B=blue, G= green) (Hartley et al., 1963). Two other aflatoxins M1 and M2 were isolated from urine and milk and identified as mammalian metabolites of AFB1 and AFB2 respectively (Zain, 2011).

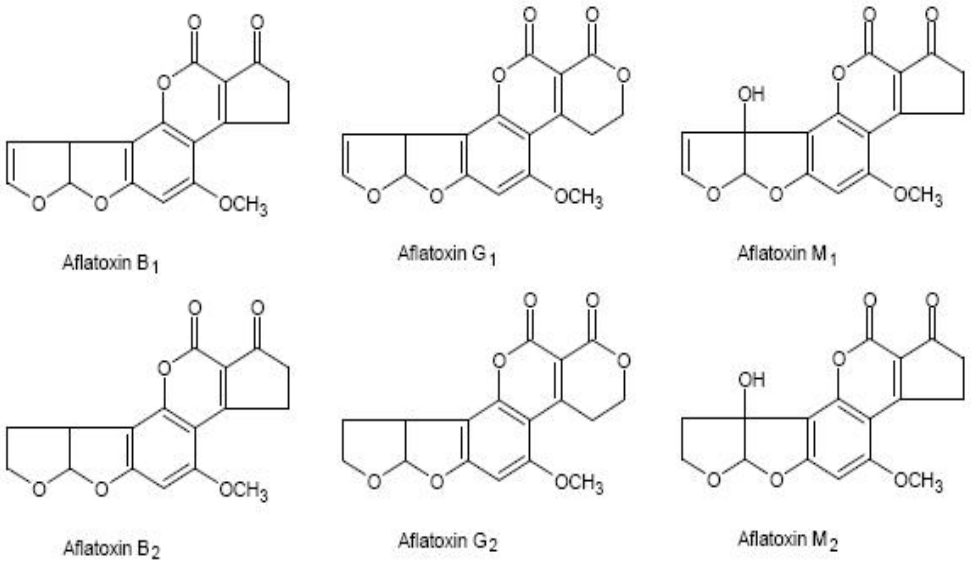


Figure 2.2 Structure of Aflatoxins (Zain, 2011)

2.3.3. Conditions for the occurrence of Aflatoxins

Crops grown under warm and moist weather in tropical or subtropical countries are especially more prone to aflatoxin contamination than those in temperate zones. According to Hesseltine (1983), occurrence of aflatoxins in a certain food and feed depends up on biological, chemical and physical factors. Biological factors are those associated with the host species, the chemical factors include the composition of the air and the nature of the substrate and the physical factors include temperature and moisture. Water stress, high-temperature stress and insect damage of the host plant are major determining factors in mould infestation and toxin production. Similarly, specific crop growth stages, poor fertility, high crop densities and weed competition have been associated with increased mould growth and toxin production (Hesseltine, 1983). Specific nutrients, such as minerals (especially zinc), vitamins, fatty acids, amino acids and energy source (preferably in the form of starch) are required for aflatoxins formation. The limiting temperatures for the production of aflatoxins by *A. flavus* and *A. parasiticus* are reported as 12 to 41°C, with optimum production occurring between 25 and 32°C (Lillehoj, 1983). Synthesis of aflatoxins in feeds are increased at temperatures above 27°C (80°F), humidity levels greater than 62% and moisture levels in the feed above 14% (Royes and Yanong, 2002). The moisture content of the substrate and temperature are the main factors regulating the fungal growth and toxin formation. A moisture content of 18% for starchy cereal grains and 9-10% for oil-rich nuts and seeds has been established for maximum production of the toxin (WHO, 1979).

2.3.4. Metabolism and Mutagenicity of Aflatoxins in relation to their toxicity

Carcinogenic AFB1 involves metabolic activation for its toxicity. The toxicity of each metabolite has been of great interest in the search for the active molecular species. According to in vivo study on Mutagenicity tests of aflatoxin metabolites with the *Salmonella* mutagen assay, containing mammalian microsomes, developed by Dr. Bruce N. Ames, AFB1 was found to be the most mutagenic of the four naturally occurring AFs while AFG1 was less mutagenic. AFB2 and AFG2 exhibited activity lower than that of controls, and assessed as non mutagenic (Jeffrey et al., 1976).

Aflatoxin B1 metabolism takes place in the microsome of the liver and is mediated by mixed function monooxygenases belonging to the cytochrome P450 super family of enzymes (Guengerich, 1998). In humans, cytochrome P450 enzymes, CYP1A2 and CYP3A4, catabolize aflatoxin B1 through two separate electron transfer oxidation reactions. While CYP1A2 breaks down aflatoxin B1 to exoepoxide, endoepoxide, and aflatoxin M1, CYP3A4 breaks down aflatoxin B1 to aflatoxin B1-exo- 8,9-epoxide and aflatoxin Q1. Aflatoxins M1 and Q1 are not broken any further but are excreted in the urine. Aflatoxin B1-exo-8,9-epoxide converted either to aflatoxin mercapturic acid via the Glutathione *S*-transferase- (GST-) conjugate mediated route or into aflatoxin-glucuronide via the aflatoxin-dihydrodiol route as follows. The activated form of aflatoxin B1 (*exo*epoxides and *endo*epoxides) is detoxified through glutathione *S*-transferase- (GST-) mediated conjugation by using reduced glutathione (GSH) to form AFB1 *exo*epoxide-GSH and *endo*epoxide-GSH conjugates, respectively (Johnson, 1997). The reactive *exo*epoxides and *endo*epoxides also undergo rapid non enzymatic hydrolysis to aflatoxin B1-8,9-dihydrodiol that slowly transforms into a dialdehyde phenolate ion (Wild and Turner, 2002)(Johnson, 1996). Dialdehyde phenolate ion is subsequently hydrolyzed by aflatoxin aldehyde reductase (AFAR) to a dialcohol, in the NADPH-dependent reduction reaction. Thereafter, dialcohol is excreted in urine as aflatoxin-glucuronide (Hayes et al., 1993)(Knight et al., 1999). Aflatoxin B1 dialdehydes also form Schiff bases with primary amine groups of amino acid residues such as lysine of such a protein as albumin to form aflatoxin B1-albumin conjugate. This conjugate persists in the systemic blood as permanent and irreversible aflatoxin B1-albumin adducts and is thus considered one of the factors accounting for the low excretion of aflatoxins and their metabolites in urine (Wild and Turner, 2002)(Nassar et al., 1982).

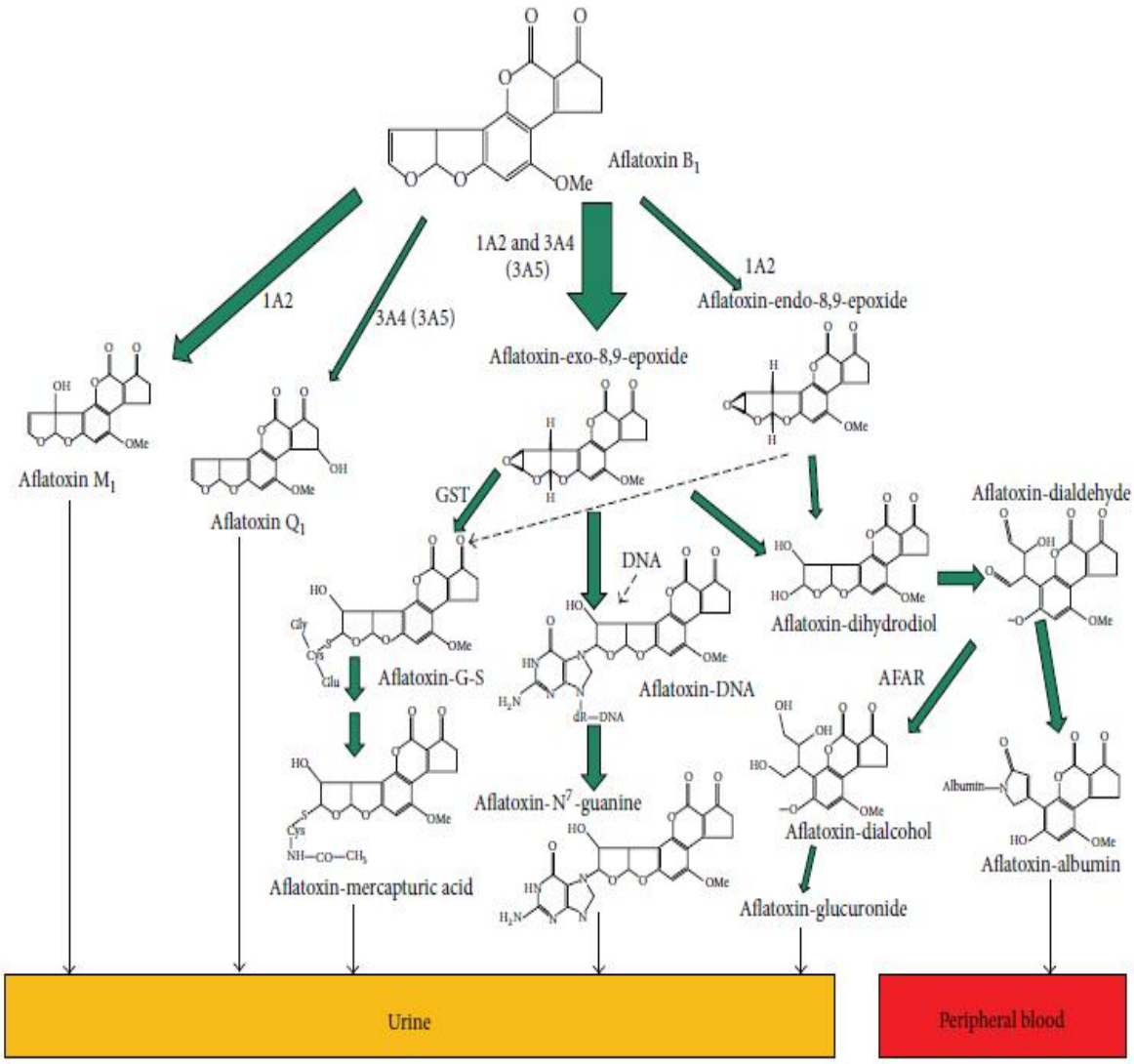


Figure 2.3 Metabolism of Aflatoxin B1

Primary derivatives of aflatoxin B1 biotransformation, aflatoxin M1 and aflatoxin-exo-8,9-epoxide (products of CYP1A2 activity) and aflatoxin Q1 and aflatoxin-exo-8,9-epoxide (products of CYP3A4 activity). Aflatoxins M1 and Q1, although toxic, are less reactive with other molecules and are easily eliminated from the body in the urine (Wild and Turner, 2002). However, aflatoxin B1- 8,9-exo-epoxide is a known mutagen, which is extremely electrophilic and covalently reacts with nucleophilic sites of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or proteins (Oliveira and Germano, 1997), thereby introducing mutations

that may affect the normal function of cells. The formation of aflatoxin B1-DNA adducts is extremely associated with the carcinogenicity of aflatoxin B1. Typically, aflatoxin B1 reacts with DNA (methylation) resulting in G→T transversion mutation as described below on Figure- 2.4. Such mutation has been associated with hepatocellular carcinoma, a type of cancer whereby aflatoxin B1 promotes AGG→AGT (Arg→Ser) transversion point mutation of p53 gene at codon 249 that alters *p53* gene, which is responsible for DNA repair (Aguilar et al., 1993). Apart from G→T transversions, G→C transversions and G→A transitions have also been reported (Levy et al., 1992).

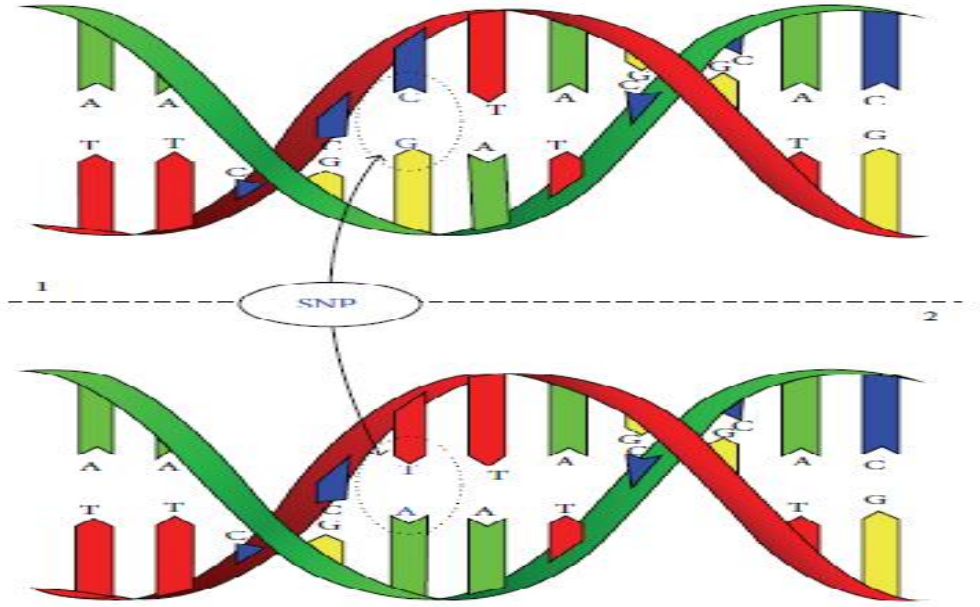


Figure 2. 4 Point mutation of G → T at codon 249 in the p53 gene resulting in aflatoxin induced hepatocellular cancer (Bbosa et al., 2013)

Nucleic acids and proteins interact covalently with aflatoxins and this results in alteration in base sequences in nucleic acids (both DNA and RNA) and in protein structures, leading to impairment of their activity. The highly reactive aflatoxin B1-8,9-exo-epoxide and its hydration product, dihydrodiol, bind covalently to DNA, RNA, and proteins to inhibit protein synthesis (Eaton and Gallagher, 1994). While the epoxide reacts at the N^7 position of guanine of both DNA and RNA, the dihydrodiol reacts with the amino groups of the bases forming a Schiff base. Aflatoxin B1 has also been reported to negatively impact carbohydrate metabolism, which results in both the

reduction in hepatic glycogen and also the increased blood glucose levels (McLean and Dutton, 1995).

2.4. Regulatory Limits for Mycotoxins

Throughout the world there are many advisory bodies concerned with food safety issues, including the World Health Organization (WHO), Codex Alimentarius Joint Expert Committee for Food Additives and Contaminants (JECFA), and the European Food Safety Authority (EFSA).They regularly assess the risk from mycotoxins and advise on controls to reduce consumer exposure. In 2008, the 31st session of the Codex Alimentarius Commission adopted guidelines for the maximum levels for OTA in raw wheat, barley, and rye, as well as maximum levels for aflatoxins in almonds, hazelnuts, and pistachios (for further processing and ready-to-eat) (Codex, 2008). In the UK, the Food Standards Agency (FSA) is responsible for ensuring mycotoxin safety (UKFSA, 2002). The UK legislation on mycotoxins is harmonized with the European Union. In the EU, regulatory limits for mycotoxins permitted in food and animal feed are set by a range of directives and Commission regulations as follows.

Commission Regulation (EC) No. 466/2001 sets maximum permitted levels for mycotoxins in food and feed and new limits are introduced by amendments to this legislation. For example, EC Regulation No. 1881/2006 set limits for aflatoxins. Maximum EU limits for oilseeds 2 ppb (µg/kg) for aflatoxin B1 and 4 ppb for total aflatoxins. Oilseeds for further processing with limits of 8 ppb and 15 ppb for aflatoxin B1 and total aflatoxins respectively (these limits do not apply to oilseeds including groundnuts for crushing for refined vegetable oil production). Aflatoxin M1 in baby food is allowed in the level of 0.1ppb and 0.05 ppb in milk. The limit for aflatoxins in rice for direct consumption is 2 ppb for aflatoxin B1 and 4 ppb for total aflatoxins. The maximum limit for OTA (Ocratoxin A) is 10 ppb in dried vine fruits (currants, raisins, sultanas) and in soluble coffee, 5 ppb in roasted coffee, 3 ppb in all products from cereals intended for direct human consumption, 2 ppb for wines, grape juice, grape must and grape must concentrate, and 0.5 ppb for baby foods processed cereal-based foods for infants and young children.

