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Study on *Aspergillus species* and Aflatoxin Levels in Sorghum (*sorghum bicolor L.*) stored at different period and storage system in Kewet Districts, Northern Shewa, Ethiopia.

By

Geremew Tassew

Advisors: Mr. Tilahun Bekele (Assistant professor)

Dr. Ashagrie Zewedu (Assistant professor)

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Study on *Aspergillus species* and Aflatoxin Levels in Sorghum *(sorghum bicolor L.)* grain stored at different period and storage system: The case of Kewet woreda, Northern Shewa, Ethiopia.

Msc. Thesis

By: Geremew Tassew W/semayat

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Approved by Examining Board

Name	**Signature**	**Date**
Examiner: Dr Emana Getu	_____	_____
Examiner: Dr Kaleab Baye	_____	_____
Chairman: Mr. Aynadis Tamene	_____	_____

DECLARATION

I, the undersigned, declare that this thesis is my original work. It has never been submitted in any institution and that all sources of materials used for the thesis have been dully acknowledged.

Name: Geremew Tassew W/semayat

Place: Addis Ababa University

Signature: _____

Date: _____

The thesis has been approved for submission by:

Name of Advisors	**Signature**	**Date**
Dr. Ashagrie Zewedu	_____	_____
Mr. Tilahun Bekele	_____	_____

DEDICATION

This thesis is dedicated to all those who try to work hard towards solving problems of societies in the field of food science and nutrition.

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List of Abbreviations

AF- Aflatoxin

AFB1-Aflatoxin B1

AFB2- Aflatoxin B2

AFG1- Aflatoxin G1

AFG2- Aflatoxin G2

AFM1- Aflatoxin M1

AOAC- Association of Official Analytical Chemists

ARSO- East African Regional standard organization

CSA- Central Statics Agency

EFSA European Food Safety Authority

EFSA -European Food Safety Authority

ELISA-Enzyme Linked Immunosorbent Assay

EU-European Union

FAO- Food and Agricultural Organization

FDA- US Food and drug Administration

FEHD- Food and Environmental Hygiene Department

HCC-Hepatocellular carcinoma

HKSAR- Hong Kong special administrative region

HPLC- FLD-High performance Liquid chromatography coupled with florescence Detector

IARC-International Agency for Research on cancer

JECFA- Joint Expert Committee on Food Additives

LB-- Lower Bound

LOD --Limit of Detection

LOQ --Limit of Quantification

PAD--Potato Dextrose Agar

PP^b – parts per billion

RSD-Relative standard Deviation

TLC- Thin Layer Chromatography

USAID-United States Agency for International Development

WHO- World Health Organization

Abstract

Sorghum serves as staple food for over 100 million people in Sub-Saharan African countries. It is the most important nutritional security crop and ranks third among major cereal crops in terms of area and production next to teff and maize in Ethiopia. However, sorghum is susceptible to contamination by fungal species mainly Aspergillui flavus and Aspergillui parasiticus species producing mycotoxins, aflatoxins that have hepatotoxic and carcinogenic effects on humans and animal. Study was conducted to assess Aspergillus species and aflatoxin levels in sorghum (sorghum bicolor L.) stored at different period and storage system. Thirty Samples were analyzed for aflatoxins contamination using HPLC with fluorescent detection and Aspergillus species were isolated and identified using culture media. About 56.7%, 16.7%, and 23.3% of the sorghum samples were found to be contaminated with Aspergillus flavus, Aspergillus niger and Aspergillus parasiticus, respectively. Total aflatoxin and AFB1 contamination occurred in sorghum with a maximum concentration range of 11.44 to 344.26µg/kg and 3.95 to 153.72µg/kg, respectively. The levels of Aflatoxins detected in sorghum were within the range of 1.17 to 91.82µg/kg for AFB2, 3.22 to 52.02µg/kg for AFG2 and 9.87 to 139.64µg/kg for AFG1. Sorghum stored for ≥ 2 years had high level of the aflatoxin B1 (52.19 µg/kg) followed by sorghum stored for < 12 months (38.24 µg/kg). Although storage period had resulted in no significant difference in AFB2, AFG1, and AFG2 concentrations, AFB1 varied significantly ($P<0.05$). On the other hand, the concentration of aflatoxins in all sorghum samples surpassed the maximum level set by the European commission, 10µg/kg for total aflatoxin and 5µg/kg for aflatoxin B1.Therefore, this situation clearly demands wider national or international programs for the control of aflatoxin contamination in sorghum. In conclusion, creating unfavorable conditions for fungal growth with storage system and use of proper storage methods may help in minimizing aflatoxin contamination.

Keywords: *Sorghum, Aflatoxin, Aspergillus spp., Storage*

1. Introduction

1.1. Background

Sorghum (*Sorghum bicolor L. Moench*) is the world's fifth most important cereal crop. In the year 2005, sorghum was grown worldwide on 43,727,353ha with an output of 58,884,425 metric tons (FAO, 2005). It is grown for grain and fodder in the semi-arid tropics, mainly in Asian and African countries (Frederiksen & Odvody 2000). Sorghum is also used as a major food and nutritional security crop to more than 100 million people in the Horn of Africa, outstanding due to its resistance to drought and other production constrains (Katile *et al.,* 2010).

In Ethiopia, sorghum ranks third among major cereal crops in terms of area and production next to teff (*Eragrostis teff*) and maize (*Zea mays*) (Tigabu *et al.,*2012). Currently, the total area devoted for sorghum production in Ethiopia is estimated to be more than 1.6 million hectare of land (CSA, 2010). It provides one third of the cereal diet and is grown almost entirely by the subsistence farmers (Serna-Saldivar *et al*., 1995). Sorghum is a staple food crop on which the lives of millions of poor Ethiopians depend. Besides, being a major source of staple food for humans, it serves as an important source of feed and fodder for animals (Tekle and Zemach, 2014). However, sorghum crops have been affected by numerous diseases and the grains are subjected to a broad range of mould contamination both in the field and after harvest (Brandfass & Karlovsky, 2008). These moulds are known to produce mycotoxins that cause serious health risks to humans and animals that feed on contaminated grains (Pestka & Smolinsky, 2005; Katile *et al*., 2010).

Aflatoxins are naturally occurring toxic secondary metabolites of the storage fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) which are produced in most agricultural products stored at inappropriate places, temperatures and water activities. These two species are common and widely distributed in tropical and sub-tropical parts of the world. Aflatoxin has been found as contaminant in agricultural food products especially in cereals and animal feeds (Rajarajan *et al*., 2013).

Aspergillus flavus is common and widespread in nature and is most often found when certain grains are grown under stressful conditions such as draught. Additionally, mould occurs in soil,

decaying vegetation, hay and grains undergoing microbiological deterioration and invades all types of organic substrates whenever and wherever the conditions are favorable for its growth (Rajarajan *et al.,* 2013). *Aspergillus* infection and aflatoxin contamination is frequently reported in sorghum seeds or grains all over the world (Frederiksen & Odvody, 2000).

Numerous reports have indicated the presence of association between consumption of aflatoxin contaminated food and liver related diseases including *Hepatocellular carcinoma* (HCC), or liver cancer (Gourama *et al.,* 1995; Nissen and Martin, 2002; Williams et al., 2004; Jian *et al*., 2005). Moreover, acute illness and death, immune system suppression, stunting and several other nutritional illnesses are also related to dietary exposure to aflatoxins (USAID, 2012). The extent to which mycotoxins affect human health is difficult to investigate in countries whose health system lack capacity and resources are limited. For instance, factors such as immune suppression contributing to the overall burden of infectious diseases is difficult to quantify, but is undoubtedly significant. However, food safety remains an important opportunity for addressing current health problems in developing countries (Shepard, 2008).

Ethiopia being the centre of origin and diversity for sorghum, the crop has been cultivated for many thousand years (Mekbib, 2009). It has various agro-climatic regions produces a variety of crops. Most of the produces are stored under poor and unsatisfactory storage conditions for considerable periods. Traditional storage structures in Ethiopia are usually made up of mud, bamboo strips and underground pits. In addition to these structures grains are stored in polyethylene and gunny bags. Comprehensive storage under inadequate storage conditions influenced to produce the growth of fungi and productions of mycotoxins (Lal, 1986; Mashilla, 2004).

Likewise in kewet woreda, sorghum grains are stored in underground pits and above ground storage system such as silos (*Gotera*) for a long period of time. These storage systems are believed to protect against insect damage and theft, fire, domestic and wild animals and improve the quality of sorghum as well. The community also produces pan-cake food known as *injera* which is prepared from this stored sorghum blended with other cereal grains. However, these sorghum grains are stored under unhygienic conditions and very often spoiled by moulds.

1.2. Statement of the problem

Cereals and its products are the main food for human consumption throughout the world. The cereal grains belong to corn, sorghum rice, wheat, and barley are found susceptible to aflatoxin accumulation by *aflatoxigenic* fungi. The aflatoxin problem in cereals is not restricted to any geographic or climatic region. Aflatoxin produce toxins on cereals, both in the field and in storage; they involve both the grain and the whole plant (Hesseltine, 1974). Mycotoxin contamination of human food and livestock feeds has come to the attention of the World Health Organization (WHO) due to global concern in relation to human and animal health. Although the limits for mycotoxin contamination can be established, these are difficult to achieve in practice, and contamination may not be avoidable without unacceptable losses of food and feeds (Maryam, 1999).

Aflatoxin contamination of food causes *hepatotoxicity*, *carcinogenicity*, *immunosuppression*, *mutagenic* and *teratogenic*. It is a serious health problem because factors which encourage the production of these toxins by mycotoxin (*aspergillus flavus* and *aspergillus parasiticus*) abound in Africa. Because their presence in food interferes with micronutrients absorption and status in the body and therefore it affect immunity and development (Iyanda *et al.,* 2014). Aflatoxin B1 is one of the most potent naturally occurring animals carcinogenic as well as immunosuppressive and is formed in corn, corn silage, all cereal grains, sorghum, peanuts, and other oilseeds. It is increasingly implicated in human and animal pathology (IARC 1993; Tchana *et al.,* 2010). In addition, *aflatoxicosis* causes induction of tumors, impaired central nervous system, skin disorders, and hormonal defects and decreased bone strength (Saleemullah *et al.,* 2006).

Aflatoxin exposure has also been associated with childhood stunting and it is a chronic form of malnutrition, is potentially associated with many health problems, including an increased rate of infectious illnesses, impaired learning capabilities, and reduced work productivity (Black *et al.,* 2013). On the other hand, aflatoxins can result in major economic losses and can negatively affect animal and human health. In 2004, several hundred Kenyans became severely ill, and 125 died, of acute *aflatoxicosis*: a disease of liver failure associated with consuming extremely high levels of aflatoxin in food. It has been estimated that more than 5 billion people in developing

countries worldwide are at risk of chronic exposure to aflatoxins through contaminated foods (Strosnider *et al.,* 2006).

In Ethiopia, the problem of aflatoxin contamination in agricultural commodities is much more serious than commonly visualized. The growth and propagation of aflatoxigenic fungi depends on several factors including high temperature and humidity under which natural substrates are stored. Even though, particular social conditions and behavior including methods of storage, preservation of food products and traditional feeding may play a significant role. In all these aspects, Ethiopia is considered to provide a favorable situation for *aflatoxigenic* mould production of agricultural products. Previous reports have been made on aflatoxin contamination of cereals and pulses such as sorghum, teff, wheat, maize, peanut, legumes collected from silos, warehouses, shops and market places (Rehima, 2006; Kumeda and Asao, 2001).

In *Kewet* woreda (*Teri, Yelen, Rassa, Gerenefara and Ashege ena Yegeda kebele*), the farmers are using underground storage system and above ground storage (*Gotera* are prepared from different plants, clay, grass, ash, and caw dung). These practices is firstly washing the underground pits with water and immediately the sorghum grain until they fill, then the cover with grass, clay and flatted stone. Some farmers are using cleaning, insecticides and fumigants to prevent insect damage and adding the sorghum grain in to the pits. The farmers store this grain until they need for consumption and market. The stored grain elongated for one and more than three years until the food scarce is occur. Therefore, this kind of storage practice may develop mycotoxin contamination.

Aflatoxins are extremely persistent under most conditions of storage, handling and processing. It is impossible to eliminate them once the food stuffs are contaminated (Ubwa *et al.,* 2014). Many investigators have detected aflatoxins and other mycotoxins in many agricultural foods such as maize, teff, broad bean, sorghum, berebere, traditional spices (mitten shiro) and wheat in Ethiopia (Geyid and Maru, 1987, Habtamu and Kelbesa, 1996, 2001, Chala *et al.,* 2014, Besrat and Gebre, 1981, Ayalew *et al.,* 2006, Abate and Gashe, 1985). Those researchers were did not analyze *Aspergillus spp.* and aflatoxin contamination from sorghum grain stored under aboveground (*Gotera*) and underground pit for different periods. So far quite numerous pieces of work have been done on aflatoxin contamination of Agricultural products. However, research is

done to evaluate the level of *aspergillus species* and aflatoxin contamination in sorghum *(sorghum bicolor L.)* stored at different period and storage system. Therefore, this particular research were endeavored to analyze the occurrence of *aspergillus species* and aflatoxins content in sorghum grain stored at different period and storage system.

1.3. Significance of the study

The findings of this study will be helpful in safeguarding the public health of the nation by increasing knowledge and awareness of the mycotoxin contamination on agricultural food products. It is also reduce the healthcare costs as well as increase the well-bigness and healthy life style of the consumers. On the other hand, it is an input for Agricultural sectors, Policy and Decision makers to create and implement training programs. Researchers and academicians of the field can use the findings of the study as a reference material. Generally, the result will be used to strengthen the development of Food security, agriculture, food safety and healthcare strategies in Ethiopia.

1.4. Objective

1.4.1. General Objective

To evaluate the incidence of *Aspergillus species* and aflatoxin levels in sorghum *bicolor* stored at different period and storage system in Kewet District, Northern Showa, Ethiopia.

1.4.2. Specific Objectives

- To assess the incidence and distribution of *Aspergillus species* in sorghum stored at different period and storage system.
- To analyze the aflatoxins *(B1, B2, G1, and G2)* content of sorghum stored at different period and storage system.
- To evaluate the association between aflatoxin levels with storage periods
- To evaluate the association between aflatoxin levels with storage system

2. Literature Review

2.1. Overview of Sorghum Production

Sorghum *(bicolor)* is a viable food grain for many of the World's most food insecure people who live in marginal areas with poor and irregular rains and often poor soils. Sorghum is the fifth most important cereal crop in the world (Tekle and Zemach, 2014) and fourth following wheat, rice and maize in Africa. The sorghum grains are used as a staple food for more than 500 million people in the semi-arid tropics of Africa and Asia and more than 80% of the world area of production is confined to these two continents. Moreover, over 100 million people in sub-Saharan Africa countries depend on sorghum as staple food (Serna-Saldivar and Rooney, 1995; Smith and Frederiksen, 2000). In West Africa, farmers are cultivating sorghum to adapt the harsh and unpredictable conditions. Sorghum crop are grown predominantly in low-rainfall, arid to semi-arid environments. This crop is typically produced under adverse conditions such as low input use and marginal lands (Lacy *et al.,* 2006; Tekle and Zemach, 2014). Due to its drought tolerance and adaptation attributes, this crop is grown in eastern Africa, where agricultural and environmental conditions are unfavorable for the production of other crops. All lines of evidence point to the north-east quadrant of Africa, mainly Ethiopia, as the centre of domestication of sorghum (Masresha *et al.,* 2010).

2.2. Production of Sorghum in Ethiopia

Ethiopia is the centre of origin for sorghum. It has been cultivated for many thousands of years and hence indigenous knowledge based sorghum classification and naming has a long tradition (Firew Mekbib, 2007). Sorghum is one of the major staple crops grown in the poorest and most food insecure regions of the country. It is grown on 1,468,070 ha with a total production of 2,173,598 Mt. The crop is typically produced under adverse conditions such as low input use and marginal lands. It is well adapted to a wide range of precipitation and temperature levels and is produced from sea level to above 2000m (Fetene, 2011; Abu Tefera, 2013).

Sorghum ranks third among major cereal crops in terms of area and production next to teff (*Eragrostis teff*) and maize (*Zea mays*) (Tigabu *et al.,*2012). Currently, the total area devoted for sorghum production in Ethiopia is estimated to be more than 1.6 million hectare of land (CSA,

2010). Moreover, Ethiopia is the largest sorghum producing country in eastern and southern Africa next to northern Sudan. Sorghum is the second most important cereal produced in Ethiopia, accounting for 19 % of the total cereal produced in the country and covering some about 20 % of the total area under cereals. Production of sorghum grain stands third next to teff and maize in area and the second in total production next to maize and show an increasing trend in the past 15 years. It covers 16% of the total area allocated to grains (cereals, pulses, and oil crops) and 20% of the area covered by cereals (FAO, 2013). However, low productivity of sorghum in Ethiopia could be attributed to biotic and edaphic factors (physical or chemical composition of soil) affecting directly and indirectly sorghum production (Tekle and Zemach, 2014).

Sorghum is growing in all regions of Ethiopia in altitude ranges of 400m to 2500m. Oromia, Amhara and Tigray regions are the three major producers of sorghum covering 86.4% of the total area and 89% of the total production in the last nine years (Matthijs Kool and Frank van Steenbergen, 2014). It is nearly 4.5 million smallholders located in the eastern and northwest parts of the country, where the weather is dry and soil fertility is poor. Table 1 show that, the main sorghum producing regions are Oromia and Amhara, accounting for nearly 80 percent of the total production. The leading sorghum producing zones are East and West Hararge in Oromiya and North Gondar and North Showa in Amhara. Two regions, SNNPR and Tigray, are relatively less important, contributing 11 and 4 percent of the national production, respectively. Ethiopia is the second largest producer of sorghum, after the Sudan (FAO, 2013).