Since EC regulation No. 1881/2006 was issued there have been several amendments to the permitted levels of various mycotoxins in miscellaneous food products. These amendments are Commission Regulation (EU) No. 105/2010 which set maximum levels for certain contaminants

in foodstuffs as regards ochratoxin A. Commission Regulation (EU) No. 165/2010. Setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins and amending regulation (EC) No. 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs

Table 2. 7 Regulatory limit of aflatoxins in EU and some countries (Codex, 2008)

Country/region	**Foodstuff**	**Total aflatoxins (µg/kg)**
Australia	Peanuts/tree nuts	15
Canada	Nuts and nut products	15
European Union	Peanuts, tree nuts, dried fruits	4
India	Cereals, food grains	30
South Africa	Peanuts	15
USA	Nuts and nut products	30

East African Community (EAC) regionally harmonized national standards that have been extrapolated from the international standards setting body, the Codex Alimentarius Commission (Codex), or adopted from other countries outside of the region, rather than methodically developed based on rigorous risk assessment and analysis of actual consumption levels of aflatoxins prone foods by the EAC. Due to the absence of consensus on aflatoxins maximum limits at Codex for these foods, countries and regions have formulated national or regional maximum limits. The United States has a guideline level of 20 µg/kg for total aflatoxins in all foods for human consumption. In developing countries, maximum limits for total aflatoxins range from 10 to 20 µg/kg, with 10 µg/kg being the most frequently set level. The setting of maximum limits for aflatoxins in food standards in countries of the EAC began in the 1990s, when most of these countries started setting standards for specific foods. To date, the EAC partner states use maximum limit of 5 µg/kg for aflatoxin B1 and 20 µg/kg for total aflatoxins in selected foods, cereals, and pulses (Yun Yun et al., 2015).

2.5. Overview of Studies on Aflatoxins occurrence in Sesame seed

Different authors conducted a number of studies and detected aflatoxins from different agricultural commodities, food and feeds at different parts of the world after the discovery and isolation of aflatoxin from the mysterious Turkey-X disease of 1960. Which resulted in the loss of approximately 100,000 turkey poults in the United Kingdom, due to aflatoxin contaminated peanut meal (Blount, 1961). According to a survey of Aflatoxin on imported sesame in Iran by Hosseininia et al., (2013), the result showed that out of 269 sub-samples aflatoxins detected in 50% of the total samples at the level >1 µg/kg, but at lower levels in most cases, which is illustrated by mean AFB1 and total AFs levels of 1.25±3.70 and 1.43±4.38 µg/kg, respectively. A few (1.9 %) samples exceeded the National Iranian standard maximum accepted level for AFB1 (5 µg/kg) or total AF (15 µg/kg); the maximum total AF level found in one sample was 48 µg/kg (Hosseininia et al., 2014). Recent study on Aflatoxin B1 in sesame seeds and sesame products from the Greek market by Eleni et. al.,(2016), indicated that, thirty samples of sesame products were examined for the presence of AFB1 and 77.6% of the samples were found contaminated. Eight samples exceeded the European Union (EU) limit for individual aflatoxin in oilseeds (2 µg/kg). The most contaminated is (14.49 µg/kg AFB1) (Eleni et al., 2016). In another study on determination of aflatoxins in Sesame, Rice, Millet and Acha, from Nigeria by Makun et. al., (2014), reported that AFB1 was detected within the range of 14.71-140.9 µg/kg from eight sesame samples (Makun et al., 2014).

2.6. Overview of studies in Ethiopia on aflatoxins contamination

In Ethiopia various studies conducted on aflatoxins contamination and detected from different agricultural commodities, food and feeds by different authors but there is no single study on aflatoxins contamination on Ethiopian sesame seed so far. Early study by Abate and Gashe (1985), on the mycoflora of the Ethiopian cereals with a special emphasis on the prevalence of toxicogenic fungal groups and they have found the fungus associated more with sorghum and maize than with teff and barley and concluded that those people who consume maize and sorghum from hot and humid regions of Ethiopia are likely to have a higher level of aflatoxin in their diet (Abate and Gashe, 1985). Fufa and Urga, (1996) determined aflatoxins contamination in shiro and ground red pepper in Addis Ababa and found aflatoxins levels ranged from 100 to

500 µg/kg and 250 to 525 µg/kg, in Shiro and ground red pepper positive sample respectively, with a significant higher mean levels of contamination in ground red pepper than that of Shiro. They conclude that ground red pepper and shiro traded in Addis Ababa can be considered as high risk commodities and recommend routine survey of aflatoxins to be done (Fufa and Urga, 1996).

In recent study by Wondimeneh et al., (2016), Aflatoxin B1 was detected at levels ranging from <LOD to 33.10 µg/kg in sorghum grain (Wondimeneh et al., 2016). On the study conducted to assess Aspergillus species and aflatoxins level in *Sorghum* (*Sorghum bicolor L.*) stored under different storage system for different storage period in Northern Shewa, Ethiopia by Weledesemayat et al., (2016), reported the level of total aflatoxins, aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 were in the range of 11.44 to 344.26µg/kg, 3.95 to 153.72µg/kg, 1.17 to 91.82µg/ kg, 9.87 to 139.64µg/kg, and 3.22 to 52.02µg/kg, respectively (Weledesemayat et al., 2016). In another study on aflatoxin determination from groundnut from Eastern Ethiopia by Alemayehu Chala et al., (2012) reported that 93 samples out of 120 groundnut samples were positive for aflatoxins and the mean aflatoxins content is varied between 15 mg/kg and 11,900 mg/kg. The result reveals heavy aflatoxins contamination of groundnuts in Ethiopia. Which is far beyond the Food and Agricultural Organization of the United Nations/World Health Organization (FAO/WHO) standard (15 µg/kg) and the European Union (EU) limit i.e. 4-15 µg/kg (Alemayehu et al., 2012).

2.7. Methods to control occurrence Aflatoxigenic Fungi

A number of methods have been developed to control occurrence of Aflatoxigenic fungi and minimize the production of aflatoxins on different agricultural commodities on pre- and post-harvest stages. These methods can be grouped as biological, chemical and physical. The use of available physical and chemical methods for the detoxification of agricultural products contaminated with mycotoxins is restricted due to problems concerning safety issues, possible losses in the nutritional quality of treated commodities, coupled with limited efficacy and cost implications (Köhl et al., 2011).

A number of biological methods like bacteria, yeasts, atoxigenic *Aspergillus* strains and chemical control agents (i.e. fungicides) have been reported to inhibit growth of aflatoxigenic fungi and

subsequent aflatoxin biosynthesis. Application of competitive atoxigenic strains of *A. flavus* and *A. parasiticus* can successfully reduce aflatoxins contamination of agricultural products like Pistachio, rice, maize and peanuts (Dorner et al., 1999)(Dorner et al., 2003). The effect is mediated through competition for substrate and through the potential production of inhibitory metabolites. Various studies indicated that application of atoxigenic strains of *A. parasiticus* in field soil reduced the level of aflatoxins significantly. According to study by Brown et al., (1991) application of non-aflatoxigenic strain of *A. flavus* reduced aflatoxin contamination by 80-95% in maize (Brown et al., 1991). In another study for three consecutive years in peanut crops, the level of aflatoxin decreased from 531, 96 and 241 µg/kg in untreated soil to levels of 11, 1, and 40 µg/kg for atoxigenic *A. parasiticus* treated soil in 1987, 1988, and 1989 respectively (Dorner et al., 1992). A study by Cotty, (1994) indicated that application of *A. flavus* to soil of cotton seed field was very effective on controlling aflatoxins production in cotton (Cotty, 1994).

Two atoxigenic *A. flavus* strains, AF36 and NRRL 21882, are currently being used in USA to minimize aflatoxins contamination in crops. AF36 has been registered as biopesticide by the U.S Environmental Protection Agency and it is being used for the management of aflatoxins production on cotton field. Whereas NRRL 21882 is being used to prevent aflatoxins contamination in peanut field (Chang and Hua, 2007).

For effective reduction of aflatoxins contamination, atoxigenic strains of *Aspergillus* species must be applied in a method and at a time that allows successful competition with aflatoxin producing *Aspergillus* strains. They can be applied once per growing season (Cleveland et al., 2003)(Cotty and Mellon, 2006). Besides atoxigenic *Aspergillus* strains there are a number of reports regarding the antifungal properties of various lactic acid bacteria (LAB), which exhibit activities against several toxic *Aspergillus* species and broad range of other mycotoxigenic fungi. They produce antimicrobial compounds like lactic acid, acetic acids, hydrogen peroxide, bacteriocins, and low molecular weight proteinacious compounds during carbon source metabolism and they compete with other species by acidifying the environment and deplete the available nutrients rapidly (De Muynck et al., 2004) (Kabak and Dobson, 2009). Study reports indicate that the Anti-fungal activity of *Lactobacillus casei* inhibited both growth and aflatoxin

production in *A. parasiticus* (El-Gendy and Marth, 1981). In another study Aflatoxins production by *Aspergillus flavus* reduced significantly by the activity of *Streptococcus lactis* cultures (Suzuki et al., 1991). In Addition to the above biological strategies there are also fungal and bacterial species such as *Trichoderma spp., Phoma spp., Rhizopus spp.,* and *Alternaria spp.,* have been shown to degrade aflatoxin B1 by 65-99% in 5 days at 28 ± 2°C (Shantha, 1999).

The biological methods of controlling aflatoxins producing mold are less applicable in developing countries like Ethiopia due to their economic power. In such cases it is better to work on preventive methods that reduce the accesses which create conducive environment for the occurrence of aflatoxin producing molds on different agricultural commodities. Warm and humid climate provides congenial atmosphere for the growth of fungi and production of toxins. *Aspergillus flavus* which is known as storage fungi may infect and produce aflatoxins in crops that stored in improper conditions. Faulty storage conditions may enhance the chance of microbial attack and production of toxins. Thus starting from harvesting of the crop till the food or food products consumed by consumers. Factors that play great role on mycotoxin contamination are temperature, humidity, moisture, insect infestation and mineral deficiencies. These factors favour the occurrence of aflatoxins producing molds on agricultural commodities on field as well as off field (Srivastava, 1987).

The main strategies used for prevention of mycotoxin at pre-harvest stage include choosing crops with resistance to drought, disease, and pests and choosing strains of that crop which are genetically more resistant to the growth of the fungus and the production of aflatoxins (Brown et al., 2001) (Cleveland and Dowd, 2003). In post-harvest interventions before storage, crops should be properly dried to prevent the development of aflatoxins. Sorting and disposing of visibly moldy or damaged kernels before storage has proven to be an effective method for reducing, but not eliminating, the development of aflatoxins. During storage, moisture, insect, and rodent control can prevent damage to the crop and reduce aflatoxins development (Fandohan and Zoumenou, 2005)(Turner and Sylla, 2005). During preparation of food for consumption it involves removing contaminated portions of food, diluting contaminated food with uncontaminated food, neutralizing aflatoxins present in food. Aflatoxins are not largely affected by routine cooking temperatures, but simple food preparation methods such as sorting, washing,

crushing, and dehulling may reduce aflatoxins levels (Fandohan and Zoumenou, 2005)(Park, 2002).

2.8. Methods for detection of Aflatoxins

A range of well-developed techniques are available for detection of aflatoxins from different food and feeds. Analytical techniques for detection of aflatoxins including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography-mass spectroscopy (LC–MS), liquid chromatography couple mass spectroscopes (LC–MS/MS) and immunochemical methods such as enzyme linked immunosorbent assay (ELISA) (Turner et al., 2009). The criteria for choosing a suitable method include available time and equipment, specificity and sensitivity. Immunological method (ELISA) and chromatographic methods (GC/LC) are the most widely used techniques for aflatoxins analysis.

2.8.1. Enzyme linked immunosorbent Assay (ELISA)

ELISA is the technique uses antibody antigen binding principle. It is the most commonly used technique for aflatoxins analysis due to its simplicity, sensitivity and adaptability. This method use for screening purposes and in the field. Different versions exist that vary in the rapidity of the procedure and the quantification of the results, although all of them require enzymatic reactions, washing, and separation of bound and free label. A very important step is validation of detection matrix effects, and since false positives can occur, positive results could require confirmation by more expensive chromatographic methods (Coker and Jones, 1988).

2.8.2. Thin-layer chromatography (TLC)

Thin-layer chromatography consists of a stationary phase immobilized on a glass or plastic plate and a solvent acting as a mobile phase. The sample, either liquid or dissolved in a volatile solvent, is applied in the form of a spot on the stationary phase. Then the chromatographic plate is placed vertically in a solvent reservoir and the solvent moves up the plate by capillary action. When the solvent front reaches a certain limit of the stationary phase, the plate is removed from

the solvent reservoir. The separated spots are then visualized with ultraviolet light or by spraying with a suitable reagent. The contents of a sample can be identified by running standards simultaneously with the unknown spots. The different components in a mixture move up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase.TLC can identify and quantify aflatoxins at levels as low as 1ng/ g (Hussain, 2011).

2.8.3. High performance thin-layer chromatography (HP-TLC)

There is lack of precision associated with TLC procedures due to the introduction of possible errors during the sample application, plate development, and plate interpretation steps. High performance thin-layer chromatography methods improve the precision by automating the sample application and plate interpretation steps. This technique is less commonly used as compared to HPLC, which is more sophisticated as compared to this (Hussain, 2011).

2.8.4. Liquid chromatography with mass spectrometric detection (LC-MS)

It is one of the most advanced techniques, time-consuming and it requires expert knowledge. In LC-MS, the HPLC effluent enters an ionization chamber via a nebulizer. Fragmentation takes place in a collision chamber. The fragments then enter the high vacuum region of the mass spectrometer, where detection takes place. Several set-ups are available for optimal identification and quantification. The instruments provide better information for quantification with faster scanning and higher sensitivity. Detection can be made at pico-grams levels (Hussain, 2011).

2.8.5. High performance liquid chromatography (HPLC)

In HPLC, a liquid mobile phase or solvent is used to move the sample through the column. An immobilized stationary phase is packed in the column. The analyte is then partitioned between the two phases as it passes through the column and thus leading to the separation of compounds due to the difference in partitioning coefficients. Two types of HPLC methods are commonly used i.e., normal phase chromatography and reversed phase chromatography. In normal phase chromatography, a polar stationary phase e.g. silica gel and a non-polar solvent e.g. hexane are used. Whereas reversed-phase chromatography (RP-HPLC) employs non-polar stationary phase

e.g., C-8 or C-18 hydrocarbons and polar mobile phase e.g. water, methanol or acetonitrile. In HPLC, detection it is mainly accomplished by using ultra violet (UV) detector, diode array detector (DAD) or a fluorescence detector (FLD). Fluorescence detection utilizes the emission of light (435 nm) from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation (365 nm) for aflatoxins. Fluorescence detection has superior sensitivity than other detection systems and sometimes derivitization of the analyte has to be performed which enhances the sensitivity. Fluorescence detection is possible in the range of microgram/kg. Choice of detector usually depends on the nature of the sample. HPLC system gives result in the form of chromatogram (Hussain, 2011).

Sample Clean up techniques Prior to Analysis

Prior to chromatographic analysis proper sample clean up is required to get reliable results. Most widely used sample cleanup procedures includes IAC(Immunoaffinity) and SPE(Solid phase extraction) methods have increased importance as sample preparation techniques prior to instrumental analysis (Zheng et al., 2006).

Solid Phase Extraction (SPE) Vs. Immuno Affinity Column

Mostly cleaning of samples can be performed by using immunoaffinity column. The need for quick, simple sample clean up brought to Solid phase extraction called Supel Tox Aflazea cartridge, which reduces the sample preparation time significantly, increase reproducibility, and are more user friendly as compared to standard immunoaffinity column. Immunoaffinity column works by the principle of "bind and elute", the multiple stapes strategy. Unlike the immunoaffinity column, Supel Tox Aflazea cartridge employ an "interference removal" strategy. Supel Tox Aflazea cartridge have active site that adsorb interferences associated with aflatoxin like various fat, pigments, carbohydrate, protein interferences and retain large molecules. In addition this approach it requires less equipment and fewer consumables, providing an additional cost savings (Espenschied et al., 2015).

Table 2.8 Immunoaffinity column Vs Supel Tox Aflazea cartridge

Immunoaffinity column	Supel™ Tox Aflazea Cartridge
Works according to 'bind and elute' principle	Removes interferences associated with aflatoxin
Sample preparation takes up to 60 minutes	Sample preparation takes only 6 minutes
Process only 8 samples/day (if processing 1 at a time)	Process about 80 samples/day (if processing 1 at a time)
Sample preparation need complicated stapes	Steps few and not complicated
Must be refrigerated, brought to room temp before use	Column does not require special storage conditions

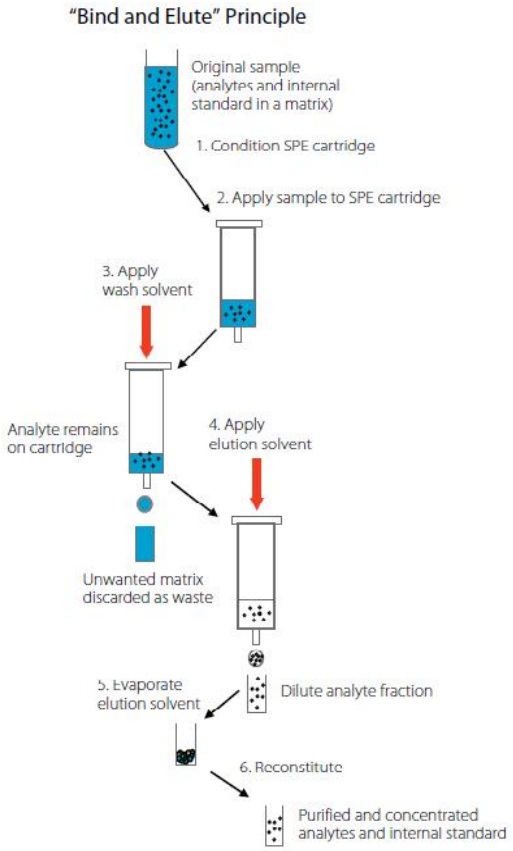


Figure 2. 5 Steps to use immunoaffinity column

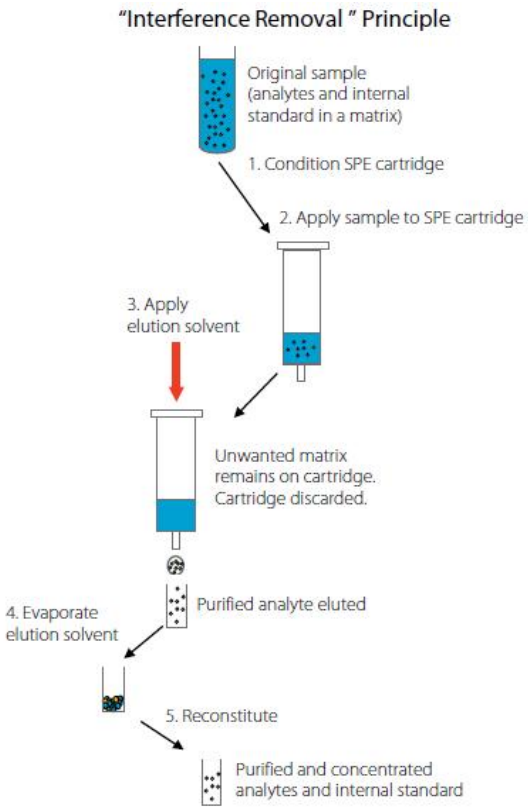


Figure 2.6 Steps to use Supel Tox Aflazea

CHAPTER THREE

3. Materials and Methods

3.1. Study Area

The study was conducted at Addis Ababa, the capital city of Ethiopia and the experiments were carried out from November 2016 to April 2017 in Addis Ababa University, Center for Food Science and Nutrition, Food toxicology and Food Microbiology Laboratories. Sesame samples collected from Ethiopian agricultural commodities warehouse service enterprise located in Addis Ababa.

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

A total of 30 sesame exporters participated in the survey. Purposive sampling technique and Semi-structured questionnaires (Appendix-1) were used to get more information. The questionnaire evaluation carried out for knowledge attitude and practice assessments related to quality and food safety issues of the sesame exporters based on the purposive sampling.

3.3. Sample and sampling

Twenty seven white and red Humera and Wollega sesame samples were collected purposively in accordance with the objective of the study from Ethiopian Agricultural Commodity Warehouse Service Enterprise (EACWSE), Located in Addis Ababa. Nine samples from white Humera sesame varieties, those cultivated in different areas and grouped as white Humera sesame, nine white Wollega sesame varieties from different areas and grouped as white Wollega by Ethiopian commodity exchange authority and nine Red sesame seed varieties. The sesame seeds collected in plastic bag by taking preventive measures to avoid contamination. The sesame seeds were separated from foreign matter and milled using miller and sieved to pass through 1 mm mesh size. The flour was packaged in tight polyethylene bags and stored in cool dry place until the analysis.

Figure 3.1 Sampling of Sesame seed from Ethiopian Agricultural Commodities Warehouse Service Enterprise (EACWSE)

3.4. Sesame seed characteristics

3.4.1. Thousand seed weight

Thousand seed weight was determined by using electronic grain counter (Numigral, CHOPIN). The mass of 1000 seeds counted were measured on electronic balance (ISTA, 195).

3.4.2. Moisture content

The moisture content of sesame seeds analyzed by using drying oven (Model: DHG-9055A: 2007, Shanghai China). The analysis has been conducted according to AOCS, (1995). Three gram of sesame seed sample was dried at 105˚C for 6 h (AOAC, 1985).

$$\text{Moisture content (\%)} = \left(\frac{W2-W3}{W2-W1}\right) \times 100 \qquad \textit{Eq-1}$$

Where w_1- Weight of crucible

w_2- Weight of crucible and fresh sample

w_3- Weight of crucible and sample after moisture removed

3.4.3. Crude fat (%)

Crude fat/ oil content of sesame seed was determined by Soxhelat extraction (Model: EV 16, SN: 4002824, Germany). By taking 2.0g finely ground sesame seed sample and by using petroleum ether as an extraction solvent according to (AOAC, 1985).

$$\text{Crude fat (\%)} = \left(\frac{W3-W1}{W2-W1}\right) \times 100 \qquad \textit{Eq-2}$$

Where w_1- Weight of crucible

w_2- Weight of crucible and fresh sample

w_3- Weight of crucible and sample after extraction

3.4.4. Peroxide value analysis

Three gram of the sample accurately weighed and transferred, into a 250 ml Erlenmeyer flask with glass stopper. Then 50ml of the appropriate solvent mixture of glacial acetic acid:

chloroform in 3:2 ratio and 1ml of freshly prepared saturated potassium iodide solution added and allowed to react for 60 ± 1 second and shake thoroughly during this period. Then 100 ml water added and shake. Finally titrated with 0.01 mol/l sodium thiosulfate solution, or 0.1 mol/l sodium thiosulfate solution at higher usages, using 1 ml starch solution or 0.1 g of Thyodene indicator. The indicator should be added towards the end of the titration but while the pale straw colour is still present. During titration shake until the blue colour disappears. Additionally blank titration carried out under the same conditions. No more than 0.5 ml of 0.01 mol/l sodium thiosulfate solution should be consumed for this purpose.

Calculation of the peroxide value

$$POV\ (meq/Kg) = \frac{(V1-V0)\,x\,C\,x\,1000\,x\,T}{m} \qquad Eq\text{-}3$$

Where

POV- Peroxide value

V_1- Consumption of 0.01 mol/l or 0.1 mol/l sodium thiosulfate solution in the main test

V_0- Consumption of 0.01 mol/l or 0.1 mol/l sodium thiosulfate solution in the blank test

C- Molar concentration (molarity) of the sodium thiosulfate solution

T- Titer of Sodium thiosulfate solution

m- weighed portion of substance in grams

3.5. Procedure for Aflatoxin analysis

3.5.1. Materials and Chemicals

Chemical and reagents:

HPLC grade Acetonitrile and methanol, N-hexane, deionized water and Aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 and mixed Aflatoxin standards purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored in dark place at 4°C.

Apparatus

Supel™ Tox Aflazea cartridges 6ml, lab stand with clump, Volumetric and Graduated pipettes (1ml, 5ml, 10ml, 25ml and 50ml), volumetric flask (10ml, 25ml, 50ml, 100ml, 500ml and 1000ml), Measuring cylinder (50ml and 100ml), Beaker (50ml, 100ml and 500ml), Erlenmeyer flask(250, 500 and 1000ml), Mixer, Stirrer, Ultra bath Sonicator, Wash bottle, Micropipettes, Micropipette tips, Millipore Filter, Filter paper, Electronic balance, syringes(5ml and 10ml), Parafilm, Sample label, Vials with screw cap. HPLC system setup contains auto sampler, injector, oven, column, Link, Degasser, fluorescence detector and desktop computer with Lab solution chromatography software.

Mobile phase:

The mobile phase was water-methanol-acetonitrile (60:25:15, v/v/v) isocratic method for better resolutions of the aflatoxin peaks. The mobile phase was filtered by applying vacuum in a filter unit and degassed before use.

Supel Tox Aflazea Cartridge

6 ml Supel Tox Aflazea cartridge in employed as Solid phase extraction (SPE) of Sesame seed to clean up the extract before aflatoxin analysis.

Figure 3.2 Supel Tox Aflazea cartridge

3.5.2. Method validations

To evaluate the analytical performance of the instrument and validity of the method, first identification, accuracy, precession, linearity, asserting the working range, LOD and LOQ were done.

Identification

Identification of Aflatoxins was done based on their retention time of individual and mixed aflatoxins (AFG2, AFG1, AGB2 and AFB1) injected at the same condition and its precision determined by percent relative standard deviation (%RSD).

Accuracy

Accuracy check of the analytical method was done by spiking 50ppb and 100ppb aflatoxin mix standard with sesame seed sample and by injecting Aflatoxin reference standard that obtained from Ethiopian Conformity Assessment Enterprise and comparing its result with aflatoxin working standard.

Precision

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

Linearity

Linearity determined by injecting a series of (2, 5, 10, 20, 30, 50, 100 and 250) ppb Mixed aflatoxin standards. The concentration range (2-250) ppb and regression equation was found by plotting the peak area (Y) versus the aflatoxins concentration (X) expressed in ppb.

LOD and LOQ

LOD was determined by injecting (0.01, 1, 0.01, and 0.8) ppb of individual aflatoxins G2, G1, B2 and B1 respectively to obtain the lowest amount of analyte greater than three times of noise level S/N > 3 . In the same way LOQ was determined by injecting (0.05, 2, 0.05, and 1) ppb of individual aflatoxins G2, G1, B2 and B1 respectively, to obtain the lowest amount of analyte which can be reproducibly quantitated above the baseline noise, that gives S/N >10.

3.5.3. Standards preparation

Aflatoxins standards of AFB1, AFB2, AFG1 and AFG2 obtained from Sigma-Aldrich. From the stock solution aflatoxins mixed standard which have a concentration of (2, 5, 10, 20, 30, 50, 100 and 250) ppb were prepared for method validation. Standards solutions were prepared in 10ml volumetric flasks using HPLC grade Methanol as a diluent. The prepared standards were transferred into vials and stored at 4^{o}C and protected from light to avoid deterioration of the aflatoxins in the solution.

3.5.4. Sample Extraction and purification

Sample Extraction

Sample extraction done according to Sigma Aldrich Co. (2012), with slight modifications. For every 100mL extraction solvent 25 gram of solid sample was used. 25 gram of finely grounded

sesame seed is weighted and added into 500mL Erlenmeyer flask. 100mL of extraction solution (84:16 acetonitrile: deionized water) was added into the sample and the flask was sealed with stopper and parafilm. Then the flask processed by using blender for 3 minute on high blending. After that the suspension was filtered under vacuum until all the liquid is filtered into the flask by using a Buchner funnel with filter paper in an Erlenmeyer flask with side arm and a neoprene filter adapter. Filtered extracts were transferred into sample jar and covered with lid.

Sample Purification

Supel Tox Aflazea purification cartridge was placed on lab stand with clamp and collection tubes assembled on the cartridge. 2 ml sample extract pipetted onto purification cartridge. Entire sample collected into collection tube. After the filtration by Supel Tox Aflazea cartridge the filtrate further filtered by using 0.45µm micro filter. Neat solution was transferred into 2mL autosampler vial. Finally 20µL injected into HPLC.

Figure 3.3 Sample Extraction and purification

3.5.5. HPLC determination and calculations

Chromatographic system:

Shimadzu HPLC instrument with, Auto sampling system, fluorescence detector and Lab solution software were used for analysis. A Shim-pack FC-ODS column (5µm, 250 x 4.6mm diameter) at 25^0C temperature and 1.2ml/min flow rate was used. The run time was 25 minutes, injection volume 20µl, diluent methanol and Needle wash (Water: Methanol 90:10 v/v). Aflatoxins were detected at 365 nm excitation and 440 nm emission wavelengths.

Calculations

The elute aflatoxins (B1, B2, G1 and G2) methanol solution were determined at parts per billion (ng/g) levels in sesame seed by AflaZea cartridge cleanup and high performance liquid chromatography with fluorescence detection and calculated according to the equation below.

$$\text{Final aflatoxin in ng/g} = m_a \left(\frac{Vf}{Vi}\right) * \left(\frac{1}{mt}\right) \qquad \textit{Eq-4}$$

Where:

m_a= Aflatoxin level in ng corresponding to the area or height of peak of the sample elute.

V_f= Final test solution elute volume in (µl)

V_i= Volume of elute injected into HPLC in (µl)

m_t= Weight of commodity represented by the final extract in (g)

3.6. Isolation and Identification of Aflatoxigenic Fungi

Potato Dextrose Agar (PDA), 10% sodium hypochlorite solution, and ethanol absolute (99.7%) for aflatoxigenic fungi species isolation and identification were used. Twenty sesame seeds per sample were surface sterilize with 10% sodium hypochlorite solution for 1 min, followed by immersing in sterile distilled water for 1 min. Then surface sterilized seeds were placed on freshly prepared Potato Dextrose Agar (PDA) plates and incubated for three days at 25˚C. Pure cultures of different out growing fungi were obtain by transferring fungal colonies to new PDA plates using sterile toothpicks, and the plates were incubated for 5-7 days at 25˚C. Isolates were

identified to a species level based on morphological (phenotypic) features as described by Mohammed A., and Chala A. (2014).

3.7. Experimental Design

Completely randomized experimental design was followed to see the level of aflatoxin in Ethiopian export standard white and red sesame seed of Humera and Wollega varieties.

3.8. Experimental data analysis

After collecting the results from KAP survey, thousand seed weight, moisture, crude fat and peroxide value analysis and HPLC quantification of aflatoxin level in sesame seed, all the data entered into IBM SPSS version 20.0 and the required statistical analysis were calculated. The results were reported as percentage and mean ± SD. Least Significant Difference (LSD) was utilized for mean separation and P-value < 0.05 was considered to be significant.

CHAPTER FOUR

4. Result and Discussion

4.1. KAP survey

Knowledge, attitude and practice of sesame seed exporters regarding the aflatoxins contamination was analyzed separately on tables below from table 4.1-4.3.