Table 1. Sorghum area, production and yield by Regions (2010-11) Area in hectare

Regions	**Production in tones**	**yield(t/ha)**	**Share of production (%)**
Oromia	739361 -1580545	2.1	39.9
Amhara	710732- 1533586	2.2	38.7
Tigray	216879 -466394	2.2	11.8
S.N.N.P.	107735- 175125	1.6	4.4

Source: Author's calculation based on CSA 2010/2011 data

2.3. Importance of Sorghum in Ethiopia

Sorghum is the most important nutritional security crop in Ethiopia. It is well-known for its versatility and diversity produced over a wide range of agro-ecological zones. Sorghum is also drought tolerant relative to other major cereal crops (Abu Tefera, 2013). Cereal crops in the dry land areas are Sorghum, maize, teff and millet. These are the major food crops for human's diet and also provide feed for animals. Farmers in the semi-arid regions of Ethiopia plant sorghum and maize at high seed rate to ensure enough plant stands (FAO, 2013).

Sorghum is a principal source of energy, proteins, vitamins and minerals for millions of the poorest people living in Africa, Asia and the Semi-arid tropics worldwide (Raihanatu *et al.,* 2011). It is one of the most important indigenous food cereals in Ethiopia (Benti *et al.,* 1993). In Eastern Amhara region (Waghamra, North Welo, South Welo, Oromiya and North Shewa zones), sorghum is the second most important food crop next to teff (CSA, 2000). It is also one of the leading traditional food crops in comprising 15-20% of the total cereal production in the country (Tekle and Zemach, 2014).

The main use of sorghum in Ethiopia is for making traditional bread, injera (staple food of Ethiopians'-thin pan cake) next to teff, for human consumption. It is also used for local foods and local beverage production in some parts of the country. It accounts for an average of ten percent of daily caloric intake of households living in the eastern and North West areas of the country. Lower quality sorghum grain is also used for animal feed. The stalks are an important product and are used as fire wood, fuel, fodder, and construction material for rural houses. Sorghum consumption is increasing in middle and lower class communities due to mixing of sorghum with teff to make injera due to the higher price of teff (FAO, 2013; Abu Tefera, 2013).

2.4. Traditional Storage of Sorghum and Mycotoxin contamination

Agricultural products are contaminated with aflatoxin producing moulds during harvest or at post-harvest stages, transportation and storage time under favorable conditions of temperature and humidity (Bhat, 1988). However, the higher levels of mycotoxin contamination were encountered during storage in the retail or wholesale markets than during harvest or storage through farm *(*Rajarajan *et al.,* 2013). Cereals, pulses and legume products are also the main

foods for human consumption throughout the world. Where the grains belong to corn, sorghum, barley, wheat and rice are found susceptible to aflatoxins accumulation by aflatoxigenic fungus. The problem of aflatoxins occurring naturally in cereals, especially in rice and corn, has become troublesome because of changing agricultural technology. The aflatoxin problem in cereals is not restricted to any geographic or climatic region. Toxins are produced on cereals, both in the field and in storage. They involve both the grain and the whole plant (Hesseltine, 1974).

Most Farmers in the central, northern, southern and western parts of Ethiopia commonly store their sorghum seeds and grains in the above-ground bin locally known as *Gotera*, which is made of bamboo sticks, wood and/or mud (Mashila, 2004). However, the community of Amhara region in kewet woreda, the sorghum grain stored in flask-shaped traditional underground pit storage and above ground known as Gotera (Rik). The sorghum grain stored until it is consumed or sold when the grain market improves.

Underground pits storage are usually dug in sandy or stony soils with gentle topography and where there is less water drainage problem and less hazard of soil collapsing into the pits. Justifications are given by farmers for this type of storage includes protection from insect pests, fire, theft, and domestic and wild animals. The low cost of pit preparation, especially in places where wood for above-ground bin construction is not available, is mentioned as an economic reason. Farmers also believe that grain stored in underground pit is blessed by God and bountiful. The storage pits are mostly neither lined nor plastered with any material that would reduce moisture migration into the stored grain (Gilman, 1968).

The contact of the grain with wet inner pit walls often leads to moisture entrance into the inter-granular space elevating both the grain moisture content and the relative humidity inside the pit. Besides, respiration by insect pests, mainly *Sitophilus*, *Sitotroga* and *Tribolium species*, micro-fungi, other micro-organisms and the living grain itself produces high temperature and water activity (Mashilla *et al.,* 2004). Such a pit environment leads to moulding and grain deterioration (Shashidhar, Ramakrishna & Bhat, 1992). However, the sorghum grain stored in the underground pit storage is often mixed with collapsing soil from the walls and this affects its purity, market and nutritional values. It is also difficult to remove grains for consumption or sale frequently.

Likewise, Kewot woreda (Teri, Yelen, Rassa, Gerenefara and Ashege ena Yegeda kebele), the farmers are using under the underground pit storage system and above ground storage (*Gotera* are prepared from different plants, clay, grass, ash, and caw dung). These practices is firstly washing the underground pits with plane water and immediately the sorghum grain until they fill, then the cover with grass, clay and flatted stone. Some farmers are using cleaning, insecticides and fumigants to prevent insect damage and adding the sorghum grain in to the pits. Mostly farmers store this grain until the need for consumptions and market. The stored grain may stay for more than three years until the food scarcity is happen and market place improves. Therefore, this kind of storage practice may develop mycotoxin contamination.

Photo taken by the author

Plate 1. *Above ground storage of sorghum grain at kewet woreda*

Photo taken by Photo taken by the author

Plate 2. *Underground pit storage of sorghum at kewet woreda*

2.5. Mycotoxins

Mycotoxins are natural, toxic low molecular weight secondary metabolite compounds produced by microscopic filamentous fungi that can contaminate edible food grains (sorghum, wheat, maize, etc.), during harvest or storage and in some cases on commodities of animal origin (meat products, sausages) under a wide range of climatic conditions. Mycotoxins are harmful to human health when they are absorbed through consumption, inhalation, or dermal absorption. It has been shown that ingestion of contaminated food or feed is the main source of mycotoxin exposure to both human and animals (Ajoy *et al.,* 2010; Ilunga, 2012).

From 200 different filamentous fungi species, e.g. *Aspergillus, Penicillium and Fusarium species (sp.),* have been identified. Several hundred different mycotoxins have been shown to produce toxic compounds so far, exhibiting great structural diversity, which results in different chemical and physicochemical properties. Aflatoxins and ochratoxins (produced mainly by *Aspergillus sp.*), *fumonisins, trichothecenes and zearalenone* (produced by *Fusarium sp.), patulin* (produced by *Penicillium sp*.), and ergot alkaloids (produced in the *sclerotia of Claviceps sp.*) receive the most attention due to their frequent occurrence and their severe effects on animal and human health (Krska *et al.,* 2008).

2.5.1. Toxicity of mycotoxin

Mycotoxins are potent toxins and have a wide range of actions on animals and humans, e.g. cyto-nephro-toxin and neuro-toxic, carcinogenic, mutagenic, immunosuppressive and estrogenic effects. Although mycotoxicoses caused by direct consumption of contaminated food products and feed stuffs produce undesirable effects and poses the greatest risk in human or animals. The poisoning effects of the mycotoxins are very serious. It is estimated that approximately 25% of the world's cereals are contaminated with known mycotoxins, while a higher percentage could be contaminated with unidentified mycotoxins (Devegowda *et al.,* 1998; Krska *et al.,* 2008).

The effect of toxigenic fungi on grain products has the potential of leading to significant health hazards. The production of these compounds, especially on grains, is highly dependent on environmental factors (e.g., temperature and moisture content), pre and/or postharvest handling system. Thus, when changes in the weather occur, mycotoxins will be affected. Mycotoxins are

produced when conditions of high relative humidity (100% optimum) and mean temperatures of 19-21°c favor growth of the fungi. Different toxin-producing moulds may be present in an ingredient or feed at various concentrations. Under ideal growing conditions these moulds may produce toxins in only minute quantities, but their effects can be huge. It is climate-dependent, plant and storage-associated problems, and are also affected by non-infectious factors, e.g., the bioavailability of micronutrients, insect damage, and other attack from other pests, that are in turn determined by climatic conditions. Climate represents the key agro system that drives fungal colonization and mycotoxin production (Magan *et al.,* 2003).

2.5.2. The Genus *Aspergillus*

Aspergillius are a fascinating group of fungi exhibiting huge ecological and metabolic diversity. These include notorious pathogens such as *Aspergillus flavus*, which produces aflatoxin, one of the most powerful, naturally occurring, compounds known to man. These species are common and widespread (Machida and Gomi, 2010). One include the aflatoxigenic species *A.Flavus, A. parasiticus* and more recently *A.nominus* which cause serious problems worldwide in agricultural commodities, and the other includes the non-aflatoxigenic species *A.oryzae, A.sojae and A. tamri*, traditionally used for production of fermented food in Asia (Kumeda and Asao, 2001).

The genus *Aspergillus* includes over 200 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man. Among these, *Aspergillus fumigatus* is the most commonly isolated species, followed by *Aspergillus flavus* and *Aspergillus niger. Aspergillus clavatus, Aspergillus ustus, Aspergillus glaucus group, Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, and Aspergillus versicolor* are among the other species less commonly isolated as opportunistic pathogens (Clement *et al.,* 2013 and Rahul *et al.,* 2014). Among the different types of aflatoxins identified, the major members are aflatoxins B1, B2, G1 and G2 aflatoxins are fluorescence under the UV light. Aflatoxin B1 and B2 emit blue fluorescence where as aflatoxin G1 and G2 emit green fluorescence. The quantity and relative proportion of four compounds in culture extracts varies depending on mould strain, medium composition and culture conditions. Normally, aflatoxin B1 is present in largest amounts whereas B2 and G2 are produced in small quantity (Wogan, 1966).

Fungi, such as *A. oryzae,* involved in the industrial production of soy sauce and *A. niger* used for the production of citric acid and enzymes such as glucose oxidize and lysozyme. Such is the interest in *Aspergillus* that, to date, the sequences of fifteen different *Aspergillus* genomes have been determined. They are among the most successful groups of moulds with important roles in natural ecosystems and the human economy. In addition producing numerous useful extracellular enzymes and organic acids, these moulds also produce secondary metabolites of importance in biotechnology. Some *Aspergillus species* function as plant and/or animal pathogens. *Aspergillosis* is the name given to all animal diseases caused by growth of any member of the genus on a living host. Immunosuppression is generally a prerequisite for systemic *Aspergillus* infections in humans. The incidence of systemic *Aspergillosis*, the most serious form, is on the rise and imposes an increasing medical burden upon hospitals and physicians (Machida and Gomi, 2010).

Table 2. The color of the colony in various Aspergillus species

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

Source, Rahul *et al.,* 2014

2.5.3. Aflatoxins

Aflatoxins are a group of chemically related mycotoxins and are classified as B1, B2, G1, and G2. The most common and toxic of the aflatoxin group is B1. Aflatoxin was first identified in stored grain, as a product of the fungus *Aspergillus flavus*. It was later found that similar toxins

were produced by toxigenic strains of *Aspergillus parasiticus*. Aflatoxin has been found in wheat, corn, barley, oats, coconut oil and meal, cottonseed, cassava, dry pea meal and other raw food products (Bagley, 1997).

Aflatoxins are natural occurring secondary metabolites of storage fungi, extremely toxic, mutagenic, and carcinogenic poisonous produced by *Aspergillus flavus* and *A. parasiticus* are common contaminants in several staple crops produced in tropical and sub-tropical conditions around the world (Deiner *et a1.,* 1987; Kurtzman *et al.,* 1987; Goto et al., 1996; Peterson *et al.,* 2001). They have been found in mouldy human food and animal feeds and have been implicated in numerous animal disorders (Russell and Paterson, 2007). The aflatoxin group is comprised of aflatoxin B1, B2, G1 and G2. Its order of toxicity is B1 > G1 > B2 > G2. Letters 'B' and 'G' refer to its blue and green fluorescence colors produced by these compounds under UV light. Numbers 1 and 2 indicate major and minor compounds, respectively. *A. flavus* only produces B aflatoxins, while *A. parasiticus and A. nomius* also produce G aflatoxins (Fadia *et al.*, 2014 and Harish *et al*., 2013). In addition, aflatoxin M1 (AFM1), a hydroxylated metabolite of AFB1, is excreted in the milk of dairy cows consuming an AFB1-contaminated ration. Aflatoxin B1 is the most toxic in the group and widely familiar as the majority potent hepatocarcinogenic compound and along with other certain members of the group possess additional toxic properties including mutagenicity, tetrogenicity, acute cellular toxicity and it suppresses the immune system (Rajarajan, *et al.,* 2013).

Aspergillus flavus may be the predominant producer of the B group (Russell and Paterson, 2007). Moreover, aflatoxin M1 is usually considered to be a detoxication by product of aflatoxin B1 and it is also the hydroxylated metabolite present in animal products that eat foods containing the aflatoxin B1 toxin. M1 is cytotoxic and acute toxicity is similar to that of aflatoxin B1 and was classified in Group 2B as possibly carcinogenic to humans. Aflatoxin M1 is very slightly soluble in water, freely soluble in moderately polar organic solvents and insoluble in non-polar solvents, unstable to ultraviolet light in the presence of oxygen, pH (<3, >10) and oxidizing agents. On the other hand, it has an intensely fluorescent in ultraviolet light (Khalil *et al.,* 2013).

2.5.3.1. Chemical structure and physical properties 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. Aflatoxins B1, B2, G1 and G2, distinguished by their fluorescence color under ultraviolet light. The chemical structures of some aflatoxins are shown in figure 1. Crystalline aflatoxins are extremely stable in the absence of light and particularly UV radiation, even at temperatures in excess of 100ºC. A solution prepared in chloroform or benzene is stable for years if kept cool and in the dark (Wogan, 1965).

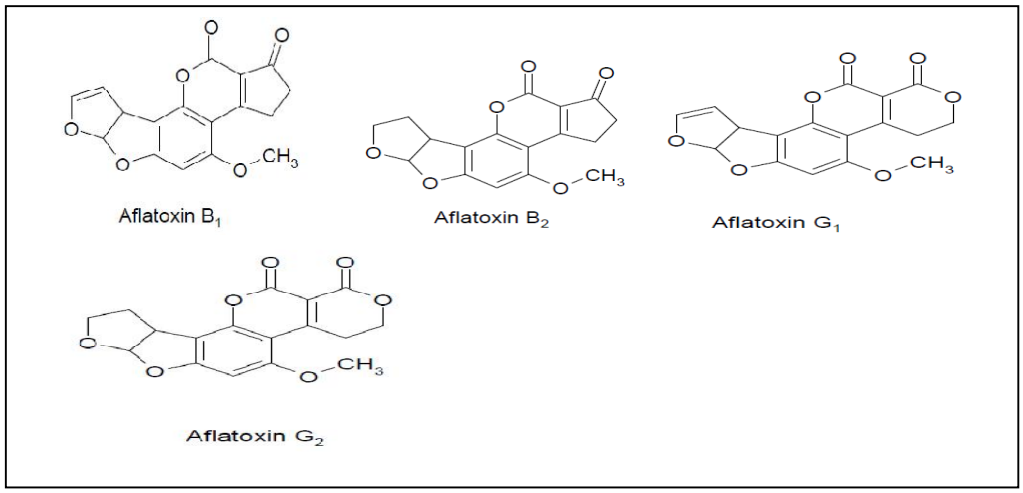


Plate 3. Chemical structure of Aflatoxins *Source; Cole and Cox, 1981.*

Aflatoxins consist of a group of approximately 20 related fungal metabolites, although only aflatoxins B1, B2, G1 and G2 are normally found in foods. Aflatoxins B2 and G2 are the dihydro derivatives of the parent compounds. Physical properties and spectral characteristics of aflatoxins summarized in Table 1.They are produced by at least three species of *Aspergillus, A. flavus, A. parasiticus* and *A.nomius*. *Aspergillus flavus* and aflatoxin are more likely in corn grown in the heat and drought stress associated with warmer climates. *Penicillium species* grow at relatively low water activities and low temperatures and are widespread in occurrence. Because both *Aspergillus* and *Penicillium* can grow at low water activities, they are considered storage fungi (Christensen *et al.,* 1977).

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

Property Aflatoxins	B1	B2	G1	G2	M1
Chemical Formula	C17H12O8	C17H14O8	C17H12O7	C17H14O7	C17H12O7
Molecular weight	312	314	328	330	328
Melting point (^{0}C)	268-269 (D)1	287-289 (D)	244-249 (D)	230	299 (D)
Sorbent Pentane, +	Chloroform	Chloroform	Chloroform	Ethyl acetate	Methanol
Fluorescence emission	425 nm	425 nm	450 nm	425 nm	425 nm

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

2.5.3.2. Factors influencing the Growth of *Aspergillus spp.* and Aflatoxins

The factor implicated in the growth of the fungus belonging to the *Aspergillus genus* in foods is as much as those relating to the environment in which they develop (PH, composition of the food or water activity) as to extrinsic factors; ambient humidity, storage temperature and microbial composition *(Zinedine and manes, 2009).*

Fungal spoilage of stored agricultural foods and aflatoxin production highly depends on several important factors including moisture content, relative humidity in the air and temperature in the environment. Although the optimal growth temperature ranges of mycotoxins production by various moulds is 25°C to 28°C and the species *A. flavus* can grow at optimal temperatures as low as 10-15^{0}C, at pH range from 2.1 to 11.2 and minimum water activity (aW) between 0.80 to 0.83. Even tough, constant temperature (25°C) is generally accepted as the temperature near the optimum for aflatoxin production (Habtamu and Kelbessa, 2001; Sweeney and Dobson, 1998).