According to the response of sesame exporters for the questions prepared to assess their knowledge about aflatoxins, below under table-4.1, more than half of sesame seed exporters participated in this survey do not know anything about aflatoxins and they cannot suspect that aflatoxins can contaminate sesame seed. Even one of the respondents replied that he know about aflatoxins but he thinks that aflatoxins can contaminate only milk. Almost all of the respondents do not know the methods to control aflatoxins contamination and the conducive conditions that favor for the occurrence of aflatoxigenic fungi on sesame seed.

Table 4.1 Knowledge survey response of sesame exporters

Exporters knowledge about Aflatoxins			
No	**Questions**	**Summarized response in percent**	
		Yes	**No**
1.	Do you have an idea about aflatoxins?	40 %	60 %
2.	Do you know that aflatoxin affect the health of human?	37 %	63 %
3.	Do you suspect that aflatoxin present in sesame seed?	10 %	90 %
4.	If yes for question 3, do you know any method of preventing aflatoxins occurrence in sesame seed?	3 %	97 %
5.	Do you know conditions that favor infestation of sesame seed by aflatoxins?	3 %	97 %
6.	Do you know that sesame with molds has been contaminated with aflatoxins?	3 %	97 %

As the result revealed below on table-4.2, all of the participants of this survey do not have any idea about the association between improper seed drying and well aerating of sesame storage area with aflatoxins contamination. Additionally, almost all of the participants do not have any idea that contamination of sesame seed with molds may reduce its overall market acceptability. Which indicates that the attitude of majority of the participants of this study towards the methods those help to prevent and minimize aflatoxins contamination is very low.

Table 4.2 Attitude survey response of sesame seed exporters

Exporters attitude on Aflatoxins contamination			
No.	**Questions**	**Summarized response in percent**	
		Yes	**No**
1.	Do you think improper drying of seed may favor aflatoxin infestation?	3 %	97 %
2.	Well aerating of sesame seed storage may reduce occurrence of molds	3 %	97 %
3.	Do you believe the presence of molds reduce acceptability of sesame seed?	3 %	97%
4.	Do you agree with the idea that entrance of rodents and insects facilitate aflatoxin contamination of seeds during storage?	3 %	97 %
5.	Do you think leakage of water into the seed storage facilitate occurrence of Aflatoxigenic fungi?	3 %	97 %

The data summarized under table-4.3. indicates that all of the participants cannot apply any method that minimize aflatoxins contamination of sesame seed. Even though the respondents replied that their warehouse is built well to prevent entrance of water, rodents, insects to sesame storage area and it support well aeration, none of them cannot conduct any kind of check up for the presence of molds on the sesame seed when they purchase and accept the sesame seeds to

their warehouse from ECX. Finally, all of them replied that they cannot conduct any kind of test for the presence of aflatoxins on sesame seed before exporting the seed.

Table 4.3 Sesame exporters practice survey response on aflatoxin

Sesame exporters Practice to prevent aflatoxin contamination			
No.	**Questions**	**Summarized responses in percent**	
		Yes	**No**
1.	Is your warehouse sealed properly to prevent water, rodents or insects entrance?	100 %	0 %
2.	Do you check the presence and absence of molds in sesame seeds before receiving?	0 %	100 %
3.	Your warehouse is well aerated?	100 %	0 %
4.	Do you check sesame seed for the occurrence of mold frequently during storage?	0 %	100 %
5.	Have you faced sesame seed infected with molds?	0 %	100 %
6.	Do you conduct aflatoxin check up for sesame seed before exporting?	0 %	100 %

As the data analyzed above on the KAP survey of sesame seed exporters indicate that there is knowledge, attitude and practice gap on the sesame exporters those participated in this survey.

4.2. Seed Characteristics

4.2.1. Thousand Seed Weight

As described below on table- 4.4., thousand seed weight of the three sesame varieties White Humera, White Wollega and Red sesame varieties were analyzed and have an average 1000 seed weight of 3.07g, which is fit with the standard that set for export standard sesame seed. There is significant difference ($p < 0.05$) on thousand seed weight of red variety and the rest white Humera and white Wollega sesame seeds. The highest 1000 seed weight is recorded for Red sesame variety 3.22g and the lowest is for white Humera variety 2.89. This finding is similar to the study by Zebib et al., (2015), on physico-chemical properties of sesame (*Sesamum indicum* L.) varieties grown in northern area, Ethiopia, which is within the range of 2.74-3.16g/1000 seed (Zebib et al., 2015). According to Eckey, (1954) the variation on the thousand seed weight is due to variation in variety and cultural conditions (Eckey, 1954).

4.2.2. Moisture Content of Sesame Seed

Moisture is one of the main factor that determine the occurrence of aflatoxigenic fungi and aflatoxins production on a certain agricultural commodity or substrate. Average moisture requirement for a feed above 14% may favor toxin production but the moisture requirement may vary depending up on the type of the commodity. For starchy cereals the moisture requirement for toxin production is 18% and 9-10% for oil-reach nuts (WHO, 1979). As described below under table-4.4, the average moisture content of white Humera, white Wollega and Red sesame variety is 4.69, 3.77 and 3.72% respectively. There is significant difference ($p < 0.05$) in the moisture content of the three sesame seed varieties of white Humera variety and the rest white Wollega and red sesame variety. These moisture levels are below the average moisture requirements for toxin production on oil-rich nut (9-10 %). When the finding of this study compared with the study conducted by Zebib et al., (2015) on the physicochemical properties of sesame varieties grown on Northern, Ethiopia the moisture content may show variation. They determined the moisture level in the range of (3.17 - 3.96%) (Zebib et al., 2015). The detail moisture content for 27 sesame samples described under appendix-2.

4.2.3. Crude fat content of sesame seed

Oil content of oilseeds is main characteristics, mostly oils are susceptibility for oxidative rancidity, which makes the oil to deteriorate easily. Sesame seed contains higher amount of oil. According to the findings of this study, the average crude fat content of white Humera, Red sesame and white Wollega sesame varieties is 51.1, 47.44, and 53.63% respectively. White Wollega sesame variety shows higher amount of oil content when compared with others. There is significant difference ($p< 0.05$) in the oil content of the three varieties described below under table 4.4. Detail for the oil content of 27 sesame samples described on appendix-3. The result of this study is close to the finding by Zebib et al., (2015), they found average crude oil content of (50.88% - 52.67%) on the sesame varieties those grow on the northern part of Ethiopia (Zebib et al., 2015)**.** In another study in Congo Brazzaville on the chemical composition of the seeds and oil of sesame (*Sesamum indicum* L.) grown in Congo-Brazzaville, the average crude fat content of sesame seed determined is 54 % (Nzikou et al., 2009).

4.4.4. Peroxide value of sesame seed

Peroxide value indicates the ability of the oil to get rancid due to oxygen absorption during storage and processing. Thus with high peroxide value are poor resistance to peroxidation. Data revealed below under table-4.4, indicates the peroxide value of sesame seed of white Humera in the range of (3-4.8 meq/kg), white Wollega sesame (4.4-8.2 meq/kg) and Red sesame varieties (2-6 meq/kg). There is significant difference ($p< 0.05$) on the peroxide value of white Wollega sesame and the rest. This finding is similar to the peroxide value of sesame seed oil in South Eastern Nigeria (1.01 to 7.61 meq/kg) (Ogbonna and Ukaan, 2013). This finding indicates the oil of sesame seeds is resistant for oxidative rancidity, it is mainly associated with its antioxidant content like sesamin, sesamol and sesamolin (Elleuch et al., 2007). The peroxide values of Sesame seeds are below the maximum acceptable value of 10 meq/kg set by the Codex Alimentarius Commission for groundnut seed oils (Abayeh et al., 1998). The production of aflatoxins is directly related to the number of double bonds in the molecules of free and esterified fatty acids, linoleic acid and trilinolein stimulate the production of aflatoxins in greater proportion than oleic acid, triolein, and other saturated fatty acids and triacylglycerols. Lipid

oxidation increases the biosynthesis of aflatoxins (Fanelli and Fabbri, 1989)(Bircan, 2006)(De Luca et al., 1995)(Jayashree and Subramanyam, 1999).

Table 4. 4. Thousand seed weight, moisture, crude fat and peroxide value of sesame seed

White Humera sesame samples				
Sample code	Thousand seed weight	Moisture in (%)	Crude fat in (%)	Peroxide value in (meq/kg)
H-1	2.98	4.99	51.5	4.5
H-2	3.06	4.33	51	3
H-3	3.02	4.44	51.2	5
H-4	3.00	5.66	49.5	4.2
H-5	2.89	4.88	52	2.3
H-6	3.01	4.66	50.5	3.4
H-7	3.03	4.21	51	4.8
H-8	3.07	4.44	51.2	3.5
H-9	3.08	4.66	52	3.7
Average	**3.01 ± 0.057a**	**4.69 ± 0.38**b	**51.1 ± 0.77b**	**3.82 ± 0.88a**
White Wollega sesame samples				
W-1	3.00	3.99	54.50	5.3
W-2	3.01	4	53	4.5
W-3	2.98	3.44	57.75	4.8
W-4	3.11	3.55	54	6.5
W-5	3.00	3.65	53.75	8.2
W-6	3.03	3.77	52.50	4.4
W-7	3.17	3.55	52.50	5.5
W-8	3.06	3.65	52.25	5
W-9	3.14	3.88	52.50	4.4
Average	**3.05 ± 0.68a**	**3.72 ± 0.34a**	**53.63 ± 1.73c**	**5.40 ± 1.24b**
Red sesame samples				
R-1	3.11	3.66	47.25	6
R-2	3.15	4.44	47.75	5.3
R-3	3.22	3.33	46.75	2
R-4	3.09	3.66	48.25	3
R-5	3.16	3.77	47.25	4
R-6	3.19	3.44	46.75	3
R-7	3.11	3.66	46.75	5
R-8	3.21	3.88	47.50	4.5
R-9	3.16	4.10	48.75	5.6
Average	**3.15 ± 0.45b**	**3.77 ± 0.34a**	**47.44 ± 0.70a**	**4.26 ± 1.36a**

4.3. Determination of aflatoxins content of sesame seed

4.3.1. Chromatographic Method Validation

Chromatographic method validation have been done by checking different parameters such as identification, accuracy, recovery, linearity, working range, LOD and LOQ that set to measure the analytical performance of the instrument and to validate the method used to analyze aflatoxins from Ethiopian export standard sesame seeds.

Identification

Identification of four aflatoxins from the test sample have been done according to their retention time on the HPLC chromatogram which is obtained after running 250ppb mixed and single aflatoxin standards. According to the result described below on table-4.5. Their order of elution is Aflatoxin G2, Aflatoxin G1, Aflatoxin B2 and Aflatoxin B1 with retention time of 9.774, 12.328, 14.261 and 18.189, respectively. Precision of the retention time measured by using percent relative standard deviation which is between (0.092-0.434%), according to FDA percent relative standard below 2% is acceptable (FDA, 2002). Chromatographic data for the retention time of each aflatoxin standards and mixed aflatoxin standard is also shown below on figure from 4.1-4.6.

Table 4.5 Aflatoxin identification retention time

Aflatoxins	Aflatoxin 250 ppb injection retention time (min)		N	Mean	Standard Deviation	% RSD
	For single run	For mixed run				
AFG2	9.781	9.768	2	9.774	0.009	0.092
AFG1	12.352	12.304	2	12.328	0.033	0.267
AFB2	14.306	14.217	2	14.261	0.062	0.434
AFB1	18.214	18.165	2	18.189	0.034	0.186