Abundant factors affect the growth of *Aspergillus fungi* and the level of aflatoxin contamination in food. Such factors are typical weather of the region, the genotype of the crop planted, soil type, minimum and maximum daily temperatures, and daily net evaporation (Wilson and Payne 1994). *Aspergillus parasiticus* are a large genus of mould which grows at an optimal range of temperature of 28-33°C, at pH range between 3.5 to 8 and water activity from 0.83 to 0.99. Both *A .flavus and A. parasiticus* are occurs in warmer parts of the world such as tropical region where temperature and moisture are high. With regard to toxinogenesis, the temperature range in

which toxins are produced is from 12 to 40^{o}C, with the optimum between 25 and 30^{o}C (Sweeney and Dobson, 1998; FEHD, 2001).

2.5.4. Human Health Effect

Humans are exposed to aflatoxin primarily through the consumption of contaminated agricultural or animal products. Human exposure to aflatoxins is principally through contaminated foods. Inhalation of the toxins may also occur occasionally due to the occupational exposure (HKSAR, 2001). Human exposure to aflatoxin has a negative impact on health. Exposure can lead to acute or chronic aflatoxicosis, based on the duration and amount of exposure, and can compound existing health issues or the risk of disease transmission. For example, the first documented reports of aflatoxins in humans occurred in western India in 1974 where 397 persons were affected and 108 persons died in relation with ingestion of the moulded maize (Krishanamaachari *et al.,* 1975).

Aflatoxins are common mycotoxins in agriculture and food commodities causing serious health hazards to humans and animals leading to great economic losses (Priyanka *et al.,* 2012).The introduction of aflatoxin can lead to several health-related conditions including acute and chronic aflatoxicosis, aflatoxin-related immune suppression, liver cancer, liver cirrhosis, as well as nutrition-related problems in children such as stunted growth. In many areas, due to widespread high-level consumption, aflatoxin contamination through food and feed is unavoidable due to the absence of alternative food and feed resources. When ingested, aflatoxin binds to liver proteins. The metabolic products may persist for 2 to 3 months or longer and can be detected through blood tests (Jolly *et al.,* 2008).

Acute Aflatoxins poisoning is caused by ingestion of high levels of aflatoxin contaminated food and can cause immediate consequences are severe liver damage, acute jaundice and hepatitis, which subsequently may result in death (Bennet and Klich, 2003). Aflatoxicosis also causes induction of tumors, impaired central nervous system, and skin disorders, and hormonal defects and decreased bone strength (Saleemullah *et al.,* 2006). Therefore, Ingestion of 2-6 mg/day of aflatoxin for a month can cause acute hepatitis and death (Patten, 1981).

Recently published results indicate that aflatoxin exposure is a relationship exists between aflatoxins and stunting. Stunting is important from a public health perspective because it is associated with effects such as increased vulnerability to infectious diseases and cognitive impairments that last well beyond childhood. In the past 20 years stunting in children under five has decreased, yet still remains a significant public health challenge in much of the developing world. It is higher in south Asia and sub-Saharan Africa than elsewhere in the world, for example, globally it affects at least 163 million children (Black *et al.,* 2013).

Aflatoxin has extensively linked to Kwashiorkor; a disease usually considered a form of malnutrition; a protein energy deficiency of young like marasmus but unlike it. The attachment of aflatoxin B1 is a major damage to the liver DNA by producing various epoxides. several serum proteins that are vital for the body's overall growth are produce in the liver hence the distraction of liver cells leads to a decrease in the protein manufactured , ultimately ending in Kwashiorkor due to protein deficiency (Bhattacharyya, 1986;Hendrickse *et al.,* 1983).

2.5.4. Economic Impact of Aflatoxins

The economic impact of aflatoxins was derived directly from crop, livestock losses, and indirectly, from the cost regulatory programs designed to reduce risks to animal and human health. The Food and Agriculture Organization (FAO) estimates that 255 of the world's food crops are affected by mycotoxins, of which the most notorious are aflatoxins (WHO, 2006). Aflatoxin contamination can result in direct economic impact through export rejections from importers with stringent aflatoxin regulations such as European Union (EU) countries. Between 2007 and 2012, the EU alone has issued 346 notifications to African countries (PACA, 2012).

Aflatoxins losses to livestock and poultry producers from aflatoxin contaminated feeds include death and the more subtle effects of immune system suppression, reduced growth rates, and losses in feed efficiency. Other adverse economic effects of aflatoxins include lower yield for food and fiber crops (Anon, 1989). FAO estimates that many basic foods could be contaminated with mycotoxin producing fungi, contributing to huge global losses of foodstuff, about 1000 million metric tons each year (Bhat *et al*., 2010).

2.5.5. Global Regulation for Aflatoxins in Foods

Mycotoxins are toxic to humans and animals when present in food and feed above tolerance limits. Reduction in mycotoxin exposure of humans and animals has necessitated regulatory interventions by some national governments and international agencies. Conventional estimates of lost crop revenues, and the cost of research and monitoring activities, range from $0.5 to $1.5 billion annually. To achieve current global regulatory compliance in mycotoxin safety levels farmers also have to spend more effort and assume higher risk. Regulatory levels vary between countries and, as a result, mycotoxins act as Technical Barrier to Trade and have led to trade disputes between nations. While food production, processing and marketing systems of developed economies have been largely successful in delivering mycotoxin safe products, regulations have been ineffective in most less-developed countries due to socio-economic, technical, institutional and policy factors. Agricultural products of the best quality are exported from less developed countries to augment export earnings, leaving poorer quality foods to be consumed locally, compromising the health of local populations. Wherever regulations cannot be implemented, the key management options for consumers and farmers are awareness and adoption of simple mycotoxin management practices (Bandyopadhyay *et al*., 2007).

The maximum levels of aflatoxins (aflatoxins B1, B2, G1, G2 and M1) in foodstuffs are laid down in Commission Regulation (EC) No 1881/20064 as amended by Commission Regulation (EU) No 165/20105 and Commission Regulation (EU) No 1058/20126. Maximum levels in legislation are specified for aflatoxin B1, aflatoxin M1, and for the sum of aflatoxins B1, B2, G1 and G2 (EFSA, 2013).

Aflatoxins have been recognized as significant contaminants by the agricultural production community since the 1960s and control strategies have mostly eliminated harmful exposures in developed countries (Guo and Widstrom *et al*. 2000). Several institutions around the world have classified and regulated Aflatoxins in food. The European Union (EU) has the most rigorous regulations concerning mycotoxins in food. The limits of AFB1 and total AF in foods are 5 µg/kg in AB1 and 10 µg/kg in total aflatoxin , respectively, in more than 75 countries around the world whilst they are 2 µg/kg in AFB1 and 4 µg/kg in total aflatoxin in the European Union (EU) (Herzallah, 2009).The US Food and Drug Administration (FDA) have established acceptance

level of 20 ppb for aflatoxin in maize for human consumption, with the level in milk being even lower (0.5 ppb) (Grybauskas *et al*., 2000).

However, different countries have different accepted levels for aflatoxins in foods. It is now realized by many developing countries that reducing aflatoxin levels in foods will present international trade advantages as well as offer long-term health benefit to the local population. The new European standards of aflatoxin level in groundnuts and cereals means that effective control must be found by developing countries for them to continue to export to the attractive European Union markets. For example, (Bankole and Adebanjo, 2003; Ayalew,2010) have proposed many solutions against aflatoxin production in food, and some of the strategies which may be applicable in West Africa by education and extension programs on radio and television discussion, food safety system, critical control (HACCP) system to produce food free of hazards. Strict food safety regulations implemented in most countries demand aflatoxin contamination less than 20 ppb. Despite availability of wide variety of diagnostic tools for estimation of aflatoxins, their utilization in most developing countries is limited due to high cost, difficulties with importation and lack of appropriate laboratory facilities and human skills (Kumar, *et al*, 2008).

There is currently harmonized European commission (EU) legislation on mycotoxins. In England this legislation is implemented by the contaminates in food (England) regulations 2003 (S.I.2003 No.1478), and sets limits for aflatoxins in food including maize, peanut and edible nut products. Parallel legislation exists for Scotland, Wales and Northern Ireland. The regulatory limits are 2μg/kg for aflatoxin B1 and 4μg/kg for total aflatoxins (Judy, 1998).

National and international institutions and organizations, such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations, have recognized the potential health risks to animals and humans posed by food and feed-borne mycotoxin intoxication and addressed this problem by adopting regulatory limits for major mycotoxin classes and selected individual mycotoxins (Krska *et al*.,2008).

On the other hand, to protect consumers and farm animals, regulatory limits have been adopted. National and International institutions and organizations such as the EC, the US Food and drug

Administration (FDA),WHO and FAO have recognized the potential health risks to animals and humans posed by consuming aflatoxin-contaminated food and feed. In an attempt to harmonize the current tolerances to aflatoxin which exist in different countries, the working group on mycotoxins of the WHO and FAO proposed maximum limits of 15µg/kg for total aflatoxins in raw ground nuts based on a sample size of 20kg (Bhat et al.,1997).

The potential economic problems associated with the level of 10µg/kg and the public health implications of a level of 15µg/kg, as compared to 10µg/kg for aflatoxin in foods, are two main issues in the setting of maximum levels for aflatoxins in groundnuts intended for further processing. Many countries considered the level of 15µg/kg to be a reasonable that could be achieved by producing countries, thus facilitating international trade, considering that a lower level would constitute a trade barrier due to the finding of the education of joint FAO /WHO Expert Committee on Food Additives (JECFA) that this may not offer a significant improvement, in public health. However, genotoxic properties of aflatoxin, uncertainties in risk assessment, the As Low As is reasonable Achievable principle, and inadequate data on the effect of a level of 10µg/kg on the availability of ground nut in world market support the continued consideration of lower level (Anna,2012).

Codex Alimentarius commission and the JECFA have adopted the maximum limit for total aflatoxins as 15µg/kg in raw peanut and 20 µg/kg for sorghum grains. This issues of food safety in Africa is one which interact with and is frequently subject to issues of food security, especially in geographic areas where food shortages are caused by recurrent natural whether phenomena such as drought. In addition, many subsistence farming communities in Africa are reliant on the consumption of home-grown crops, irrespective of the quality considerations normally applied in the developed world. Nevertheless, some African governments have instituted food safety regulations to control mycotoxin, especially aflatoxin, contamination of the national food supply and research into natural occurrence of aflatoxins in a range of local foods is Widely conducted (RASFF,2011).

East African sorghum grains specification standard (Reference No. ARS 462), sated to the sorghum grains shall comply with those maximum mycotoxin limits established by the Codex Alimentarius Commission for this commodity. In particular, total aflatoxin levels in sorghum

grains for human consumption shall not exceed 10µg/kg with B1 not exceeding 5µg/kg when tested according to ISO 16050 (ARSO, 2012).

2.6. Analytical Methods for Aflatoxin Determination in Food.

There are various analytical methods to determine aflatoxin in food. For example ELISA, TLC, LCMSMS and HPLC which are significantly different based on performance, sensitivity, and accuracy.

ELISA

ELISA is widely used in food and feed industries to determine the content of aflatoxin in food products. Though the ELISA is accurate stable and reproducible, the analysis sometimes shows false positive, or false negative due to enzyme instability and variations of enzymes reaction conditions. So the application of this method in analysis of aflatoxin remains to be improved in future (Stubblefild; 1987; Akiyama et al., 2001).

TLC

Thin Layer Chromatography is a chromatography technique used to separate mixtures, which is performed on a sheet of glass, plastic, or aluminum foil coated with a thin layer of adsorbent material usually silica gel, aluminum oxide, or cellulose. It can be used to monitor the progress of molecule migration to identify compounds present in a given substance and to determine the purity of a substance. TLC can also be used on a small semi-preparative scale to separate mixtures of up to a few hundred milligrams. The mixture is not “spotted” on the TLC plate as dots, but rather applied to the plate as a thin even layer horizontally to and just above the solvent level. For small-scale analysis, TLC can be far more efficient in term of time and cost than chromatography. To analyze the amount of aflatoxin in samples by TLC, the small-scale target can be visible at UV light under 365 nm wavelength. Therefore TLC analysis is often affected by many factors; the accuracy of this method is poor. With the improvement of extraction and isolation method as well as the application of new reagents, the TLC detection becomes a simple and widely used analysis method (Stubblefild; 1987; Akiyama et al., 2001).

HPLC

For Aflatoxin analysis several methods have been developed over the last 30 year. Because of the advance in technology, higher separation power, the better clean up prouder and the combination of both a higher sensitivity has been registered. High performance liquid Chromatography with Florescence detection becoming the most used analytical methodology in Laboratory. Other advance have regard the environment, as the replacing of chlorinated solvents, preferred in the past, by aqueous mixture of methanol and acetonitrile, that are more compatible with antibodies, recently introduced in many applications. These reagents marked a turning point in the sample preparation step as well as in the identification phase, showing a high flexibility in many practical situations in which reliable, rapid and simple analyses are required to reduce the costs (Anna, 2011).

HPLC using florescence detection has already become the most accepted chromatographic method for the determination of aflatoxins. For its specificity in the case of molecules that exhibit florescence, Commission Decision 2002/657/EC, concerning the performance of analytical methods, considers the HPLC technique coupled with florescence detector a suitable confirmatory method for aflatoxin identification. Liquid chromatographic methods for aflatoxin determination include both normal and reverse-phase separations, although current methods for aflatoxin analysis typically rely upon reverse-phase HPLC, with mixtures of methanol, water, and acetonitrile for mobile phases. Aflatoxins are naturally strongly florescent compounds, so the HPLC identification of these molecules is most often achieved by florescent detection (Stubblefild; 1987; Akiyama et al., 2001). Therefore, it is more preferable to analyze aflatoxin from sorghum sample by HPLC.

3. Materials and Methods

3.1. Description of the Study area

The study was conducted in *Kewet* Woreda; Teri, Yelen, Rassa, Gerne fara and Ashgnena yegeda Kebles which are found in North Showa Zone of Amhara Region at about 225 km to the north of Addis Ababa along the main road to Dessie.

Kewet woreda is a lowland and semi-arid area that lies at an altitude ranging between 1280 and 2700 meters above sea level. It is located at longitude and latitude of 10°00′N 39°54′E and 10.000°N 39.900°E, respectively. The average annual rainfall is around 600-700mm and temperature of 17 to 30°C. The reason for selecting this area was that farmers in the area are the major sorghum producers and also consumers of sorghum grain which is being stored under different traditional storage system and for long period of time. The vast majority of people are rural small holding who depend on cultivation of 16,046 hectares of arable land. Based on the 2007 national census conducted by the Central Statistical Agency of Ethiopia (CSA) rural Kewet woreda has a total population of 100,760. The study areas were indicated in plate 4.

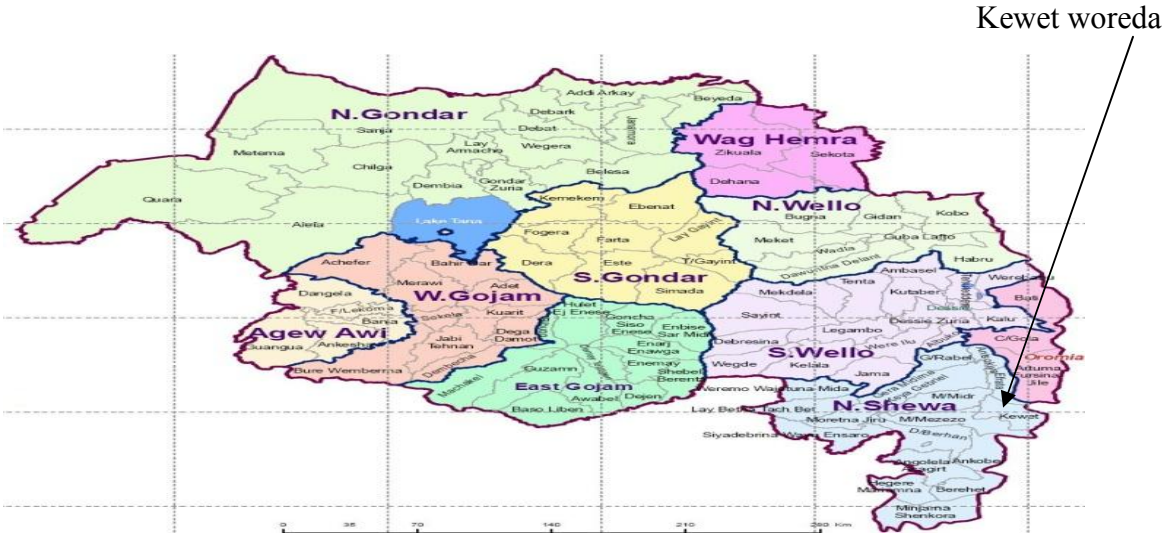


Plate 4: Map of the area where the samples were collected: Kewet Woreda found in North Showa region of Amhara.

3.2. Apparatus and reagents

SHIMADZU-HPLC system consisting of auto sampler, pump, column oven, florescence detector, PC with chromatography software, HPLC column (C18), weighing balance, refrigerator, spatula, Beakers (10ml, 25ml,100ml, 250ml and 500ml), single use syringes 5ml (pp), 10 ml (pp), 0.45 µm filter paper, centrifuge, concentrator, micropipette, Vortex and other necessary laboratory glass wares and equipments were used for the analyses of the Aflatoxin content in sorghum grain.