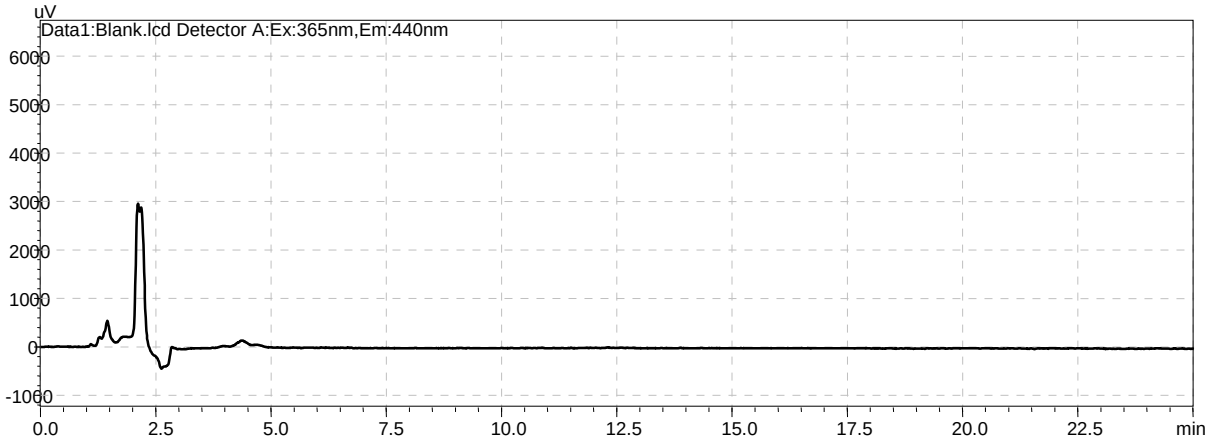


Figure 4.1 Blank Chromatogram

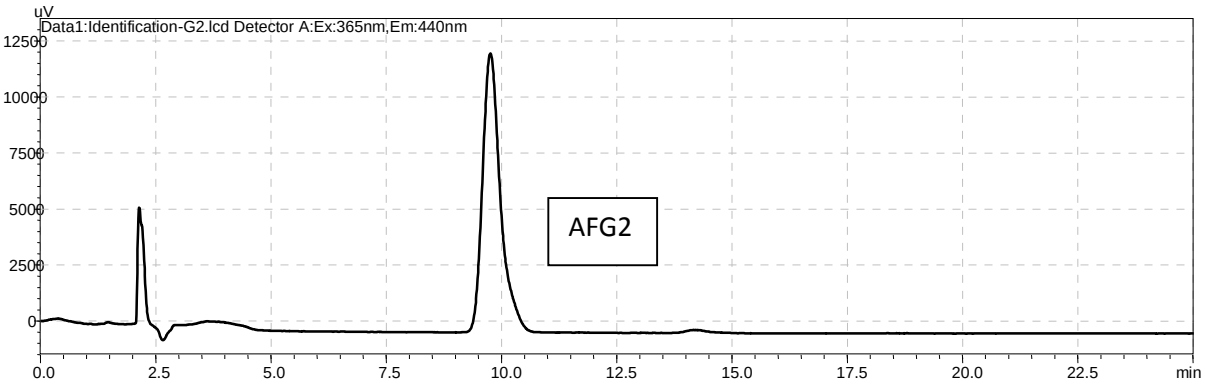


Figure 4.2 Aflatoxin G2 Standard of 100ppb Chromatogram

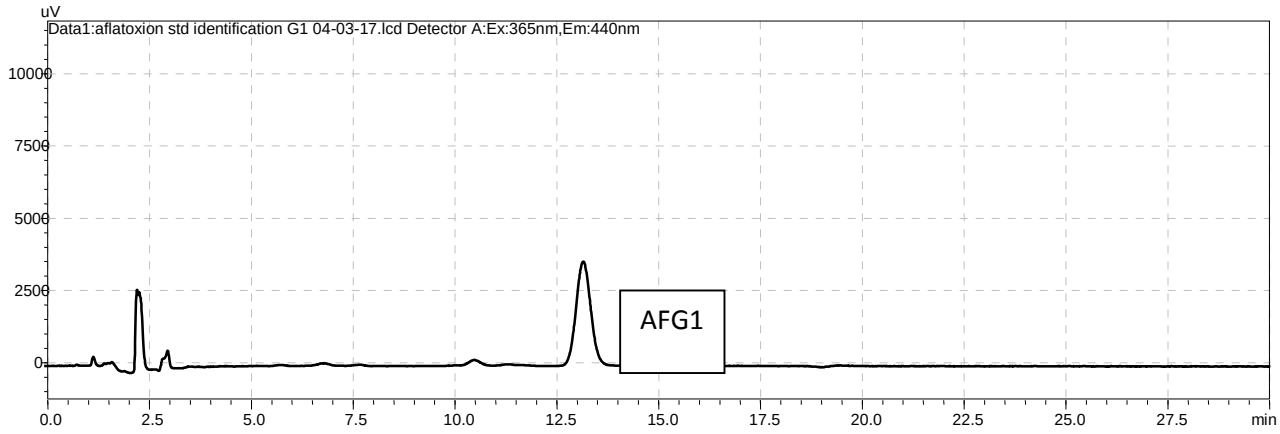


Figure 4.3 Aflatoxin G1 standard of 50ppb Chromatogram

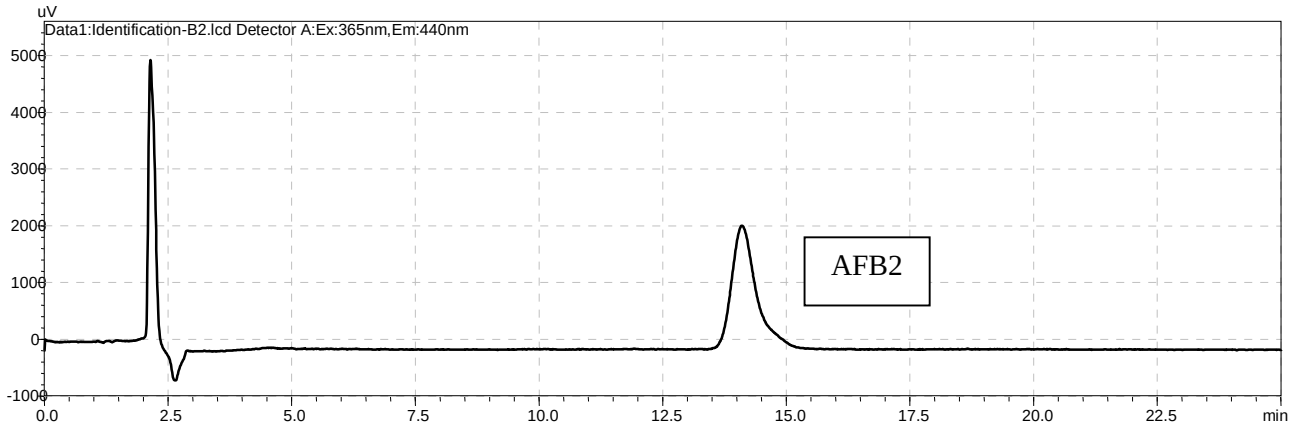


Figure 4.4 Aflatoxin B2 standard of 10ppb Chromatogram

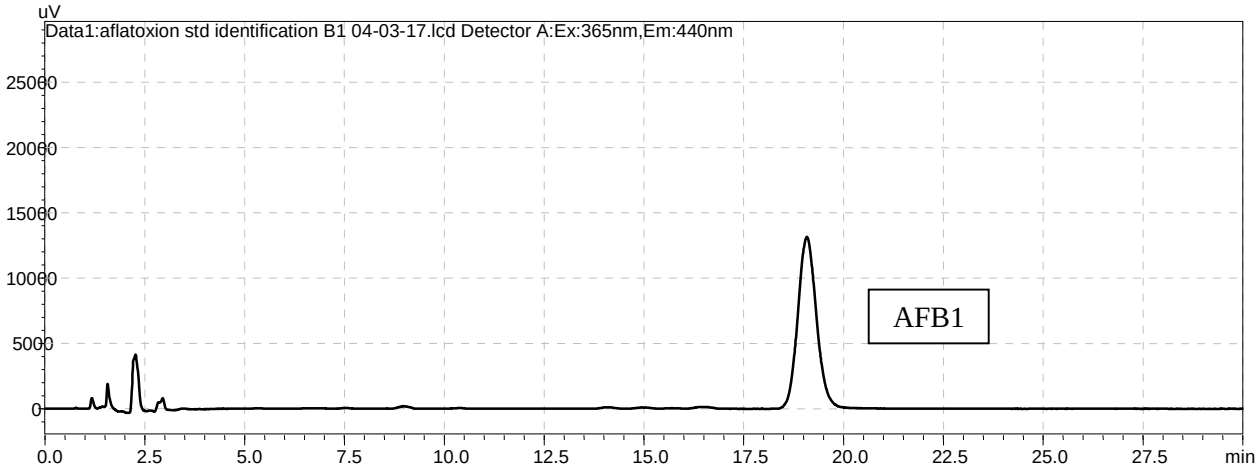


Figure 4.5 Aflatoxin B1 standard of 30ppb Chromatogram

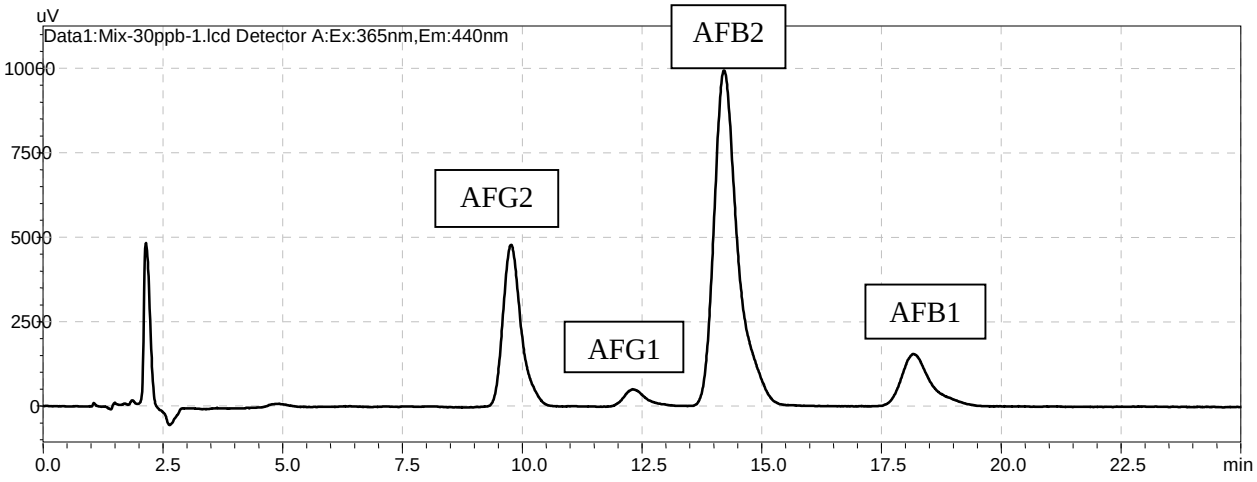


Figure 4.6 Aflatoxins mixed standard 30ppb Chromatogram

Limit of Detection and Limit of Quantification

The ability of the instrument to detect the smallest change on the analyte is 0.01, 1, 0.01, and 0.8ppb for Aflatoxin G2, G1, B2, and B1 with signal to noise ratio of 4.152, 3.186, 8.028 and 7.920 respectively. The signal to noise ratio which is greater than 3 is acceptable for limit of detection of the instrument. The limit of quantification of the instrument is 0.05, 2, 0.05, and 1 ppb for Aflatoxin G2, G1, B2 and B1 with signal to noise ratio of 10.305, 12.441, 14.295 and 11.620 respectively. The acceptable level of signal to noise ratio for the limit of quantification is greater than 10. The result shown below on table-4.6.

Table 4.6 Limit of detection and limit of quantification

Aflatoxins	LOD (ppb)	Signal to noise ratio (S/N)	LOQ (ppb)	Signal to noise ratio (S/N)
AFG2	0.01	4.152	0.05	10.305
AFG1	1	3.185	2	12.441
AFB2	0.01	8.028	0.05	14.295
AFB1	0.8	7.920	1	11.620

Accuracy and Recovery

To check the accuracy of the purification method used during sample preparation have been done by spiking known amount of analyte with sesame seed sample extract and percent recovery of the analyte is calculated. According to FDA, (2002) acceptable level of mean percent recovery is within the range of 100 ±20 range or the percent recovery lay within the range of 80-120 is acceptable level. After spiking sesame seed extract with 50ppb and 100ppb mixed aflatoxins standard, the percent recovery obtained after injecting into HPLC system and the results described below on table-4.7. Which is within the range of (86 - 97), it indicates that the method is accurate within the desired recovery range that set by FDA and the percent relative standard deviation in less than 5 (FDA, 2002).

Table 4.7 Statistics for aflatoxin recovery

Aflatoxins	Spiking Concentration		% Recovery		N	Mean	Standard deviation	% RSD
	100ppb	50ppb	100ppb	50ppb				
AFG2	10ppb	5ppb	94.4	95.33	4	94.865	0.657	0.69
AFG1	40ppb	20ppb	83.07	88.72	4	85.895	3.995	4.65
AFB2	10ppb	5ppb	95.22	99.05	4	97.135	2.708	2.77
AFB1	40ppb	20ppb	90.6	88	4	89.3	1.838	2.05

The check for accuracy of the chromatographic method have been also done by comparing the peak area value of 50ppb mix working standard with 50ppb standard aflatoxin reference. As revealed below on table-4.8, the closeness of the peak area value of mix reference and mix working standard is expressed in terms of percent relative standard deviation, which lay between (1-1.79).

Table 4.8 Statistics for the accuracy check

Aflatoxins	Conc. 50ppb	N	Peak area		Mean	Standard deviation	% RSD
			Working standard	Reference Standard			
AFG2	5ppb	2	256451	260124	258287.5	2597.2	1.00
AFG1	20ppb	2	39991	41017	40504	725.49	1.79
AFB2	5ppb	2	627777	643112	635444.5	10843.48	1.70
AFB1	20ppb	2	150005	153824	151914.5	2700.44	1.77

Precision

The repeatability of the analytical method have been tested by injecting 10 replicates of 30ppb mixed aflatoxins standard. Under the same analytical condition within the same day. The precision is expressed as % RSD. As FDA standard the acceptable level of percent relative standard deviation for precision is ≤ 5 for significant number of samples is acceptable (FDA, 2002). The result obtained after the assay is described in table-4.9. For the peak area percent relative standard is less than 2 and for retention time less than 0.05.

Table 4.9 Statistics for Precision check

Aflatoxins	Mix Conc. (30ppb)	N	Description Statistics of Peak area			Description Statistics of Retention time		
			Mean	Standard deviation	% RSD	Mean	Standard deviation	% RSD
AFG2	3	10	126880	1192.678	0.9400	9.763	0.0028	0.0029
AF G1	12	10	14976.2	278.382	1.8588	12.313	0.0059	0.0480
AFB2	3	10	340617.9	920.636	0.2702	14.209	0.0027	0.0191
AFB1	12	10	65618.5	373.056	0.5685	18.162	0.0037	0.0204
Valid N		10						

Linearity and Working range

Linearity of the analysis evaluated by injecting eight series of mix standards (2, 5, 10, 20, 30, 50, 100 and 250 ppb) to indicate the presence of direct linear relationship between the concentration of analyte and the peak area on the chromatogram. According to the International Conference on Harmonization (ICH) guidelines a minimum of five concentration levels, along with a certain specified ranges is recommended for the linearity check up. The regression equation was found by plotting the aflatoxin concentration in parts per billion (ppb) (x) versus corresponding peak area in (mv) (y) for each concentration. The standards calibration curves described below on figure-4.7.

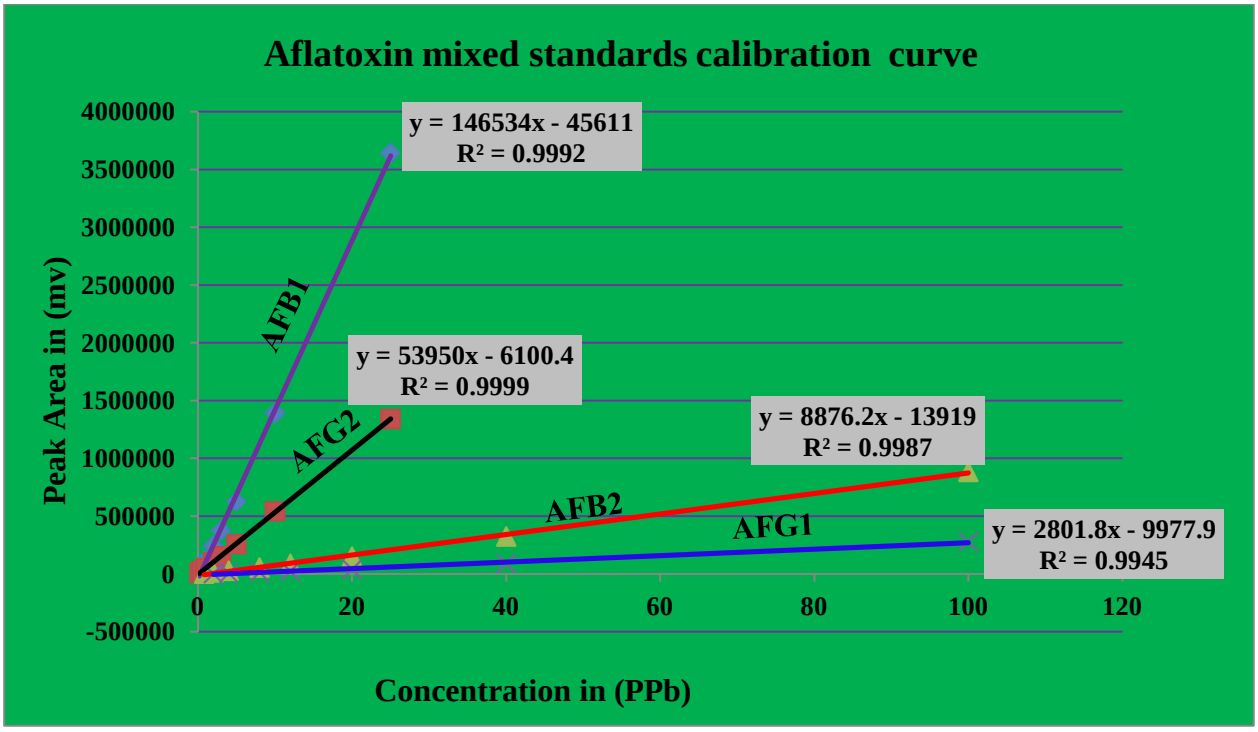


Figure 4.7 Standard calibration curve for aflatoxin B1, G2, B2 and G1

Linearity

Coefficient of determination (r^2) is the main criteria that FDA uses to check the acceptability of linearity data, which obtained from y-intercept of the linear regression line for the peak area versus concentration plot. As the data described below on table-4.10 the coefficient of determination lie between 0.9945-0.9999 that indicates the presence of strong relationship between the concentration of the analyte and peak area. The coefficient of determination > 0.998 is generally acceptable as FDA standard, for aflatoxin G1 the coefficient of determination is 0.9945 but the % RSD between the aflatoxin standards coefficient of determination is 0.24 which is by far less than 5%.

Table 4.10 Linearity check

Aflatoxins	Number of runs (N)	Calibration curve equation	R^2
Aflatoxin G2	8	Y= 53950x - 6100.4	0.9999
Aflatoxin G1	8	Y= 2801.8x - 9977.9	0.9945
Aflatoxin B2	8	Y= 8876.2x - 13919	0.9987
Aflatoxin B1	8	Y= 146534x - 45611	0.9992

The working range is obtained from the linearity study and depends on the intended application of the test method. A total of eight working ranges are used for this study 2, 5, 10, 20, 30, 50, 100 and 250ppb. The range is normally expressed in the same units as the test results obtained by the method. As the results described below in table-4.11, obtained during the linearity studies was used to assess the range of the assay method.

Table 4. 11 Working Range

No	Total Aflatoxin Concentration	Aflatoxin G2 Conc.	Aflatoxin G1 Conc.	Aflatoxin B2 Conc.	Aflatoxin B1 Conc.
1	2 ppb	0.2	0.8	0.2	0.8
2	5 ppb	0.5	2	0.5	2
3	10 ppb	1	4	1	4
4	20 ppb	2	8	2	8
5	30 ppb	3	12	3	12
6	50 ppb	5	20	5	20
7	100 ppb	10	40	10	40
8	250 ppb	25	100	25	100

4.3.2. Aflatoxin Level in Sesame Seed Sample

A total of 27 sesame seed samples from three different sesame varieties were analyzed for the presence of aflatoxins contamination. Nine samples of white Humera, nine white Wollega and

nine samples of red sesame varieties. Each sample analyzed in duplicate according to the procedure and the results described below under table-4.12. The results reported in parts per billion (ppb) or in Nanogram per gram (ng/g).

Table 4.12 Sesame sample aflatoxin content (ng/g)

Sample	AFG2	AFG1	AFB2	AFB1	Total Sum
H-1	ND	ND	7.08	1.44	8.52
H-2	ND	ND	7.24	1.32	8.56
H-3	0.64	ND	7.20	ND	7.20
H-4	1.40	ND	ND	ND	1.40
H-5	0.68	ND	ND	ND	0.68
H-6	1.28	16.2	11.2	1.36	30.04
H-7	0.56	34.68	ND	ND	35.24
H-8	1.32	31.76	7.44	ND	40.52
H-9	3.36	ND	ND	ND	3.36
Mean AF	**1.02b**	**9.18a**	**4.46a**	**0.45a**	**15.05a**
R-1	2.08	18.44	ND	1.52	22.04
R-2	1.64	31.92	7.8	1.36	42.72
R-3	2.44	32.92	ND	ND	35.36
R-4	ND	ND	0.44	ND	0.44
R-5	1.24	30.68	10.48	1.48	43.88
R-6	9.04	48.28	31.96	1.52	90.8
R-7	3.24	46.4	15.32	ND	64.96
R-8	ND	ND	ND	ND	ND
R-9	ND	ND	1.72	ND	1.72
Mean AF	**2.18b**	**23.18a**	**7.52a**	**0.65a**	**33.54a**
W-1	1.16	38.08	18.88	2.12	60.24
W-2	1.08	31.92	25.16	1.44	59.6
W-3	ND	ND	ND	ND	ND
W-4	ND	ND	ND	1.62	1.62
W-5	0.68	37.12	14.4	1.64	53.84
W-6	ND	23.72	ND	1.36	25.08
W-7	ND	22.56	8.88	1.48	32.92
W-8	ND	21.36	7.56	1.36	30.28
W-9	ND	< LOD	1.52	1.52	3.04
Mean AF	**0.32a**	**19.41a**	**8.48a**	**1.39b**	**29.62a**
Average total aflatoxin content of Sesame seed is 26.07 ng/g (ppb)					

Key: **ND**: Not detected, **< LOD**: below limit of detection

Figure-4.8 below shows the standard and sample peaks chromatogram supper imposed perfectly; this is an evidence for the sesame sample contaminated by aflatoxins. All the samples were determined by supper imposing with the standard peaks and the level of aflatoxins calculated in ng/g according to the formula.

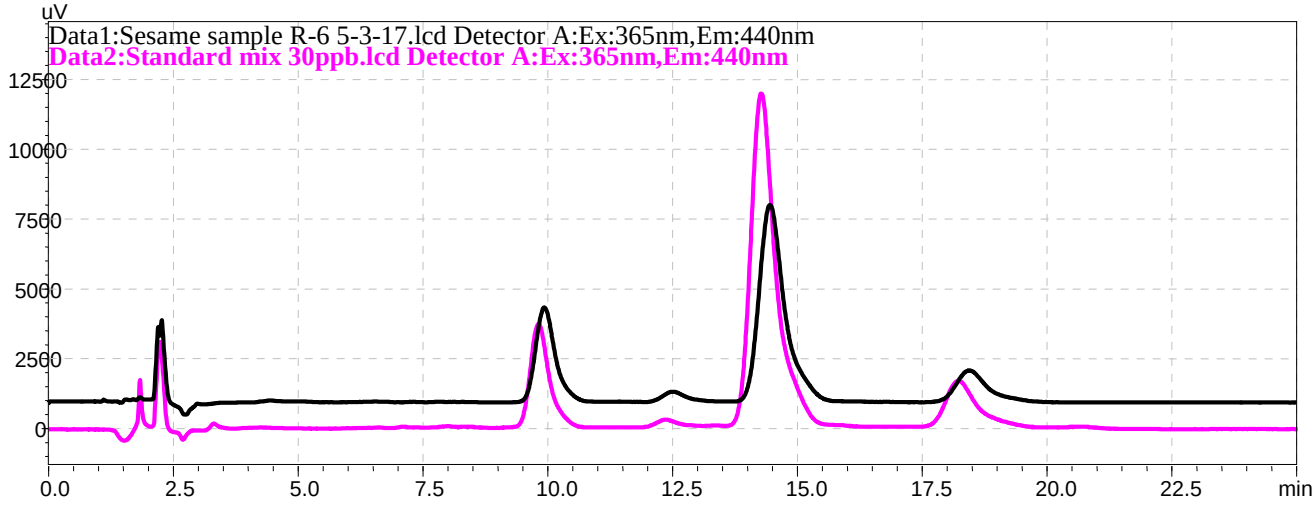


Figure 4. 8 Overlaid Chromatogram of Sesame sample and Aflatoxins mix standard

Aflatoxin B1 is the most toxic and it is known for its carcinogenic effect on human and other animals, as compared with AFB2, AFG1 and AFG2. According to the finding described above on table-4.12, out of 27 sesame samples 55 % the samples are positive for aflatoxin B1 in the range of (1.32 - 2.12 ng/g), only one of the samples shows AFB1 level above the limit of European Union for individual aflatoxin (2 ng/g) with aflatoxin content of 2.12 ng/g, since Ethiopia do not set maximum limit for aflatoxins in food and feed. 63% of the total samples are positive for aflatoxin B2 in the range of (0.44 - 31.98 ng/g), out of these 52% are above the limit European Union for AFB2. 55% of the sesame samples are positive for aflatoxin G1 within the range of (16.2 - 48.28 ng/g), all of them are above European Union standard for AFG1, and 55% of the samples are positive for aflatoxin G2 within the range of (0.56 - 9.04 ng/g), one sample have aflatoxin level above the maximum limit of European Union with aflatoxin content of 9.04 ng/g. There is significant difference ($p < 0.05$) in AFB1 and AFG2 content of White Wollega sesame and the rest White Humera and Red sesame seed varieties.

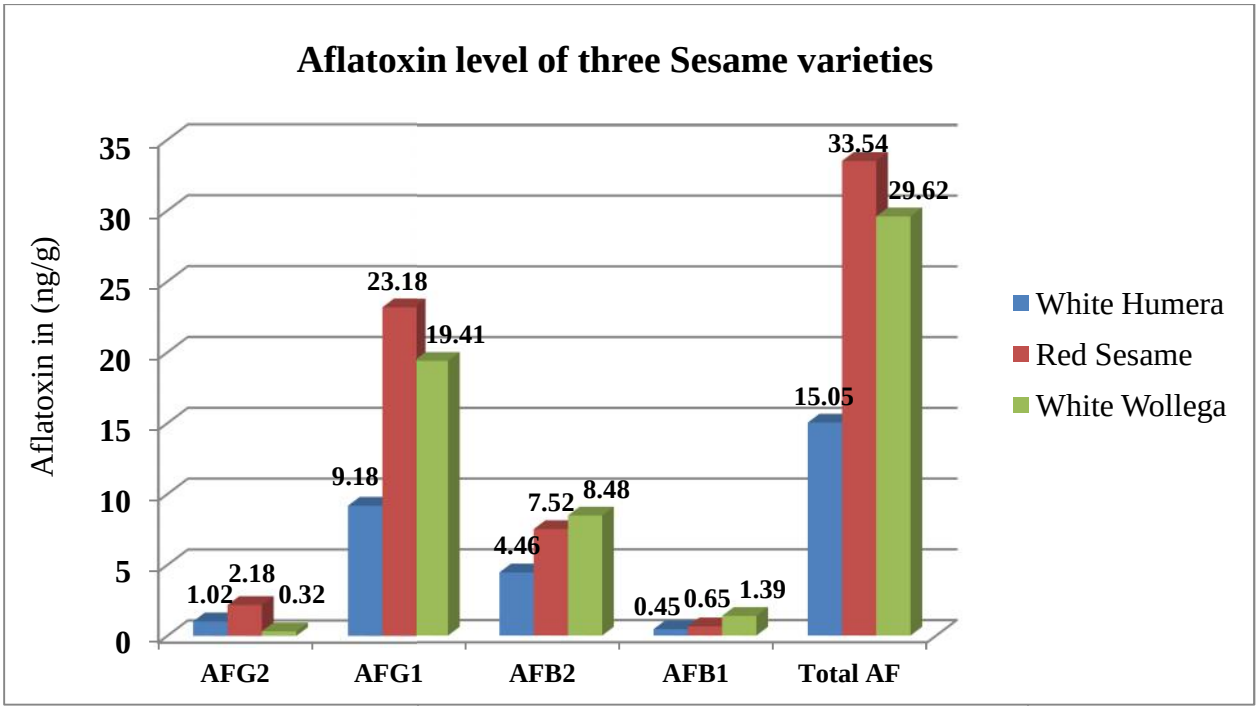


Figure 4. 9 Mean Aflatoxins content of three sesame varieties

As described above on the chart under figure-4.9, the mean aflatoxins content of white Humera variety is 0.45, 4.46, 9.18 and 1.02 ng/g for AFB1, AFB2, AFG1 and AFG2, respectively. AFB2 content is above the maximum limit of EU for individual aflatoxin (2 ng/g). The white Wollega sesame contains mean aflatoxin of 1.39, 8.48, 19.41 and 0.32 ng/g for AFB1, AFB2, AFG1 and AFG2. Aflatoxin B2 and G1 are above the European Union limit. The Red sesame variety mean aflatoxins content is 0.65 ng/g for AFB1, 7.52 ng/g for AFB2, 23.18 ng/g for AFG1 and 2.18 ng/g for AFG2. Aflatoxin B2 and G1 are above maximum permissible limit of EU for individual aflatoxin.

Out of 27 sesame samples analyzed for total aflatoxins contamination, 93% of the samples are positive for total aflatoxins with average total aflatoxins content of 26.07 ng/g, which is above the European Union maximum limit for total aflatoxins (15 ng/g). There is no significant difference in the total aflatoxins content of white Humera, white Wollega and Red sesame varieties.

White Humera sesame and White Wollega sesame seed varieties cultivated in different geographical area and also their is significant difference on their moisture content, crude fat and

peroxide value. The average aflatoxin content of Whitish Humera variety is 1.02, 9.18, 4.46, 0.45 and 15.05 ng/g for AFG2, AFG1, AFB2, AFB1 and Total Aflatoxins, respectively. Only AFG1 level is above the EU standard for individual aflatoxins. AFG2 and AFB1 are below EU limit. Whitish Wollega sesame seed contains AFG2, AFG1, AFB2, AFB1 and Total aflatoxins of 0.32, 19.41, 8.48, 1.39 and 29.62 ng/g respectively. Both AFG2 and AFB1 found at the level below EU standard, the rest AFG1, AFB2 and total aflatoxins are above the maximum limit of EU standard for individual and total aflatoxins. As the results described above, White Wollega sesame varieties indicate increased total aflatoxins contamination as compared with White Humera sesame.

Aflatoxin level of white and red sesame varieties analyzed and the result described below under figure-4.10. An average aflatoxins level of white sesame variety are 0.92, 6.47, 14.29, 0.67 and 22.33 ng/g of AFB1, AFB2, AFG1, AFG2 and total aflatoxins, respectively. The red sesame variety have aflatoxins level of 0.65, 4.46, 23.18, 2.18 and 33.54 ng/g for AFB1, AFB2, AFG1, AFG2 and total aflatoxins, respectively. Red sesame variety shows relatively higher level of contamination as compared with white sesame varieties. Since both the red and whitish sesame varieties cultivated in different geographical location and show significant differences on thousand seed weight, moisture, crude fat and peroxide value.

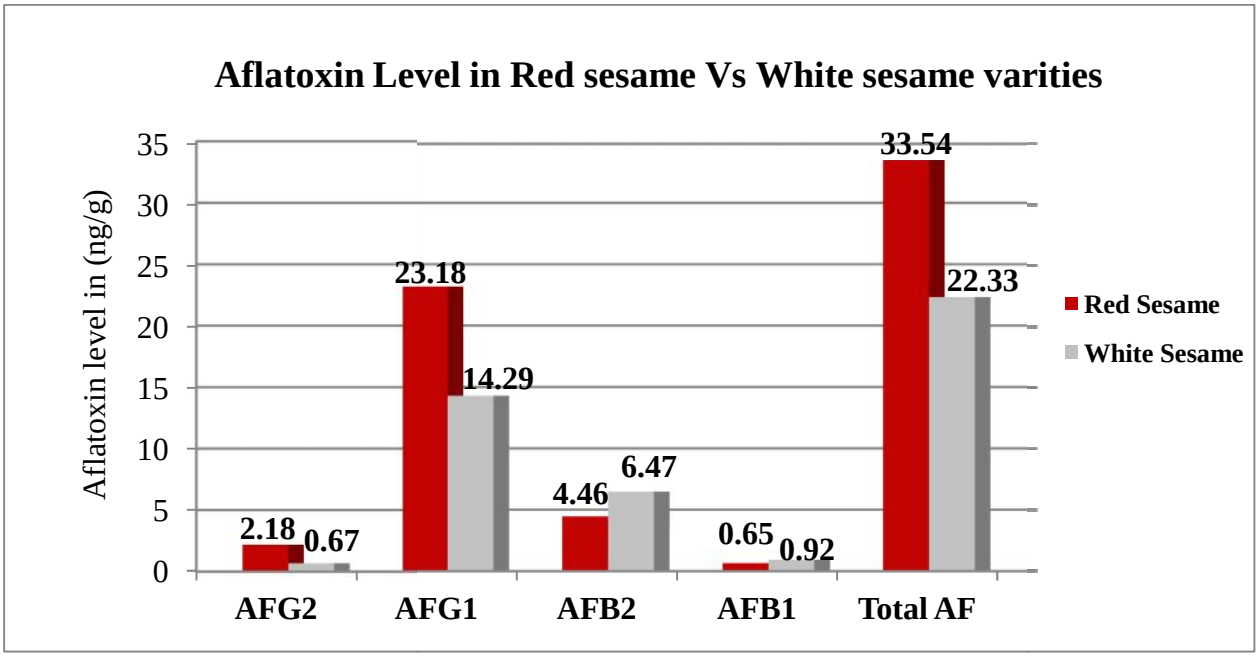


Figure 4. 10 Red and White sesame seed aflatoxins content

When comparing the findings of this study with other studies those conducted in different countries on sesame aflatoxin contamination, Ethiopian export standard sesame is less contaminated with AFB1.

A study has been conducted in Nigeria by Makun et al., (2014) on determination of aflatoxins in Sesame, rice, Millet and Acha, Aflatoxin B1 is detected from the sesame seed in the range of 14.71- 140.9 µg/kg, which is by far beyond the limit of European union (2µg/kg) and Nigerian legislated standard for AFB1 and aflatoxins (10µg/kg) in food (Makun et al., 2014).

In another survey of sesame seed for AFB1 in Greek 77.6% of the samples are positive for AFB1 with maximum content of 14.4µg/kg, Eight samples contain AFB1 above the limit of European Union limit (Eleni et al., 2016). While in a study conducted in China, 37 of 100 sesame paste samples analyzed were contaminated with AFB_1 at the levels above 20.45 ng/g (Li et al., 2009).