The chemical and reagents used for aflatoxin analysis were HPLC grade Acetonitrile, Methanol, n-hexane, MgSO4 anhydrous salt, NaCl, Aflatoxin standard and deionized water. The AFG1, AFG2, AFB1 and AFB2 standards were obtained from sigma-Aldrich (Ref A6636-118k4094). The pure reference standards were stored in dark place at 4^{o}c. Standard solution of all four aflatoxins were prepared in methanol and stored at 4^{o}c for up to 6 month. The *Aspergillus* species isolation and identification equipments and chemicals utilized were incubator, Petridis, Microscope, and Potato Dextrose Agar (PDA), 10% sodium hypochlorite solution, and ethanol absolute (99.7%), respectively.

3.3. Study Design

Nested study design was followed to see the incidence of *Aspergillus species* and Aflatoxin in Sorghum stored for different storage system and periods.

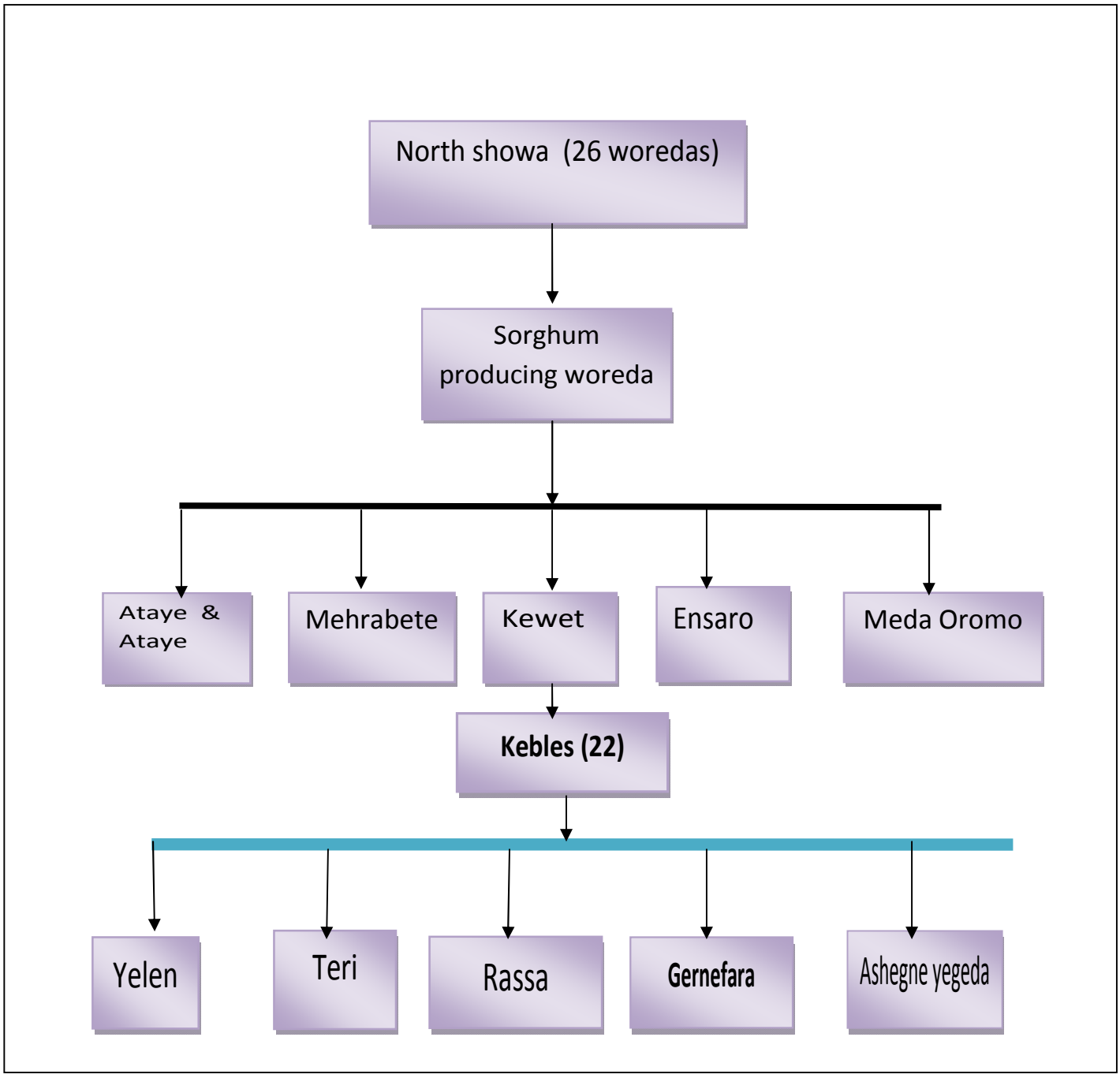


Plate 5. Nested design of sampling flow chart

In plate 5, North showa Zone contained 26 woreda and out of each woreda *Ataye, kewet merahbete, medaOromo and Ensaro* are high in sorghum production area. Based on the storage system and periods and production rate *kewet* woreda was selected. 5 Kebles were selected (*Rassa, Ashgne yegeda, Gerenefara, and Teri and Yelen*) according to sorghum storage system and production. From each of Kebles six role model farmers were proposed and selected on their production rate and one kilogram sorghum sample were collected from each role model farmers.

3.4. Sampling and sample preparation

Sorghum samples were collected and information was gathered from the study area by using questionnaire (annex1). The questionnaire was targeted on educational level, mould contamination, mould color, drying method, storage system, storage time, location of storage, cleaning and sanitation, critical problems and measure taken to control the problems, if any. The role model farmers were also chosen purposively based on the information given by the DA (Agricultural Developmental Agents) workers. The farmers were interviewed by means of one to one correspondence.

In this study, a total of 30 samples of sorghum (15 samples from each storage type or 10 samples per each storage period) were collected purposively by taking the storage periods (< 12 month, 1-2 year and ≥ year) and storage system (above ground and underground pit storage systems) into consideration. The samples were collected in plastic bags by taking preventive measures to avoid adventitious contamination and were transported to the laboratory of food science and nutrition at Addis Ababa University. The sorghum grains were separated from foreign matter, 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. However, whole grains of sorghum were utilized for *Aspergillus species* isolation and identification.

A. above ground (Gotera) storage *B. Underground pit storage*

Photo taken by the author

Plate 6. Diagram of sample collation from Kewet woreda

Plate 6 indicated that, about 1kg of sorghum samples were collected from the aboveground storage and underground pit storage system from each role model farmers.

3.5. Method Adaptation

3.5.1. Selection of HPLC-FLD

A reliable and easy method has been developed for the determination of aflatoxins in sorghum samples. High performance liquid chromatography coupled with FLD (HPLC-FLD) without post-column derivitization was used. Aflatoxin was extracted from sorghum using a QuEChERS-based extraction procedure. The optimized analytical conditions were evaluated in terms of recoveries, reproducibility, LOD, LOQ and linearity for aflatoxin (AFG2, AFG1, AFB1 and AFB2) in sorghum. Extraction, chromatographic and detection conditions were optimized in order to increase sample sensitivity.

3.5.2. Preparation of standard solution

Each of the standard reagents, aflatoxins (AFG2, AFG1, AFB2 and AFB1), was dissolved in acetonitrile and methanol (50:50) at 1mg/mL and stored at 4°C in the dark before use. To prepare the working standard for HPLC analysis, each aflatoxin stock solution was equally pipetted and transferred to a vial, and it was then diluted with the mobile phase.

3.5.3. Chromatographic conditions

The mobile phase consisted of Milli Q water (100%) and acetonitrile (CAN) / methanol (MeOH) in the ratio of 71.5/28.5, v/v at a flow rate of 1.0 ml min^{-1}. Zorbax SB RP C-18 column (4.6 x250mm, 5µm particle size) was used as the stationary phase. Wavelength range of 365 and 440 nm was selected for florescence detection. An injection volume of the sample was 20µl. The HPLC –FID gradient elution profile was indicated to in table 4.