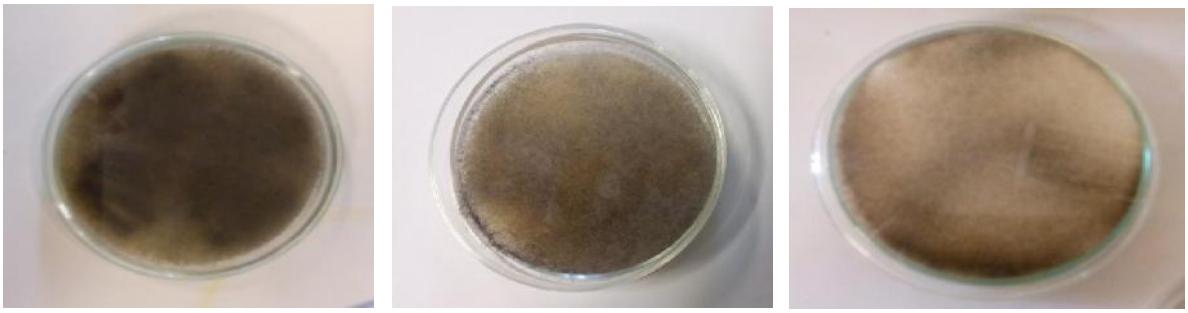
In contrary with this study, there is no another paper that deal on AFG2, AFG1, AFB2 and AFB1 separately on sesame seed. Most of the studies concentrate on AFB1 only.

Aflatoxins contamination of sesame seed compared with other oilseeds like ground nut (*Arachis hypogaea* L.), sesame seed indicates very low level of aflatoxin contamination. A study conducted in Ethiopia by Alemayehu et al., (2012), on natural occurrence of aflatoxins in groundnut (*Arachis hypogaea* L.) from eastern Ethiopia. Total aflatoxins detected in extremely higher level (15-11865 µg/kg) (Alemayehu et al., 2012). This is mainly associated with variation in the seed characteristics and storage conditions, for groundnut seed. In seed characteristics groundnut seed satisfy conditions favoring aflatoxins contamination. Moisture content of ground nut (*Arachis hypogaea* L.) is between (7-15%) (Eshetu, 2010), which is relatively higher than that of sesame seed (3-5%), moisture content of 9-10% for oil-rich nuts and seeds has been established for maximum production of the toxin (WHO, 1979).

According to the study by Eshetu, (2010), aflatoxins contamination level of newly harvested groundnuts shows reduced level of contamination when compared with groundnut seeds stored for 3-12 months. Only one sample contain aflatoxin above the codex limit (48.44ppb), but groundnut samples stored for one year shows total aflatoxins level in one sample goes up to (405.1ppb) (Eshetu, 2010).

4.4. Isolation and Identification of Aflatoxigenic fungi

For microbiological analysis surface sterilized sesame seeds were cultured on freshly prepared potato dextrose agar (PDA) media at 25°C for three days. After three days different out growing of the culture have transferred into freshly prepared PDA for further pure culturing at 25°C and cultured for 7 days. As shown below on figure-4.11, isolates were identified to a species level based on morphological (phenotypic) features as described by (Mohammed and Chala, 2014)(Cotty, 1994) (Egel et al., 1994)(Kurtzman et al., 1997)(Okuda et al., 2000). For this purpose: Isolates representing each pure culture were grown on PDA at 25°C for 5-7 days. Fungal colonies that grew rapidly and produced colors of white, yellow, yellow-brown, brown to black or shades of green, mostly consisting of a dense felt of erect conidiophores were broadly classified as *Aspergillus* spp.. *A. flavus* have biseriate conidia heads with creamy and dull white. While those that produce blue spores were considered as *Pencillium* spp. (Okuda et al., 2000). Isolates with dark green colonies, rough conidia and uniseriate conidia heads were considered as *A. parasiticus* (Klich, 2002). The major distinction currently separating *A. niger* from the other species of *Aspergillus* is the production of carbon black or very dark brown spores from biseriate phialides (Raper and Fennell, 1965). Those that showed brown colony with orange and cream reverse sides were considered *A. sojae* and *A. oryzae*, respectively (Cotty, 1994).



A B C

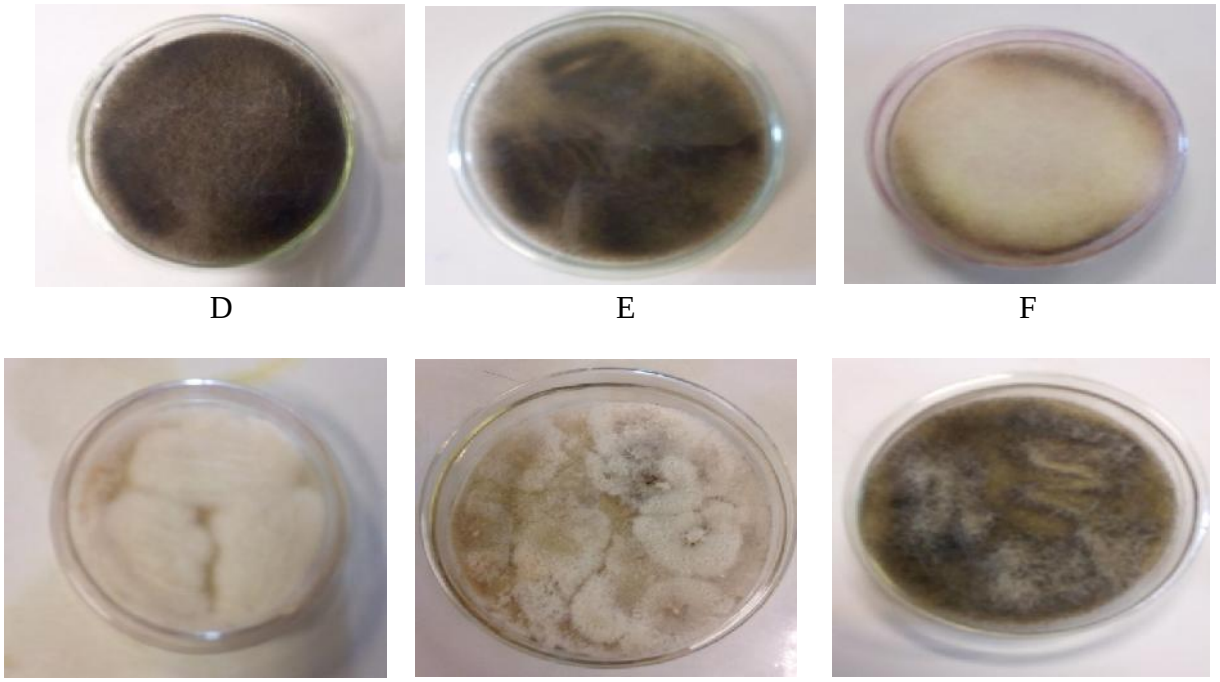


Figure 4.11 Isolates of *A. parasiticus* (A-C), *A. niger* (D), *A. flavus* (E-I)

As described above on the figure 4.11, *Aspergillus parasiticus*, *Aspergillus niger* and *Aspergillus flavus* are identified from the sesame seeds cultured on PDA. *Aspergillus flavus* was isolated from majority of the sesame seed samples cultured.

According to Hesseltine, (1983), in addition to the nature of substrate physical factors like temperature, moisture, water stress, high temperature stress and insect damage of host plant are major determining factors for mould growth and toxin production. Similarly specific crop growth stages, poor fertility of the soil, high crop densities and weed competition have been associated with increased aflatoxins contamination (Hesseltine, 1983). Even though, Seed characteristics of the sesame seeds indicates that the seed cannot satisfy the minimum requirement for the occurrence of aflatoxigenic fungi and toxins production, the finding of this study indicates aflatoxin contamination. Which is mainly associated with improper pre- and post- harvest practices on sesame seeds. To determine the root cause for aflatoxins contamination on Ethiopian export standard sesame seeds it needs further investigation on pre- and post- harvest practices on sesame seeds.

Chapter Five

5. Conclusion and Recommendations

5.1. Conclusion

KAP survey of exporters indicates there is knowledge, attitude and practice gap on the sesame exporters. Even majority of the participants do not know about aflatoxins. Additionally, exporters that know about aflatoxin do not know about the conditions that favor for the occurrence of aflatoxigenic molds and the way to control occurrence of aflatoxigenic fungi and aflatoxins production on the product.

Moisture content of sesame seed cannot make the seed susceptible for the aflatoxins contamination, when compared with other oilseeds like groundnuts because it is below the minimum moisture requirement for the production of aflatoxins on oilseeds. Peroxide value of sesame seed is in the range below the maximum acceptable peroxide value for oilseeds and the seed is resistant to oxidative rancidity, since rancid seeds are more prone to aflatoxin contamination.

In accordance with the objective of this study, 27 sesame samples analyzed for aflatoxins contamination and 93%, indicates positive result for aflatoxins in the range of (0.44-48.28 ng/g) with average total aflatoxins level of 26.07 ng/g, which is above maximum limit of European Union standards for total aflatoxins. Aflatoxin B1 and G2 detected below the limit of EU permissible limit for individual aflatoxin, Aflatoxin B2 and G1are above the European Union limit set for individual aflatoxin. Isolates of *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus parasiticus* identified from the sesame seeds cultured on potato dextrose agar medium.

According to the findings of this study, in addition to the knowledge, attitude and practice gap of the exporters the contamination level of the sesame seed is capable of interrupting Ethiopian sesame export market and affect country's economy by affecting the revenue generate from sesame export. But the extent of aflatoxins contamination of Ethiopian export standard sesame is low, as compared with aflatoxins level detected in sesame seed in Nigeria and Greek.

5.2. Recommendations

Based on the findings of this study to come up with the solutions to minimize the effect of aflatoxin contamination on the second most known national foreign export revenue generating product, the following recommendations will be made:

- ✓ According to KAP survey result more awareness creating and training programs are required for sesame exporters on aflatoxin, methods to prevent and control aflatoxin contamination and on overall food safety issues.
- ✓ Prevention of aflatoxin formation mainly relies on avoidance of contamination after harvest, buying of healthy seeds and sorting out shrivelled and discolored sesame seeds from the healthy ones. So, creating awareness for individuals on sesame value chain on the aforementioned issue may bring significant effect on prevention of aflatoxin contamination.
- ✓ It is better if the Ethiopian Commodity exchange authority and other regulating bodies may set as a regulation to test sesame seed for aflatoxin before going for export, which can reduce economic loss that may occur after exporting the product to other countries. Additionally, it is better to incorporate aflatoxin analysis as one of the parameters to grade the sesame seed.
- ✓ Due to financial limitation the designed sample size may not be enough, therefore it is better to conduct further works on this area with representative number of samples to reduce sampling error and variability on the aflatoxin level since this study is baseline for further studies.
- ✓ Controlling of aflatoxin requires a collaborative work of government bodies and individuals in sesame value chains, this may bring effective result in controlling and reducing the effect of aflatoxin.
- ✓ The government should establishment aflatoxin surveillance programs and regular monitoring and inspection of the warehouse and storage condition of the product regularly, since storage condition is the main source for post harvest aflatoxin contamination, by establishing food safety control laboratories, training of inspectors and supporting researcher to alleviate the existing problem.

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APPENDICES

Appendix-1

Questionnaire for Aflatoxin Study

Date_____

Questionnaire for Aflatoxin study

Dear respondent

Good morning/Good afternoon. Thank you for your willingness to talk with me today. I am MARKOS MAKISO who is a post graduate student of Addis Ababa University center for food science and nutrition, conducting a study on Investigation of Aflatoxins and Aflatoxigenic Fungi from export standard white and red sesame seeds of Humera and Wollega varieties, Ethiopia. The purpose of my visit today is to take information from you on the aforementioned issue. If you are willing to participate in the study, I will ask you few questions for 10-15 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 greatly appreciate your participation in the study.

Sex_____

Age_____

Aflatoxin **knowledge** assessment questions for sesame Exporters

Questions	Responses	
	Yes	**No**
1. Do you have an Idea about Aflatoxins?	☐	☐
2. Do you know that aflatoxin affect health of human ?	☐	☐
3. Do you suspect that aflatoxin present in sesame seed?	☐	☐
4. If yes, do you know any method of preventing aflatoxin occurrence in sesame seed?	☐	☐
5. Do you know conditions that favor infestation of sesame seed by aflatoxin?	☐	☐
6. Do you know that sesame with mold has been contaminated with aflatoxin?	☐	☐

Aflatoxin **Attitude** assessment questions for sesame Exporters

Questions	Responses
1. Do you think improper drying of seed may favor aflatoxin infestation?	Yes ☐ No ☐
2. Well aerating of sesame seed during storage May reduce occurrence of molds?	Yes ☐ No ☐
3. Do you believe the presence of molds reduce acceptability of sesame seed?	Yes ☐ No ☐
4. Do you agree with the idea that entrance of rodents and insects facilitate aflatoxin contamination of seeds during storage?	Yes ☐ No ☐
5. Do you think leakage of water into the seed storage facilitate occurrence of Aflatoxigenic fungi?	Yes ☐ No ☐

Aflatoxin **Practice** assessment questions for sesame Exporters

Questions	Responses
1. Is your warehouse sealed properly to prevent water, rodents or insects entrance?	Yes ☐ No ☐
2. Do you check the presence and absence of molds in sesame seeds before receiving?	Yes ☐ No ☐
3. Your warehouse is well aerated?	Yes ☐ No ☐
4. Do you check sesame seed for the occurrence of mold frequently during storage?	Yes ☐ No ☐
5. Have you faced sesame seed Infected with mold?	Yes ☐ No ☐
6. Do you conduct aflatoxin check up for sesame seed before exporting?	Yes ☐ No ☐
7. If yes, where do you conduct?	A. Analytical laboratories ☐ B. In your own laboratories ☐ C. Other ☐

If you have additional comment in this issue,_____
_____
_____
_____
_____

Thank you!

Appendix-2

Average Moisture content of sesame seed

Average moisture content of 27 sesame seed, analyzed in triplicate and the result described below in the table.

	Samples	Moisture Content (%)		
		White Humera	**White Wollega**	**Red variety**
1	Sample-1	4.99 ± 0.33	3.99 ± 0.33	3.66 ± 0.33
2	Sample-2	4.33 ± 0.33	4 ± 0	4.44 ± 0.51
3	Sample-3	4.44 ± 0.19	3.44 ± 0.38	3.33 ± 0.67
4	Sample-4	5.66 ± 0.66	3.55 ± 0.38	3.66 ± 0
5	Sample-5	4.88 ± 0.38	3.65 ± 0.35	3.77 ± 0.19
6	Sample-6	4.66 ± 0.33	3.77 ± 0.19	3.44 ± 0.50
7	Sample-7	4.21 ± 0.51	3.55 ± 0.38	3.66 ± 0.33
8	Sample-8	4.44 ± 0.38	3.65 ± 0.35	3.88 ± 0.19
9	Sample-9	4.66 ± 0.33	3.88 ± 0.76	4.10 ± 0.38
Average Moisture		**4.69 ± 0.38**b	**3.72 ± 0.34a**	**3.77 ± 0.34a**

Appendix-3

Crude fat content of sesame seed

Average fat content of White Humera, White Wollega and Red sesame varieties.

Sesame samples	White Humera variety	Red variety	White Wollega Variety
Sample-1	51.5 ± 0.70	47.25 ± 1.06	54.5 ± 0.70
Sample-2	51 ± 1.41	47.75 ± 2.47	53 ± 0
Sample-3	51.25 ± 0.35	46.75 ± 1.06	57.75 ± 0.35
Sample-4	49.5 ± 0.70	48.25 ± 1.06	54 ± 0
Sample-5	52 ± 1.41	47.25 ± 1.06	53.75 ± 0.35
Sample-6	50.5 ± 0.70	46.75 ± 1.06	52.5 ± 0
Sample-7	51 ± 1.41	46.75 ± 2.47	52.5 ± 0.70
Sample-8	51.25 ± 2.47	47.5 ± 0.70	52.25 ± 0.35
Sample-9	52 ± 0	48.75 ± 0.35	52.5 ± 0.70
Average crude fat	**51.1 ± 0.77b**	**47.44 ± 0.70a**	**53.63 ± 1.73c**

Appendix-4

Average peroxide value of white Humera, white Wollega and red sesame varieties

Sample code	White Humera	White Wollega	Red Sesame
Sample-1	4.5	5.3	6
Sample-2	3	4.5	5.3
Sample-3	5	4.8	2
Sample-4	4.2	6.5	3
Sample-5	2.3	8.2	4
Sample-6	3.4	4.4	3
Sample-7	4.8	5.5	5
Sample-8	3.5	5	4.5
Sample-9	3.7	4.4	5.6
Average POV	**3.82 ± 0.88a**	**5.40 ± 1.24b**	**4.26 ± 1.36a**

Appendix-5

Aflatoxin Study Protocol

	Institution **ADDIS ABABA UNIVESITY** **SCHOOL OF GRADUATE STUDIES**	
	Title: **Aflatoxin Study Protocol**	Page No

Types of Aflatoxins Study
DETERMINATION OF AFLATOXIN FROM SESAME SEED

Protocol prepared by

Department	Name	Signature	Date
AAU FDSN	Markos Makiso		

Protocol Approved by

Department	Name	Signature	Date
AAU FDSN	Ashagrie Zewdu (PhD)		

1. Purpose

The purpose of this standard test procedure is to determine the level of aflatoxins (AFB1, AFB2, AFG1 and AFG2) from sesame seed by using Supel Tox Aflazea cartridge for solid phase extraction (SPE). Under the specifications of international standards. Details of test conditions and test types to be done is meet compliance with International requirements of regulatory authority and evaluate toxicity of product in conformance with WHO and ISO requirements.

2. Responsibility

- ✓ Analysts who study Aflatoxins in sesame seed and other samples, by using Supel Tox Aflazea cartridge as Solid phase extraction, are responsible for using this protocol.
- ✓ All Addis Ababa University center for food Science and nutrition lecturers are responsible in supporting and monitoring the implementation of this protocol.

3. Laboratory safety and requirements

Aflatoxins degrade by light. Protect analytical materials adequately from daylight and keep Aflatoxins standard solutions protect from light by using amber vials or aluminum foil. Use of acid washed glassware for Aflatoxins aqueous solutions may cause loss of Aflatoxins. Before use, soak new glassware in dilute acid (e.g., 2M sulfuric acid; carefully add 105 ml H_2SO_4 to water to remove all traces acid).

All food sample suspected of being contaminated with Mycotoxins must be handle with care. Use disposable gloves and protective masks and goggle during sample preparation. While handling pure Aflatoxins reference material, extreme precautions are to be taken as they are electrostatic, work preferable in a hood. Swab any accidental spill of toxin with 1% sodium hypochlorite solution (NaOCl), leave 10 minutes and then add 5% aqueous acetone. Rinse all glassware exposed to Aflatoxins with methanol, add 1% sodium hypochlorite solution and after 2 hours add acetone to 5% of total volume. Let it react for 30 minutes and then wash thoroughly. Use laboratory coat or apron soaked in 5% sodium hypochlorite solution overnight and washed in water.

PREPARED BY		APPROVED BY	
Name: Markos Makiso		Name: Ashagrie Zewdu (PhD)	
Signature:	Date:	Signature:	Date:

4. Materials and Chemicals

Chemicals and reagents

HPLC grade methanol, Acetonitrile and distilled water

Solid Phase Extraction (SPE):

Solid phase extraction can be done by using the Supel Tox Aflazea cartridge with the holding capacity of 6ml.

Standards:

Aflatoxin B1, B2, G1 and G2

Apparatus:

Supel Tox Aflazea cartridge, Laboratory stand with clamp, Volumetric and Graduated pipettes (1ml, 5ml, 10ml, 25ml and 50ml), volumetric flask (10ml, 25ml, 50ml, 100ml, 500ml and 1000ml), Measuring cylinder (50ml and 100ml), Beaker (50ml, 100ml and 500ml), conical flask(250, 500 and 1000ml), Mixer, Stirrer, Ultra bath Sonicator, Wash bottle, Micropipettes, Micropipette tips, Millipore Filter, Electronic balance, syringes(5ml and 10ml), Paraffin, vacuum pump, Bucher funnel, Sample label, Vials with screw cap. HPLC system setup contains auto sampler, injector, oven, column, Link, Degasser, fluorescence detector and desktop computer with chromatography software.

Mobile phase:

The mobile phase of water-methanol-acetonitrile (65:25:15, v/v/v) which have an Isocratic elution. The mobile phases filter by applying vacuum in a filter unit and degasses.

Chromatographic system:

HPLC instrument, Auto sampling system, fluorescence detector and the instrument controlled Chromatography software. A Shim-pack FC-ODS column (5µm, 150 x 4.6mm diameter) at ambient temperature, 1.2ml min-1 f low rate and injection volume 20µl. The fluorimeter was set at 365 nm excitation and 440 nm emission wavelength.

PREPARED BY		APPROVED BY	
Name: Markos Makiso		Name: Ashagrie Zewdu (PhD)	
Signature:	Date:	Signature:	Date:

5. Procedure

Standard preparation:

Aflatoxin standards obtained from Sigma Aldrich (St. Louis, MO, USA). For each aflatoxin, a stock solution of 5mg/ml^{-1} will prepare in benzene-acetonitrile (98: 2, v/v, 2 ml) and store at 20 ^{O}C. Prepare the working standard solution as follows: an aliquot (100 µl) of each aflatoxin stock solution transfer into a 10 ml calibrated flask, evaporate under nitrogen and re-dissolve in chloroform by Ultrasonication. An aliquot (100 µl) of this solution evaporate under nitrogen and re-dissolve in the HPLC mobile phase (0.5ml), to obtain calibrant solutions at concentrations between 0.2 and 2 mg for AFB1 and, AFG1, and between 0.1 and 1 mg for AFB2 and AFG2.

Sample Extraction

25 gram of finely grounded sesame seed is weighted and added into 500mL Erlenmeyer flask. 100mL of extraction solution (84:16 acetonitrile: deionized water) was added into the sample and the flask was sealed with stopper and parafilm. Then the flask processed by using blender for 3 minute on high blending. After that the suspension was filtered under vacuum until all the liquid is filtered into the flask by using a Buchner funnel with filter paper in an Erlenmeyer flask with side arm and a neoprene filter adapter. Filtered extracts were transferred into sample jar and covered with lid.

Sample Clean up:

Supel Tox Aflazea purification cartridge was placed on lab stand with clamp and collection tubes assembled on the cartridge. 2 ml sample extract pipetted onto purification cartridge. Entire sample collected into collection tube. After the filtration by Supel Tox Aflazea cartridge the filtrate further filtered by using 0.45µm micro filter. Neat solution was transferred into 2mL autosampler vial. Finally 20µL injected into HPLC.

HPLC determination:

The elute Aflatoxins(B1, B2, G1 and G2) methanol solution will determined at parts per billion (µg/g^{-1}) levels in Sesame sample by using Supel Tox Aflazea cartridge for Solid phase extraction.

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Name: Markos Makiso		Name: Ashagrie Zewdu (PhD)	
Signature:	Date:	Signature:	Date:

6. Calculation

Final aflatoxin in ng/g= $m_a \left(\frac{Vf}{Vi}\right) * \left(\frac{1}{mt}\right)$

Where:

m_a= Aflatoxin level in ng corresponding to the area or height of peak of the sample elute.

V_f= Final test solution elute volume in (µl)

V_i= Volume of elute injected into HPLC in (µl)

m_t= Weight of commodity represented by the final extract in (g)

7. References

1. Analytical Procedures and Methods Validation. (2000). Chemistry, Manufacturing and Controls Documentation, *FDA*, *Federal Register (Notices*) 65 (169), p. 52776.

2. Sigma Aldrich

N.B: The method carries on under slight modification and validated at Addis Ababa University Center for Food science and Nutrition Addis Ababa, Ethiopia.

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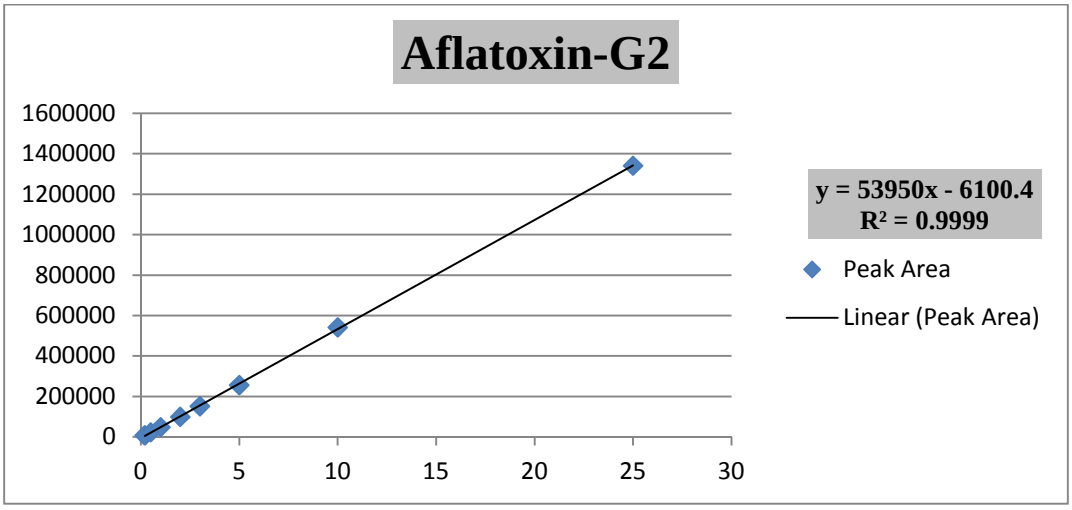
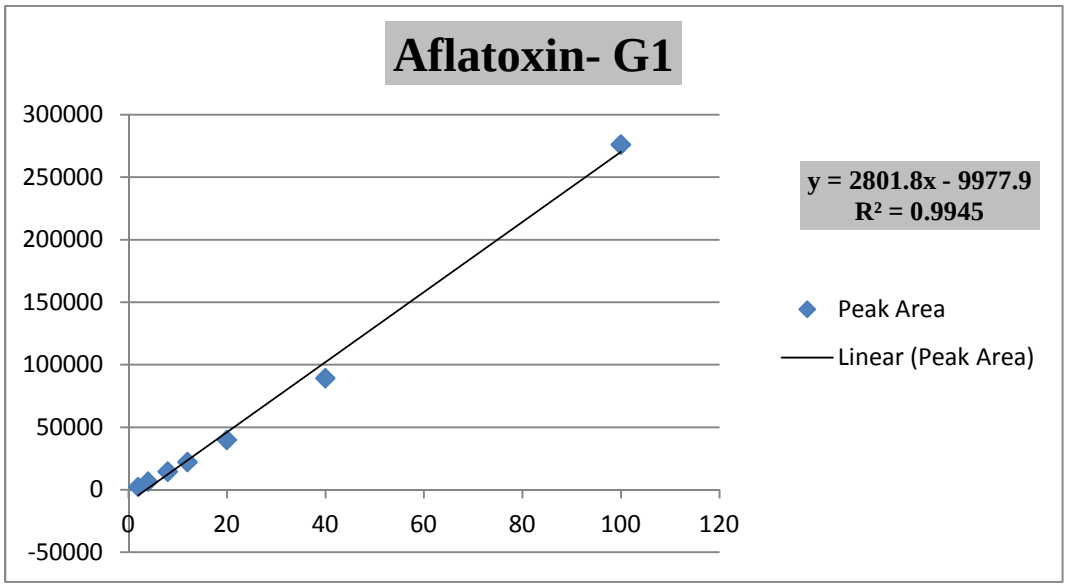
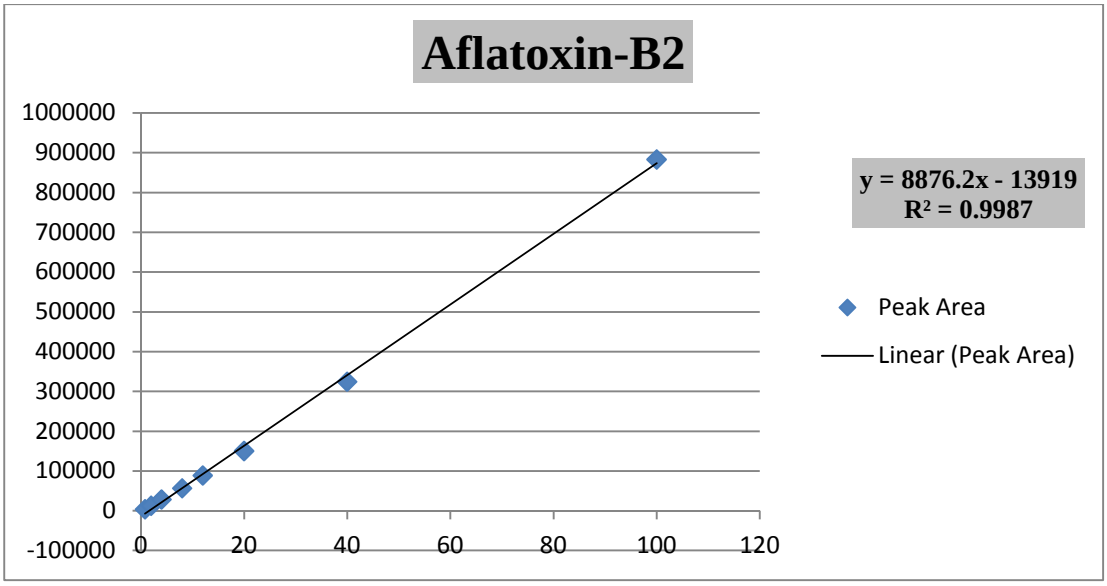
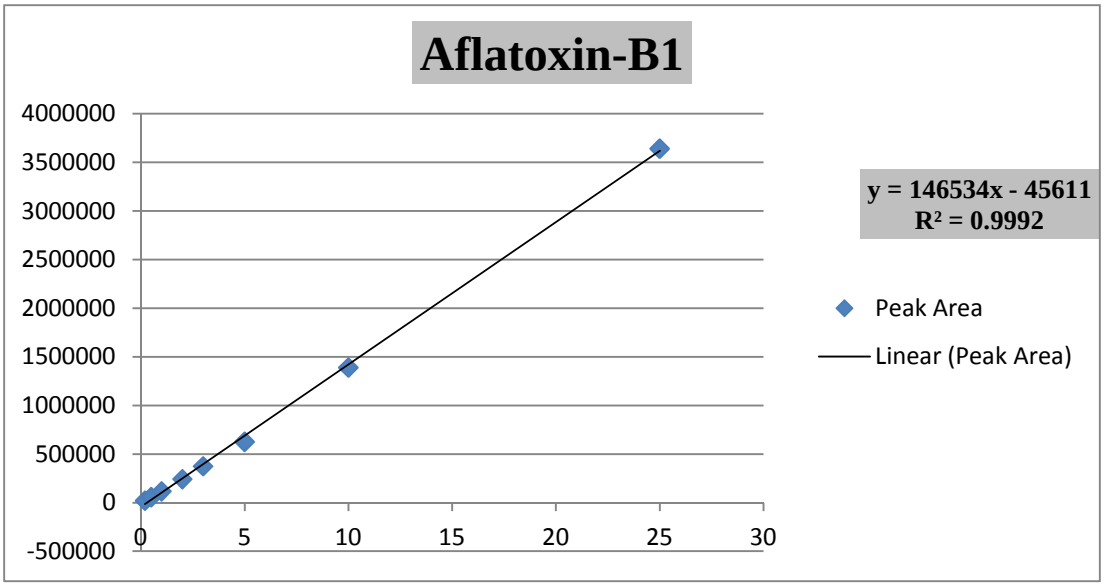
Appendix-6

Calibration curve for Aflatoxin G2, G1, B2 and B1






Aflatoxin-B2
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
-100000
0
20
40
60
80
100
120
y = 8876.2x - 13919
R² = 0.9987
Peak Area
Linear (Peak Area)


Aflatoxin-B1
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
-500000
0
5
10
15
20
25
30
y = 146534x - 45611
R² = 0.9992
Peak Area
Linear (Peak Area)