Table 4. HPLC gradient elution profile

	Water (Milli-Q)	**MeOH/ACN (71.5/28.5, v/v)**
Time (minutes)	75	25
1	75	25
12	60	40
25	50	50
28	0	100
30	0	100
32	75	25
35	75	25

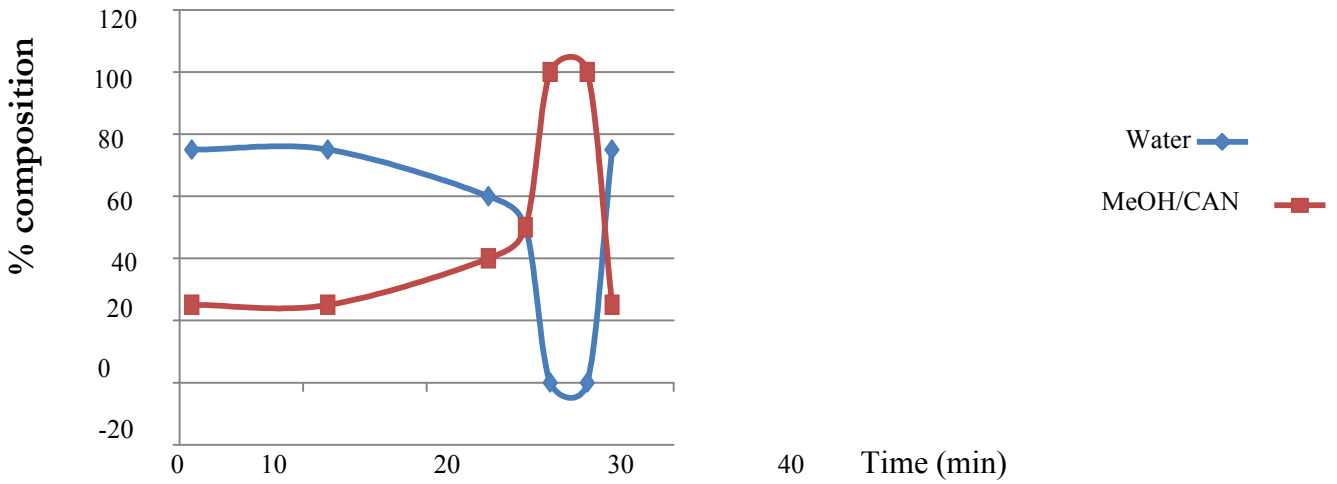


Figure 1. Graph of gradient elution profile of mobile phase

In figure 1 indicated that, the mobile phase adjusted from the above mentioned in table 4 with HPLC chromatography that the graph was performed. This showed to the working condition of four aflatoxins standard (AFB1, AFB2, AFG1 and AFG2) were occurred with respect to the retention time between 19 min and 35.1 min.

3.5.4. Validation

According to (CAC/G l 71-2009), the process of method validation is intended to demonstrate that a method is *fit-for-purpose*. This means that in the hands of a properly trained analyst using the specified equipment and materials, and following the procedures described in the method, reliable and consistent results can be obtained within specified statistical limits for the analysis of a sample.

3.5.5. Working and linear range selection

The working ranges were selected after the standard solutions to 100% (v/v) were measured. The linear range of detectability that obeys Beer's Law is dependent on the compound analyzed and detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The individual aflatoxin mixture was prepared AFB2, AFG2, AFB1 and AFG1 at concentration of 0.2µg/mL, 0.2µg/mL, 2µg/mL and 1µg/mL, respectively from the stock solution (100ppm) of total aflatoxin in methanol and acetonitrile. Then the dilution was prepared

in a volume (5µL, 10µL, 20µL, 40µL and 80µL) with 25 ml volumetric flask and make up by mobile phase (acetonitrile, water and methanol). The calibration curves were obtained with concentration of 1-16 ppb of AFG2, 5-80 of AFG1, 1- 16 ppb of AFB2 and 10 - 160 ppb of AFB1.

Each solution was injected as HPLC conditions described above and the data obtained from peak area versus each aflatoxin was treated by linear least square regression analysis. The correlation coefficient (R^2) was calculated for a standard solution and the minimum linearity was (R^2) ≥ 0.998. The standard curves were evaluated for reproducibility. Quantification of the actual aflatoxin in (ppb µg/L or µg/Kg) is based on the following formula (AOAC 2005.08):

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

Where:

A = ng of aflatoxin as eluated

T = final test solution eluate, volume (µL)

I = volume eluate into Lc (µg)

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

3.5.6. LOD and LOQ

Limit of detection is the lowest concentration of an analyte that can be detected in a sample, but not necessarily quantitated under the stated experimental conditions of the test whereas Limit of Quantititation refers to the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions of the test. The lowest concentration in a sample that can be detected, but not necessarily quantified, under test condition were determined the aflatoxin concentration yielding a signal to noise ratio of at least 3:1 (S/N ≥ 3). The limit of detection was measured for all 4 Aflatoxins by injecting standard solutions of different concentration and measuring for each substance the amount injected on the column and the S/N values.

The signal to noise ratio was determined by the equation:

S/N= 2H/h

Where:

H: height of the peak corresponding to the component

h: absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

3.5. 7. Accuracy and Precision

Precision quantifies the variation between replicated measurements on test portions from the same sample material. Precision of a method is usually expressed in terms of the within-laboratory variation (*repeatability*) and the between-laboratory variability (*reproducibility*) when the method has been subjected to a multi-laboratory trial. For a single laboratory method validation, precision should be determined from experiments conducted on different days, using a minimum of six different tissue pools, different reagent batches, preferably different equipment, etc. and preferably by different analysts. Precision of a method is usually expressed as the standard deviation. Another useful term is relative standard deviation, or coefficient of variation (the standard deviation, divided by the absolute value of the arithmetic mean). It may be reported as a percentage by multiplying by one hundred.

3.5.8. Recovery

Recovery is usually expressed as the percentage of analyte experimentally determined after fortification of sample material at a known concentration and should be assessed over concentrations which cover the analytical range of the method. The recovery of standards was processed with every batch sample. The sample was used to assess recovery with value between 60 and 120 % classed as valid (RASFF, 2011). Accordingly, the extraction recovery of AFB2, AG1, AFB1 and AFB2 from the HPLC Florence detector was measured by spiking the known 1.0g of maize sample in mix aflatoxin standard. The recovery was determined by using Eq. (1).

$$\% \text{ recovery} = \frac{\text{TOTAL ANALYTE FOUND} - \text{ANLYTE ORIGINALLY PRESENT}}{\text{ANALYTE ADDED}} \text{ X } 100 \qquad \ldots\ldots\ldots\ldots (1)$$

3.6. Method Adaptation for Analysis of Aflatoxin

In order to perform the study on aflatoxin contamination level in the 30 sorghum samples, methodology was performed as described by the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe), by extraction with Milli Q water (100%): methanol/acetonitrile (71.5/28.5, v/v), MgSO4 and NaCl, followed by centrifugation and filtration, and the quantification was carried out by HPLC-FLD, without derivitization.

The HPLC system, using a florescence detector (SHIMADZU, Japan), Rheodyne injector (20 µL), C18 column (250 mm x 4.6 mm, 5 µm) and mobile phase consisted of Milli Q water (100%) and acetonitrile (CAN) / methanol (MeOH) in the ratio of 71.5/28.5, v/v at a flow rate of 1.0 ml min^{-1}. The parameters selected for method adaptation were linearity, specificity, and accuracy, limit of detection and quantification and precision.

3.6.1. Repeatability and Reproducibility

A typical HPLC chromatogram showing the clear separation of 1 ppb standard mixture of four aflatoxins with respect to retention time G2 (19 min), G1 (21 min), B2 (23.5min) and B1 (25.8min) and the standard curve, black sample and mould infected sample was described in Figure 2 and 3 below.

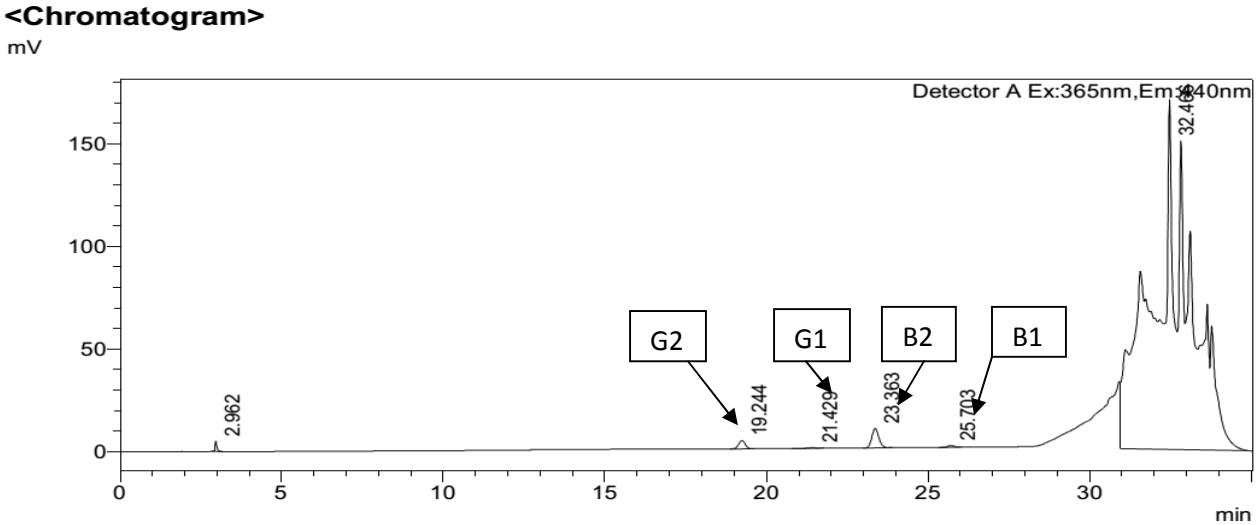


Figure 2. HPLC Chromatogram of 1 ppb standard mixture of four aflatoxins

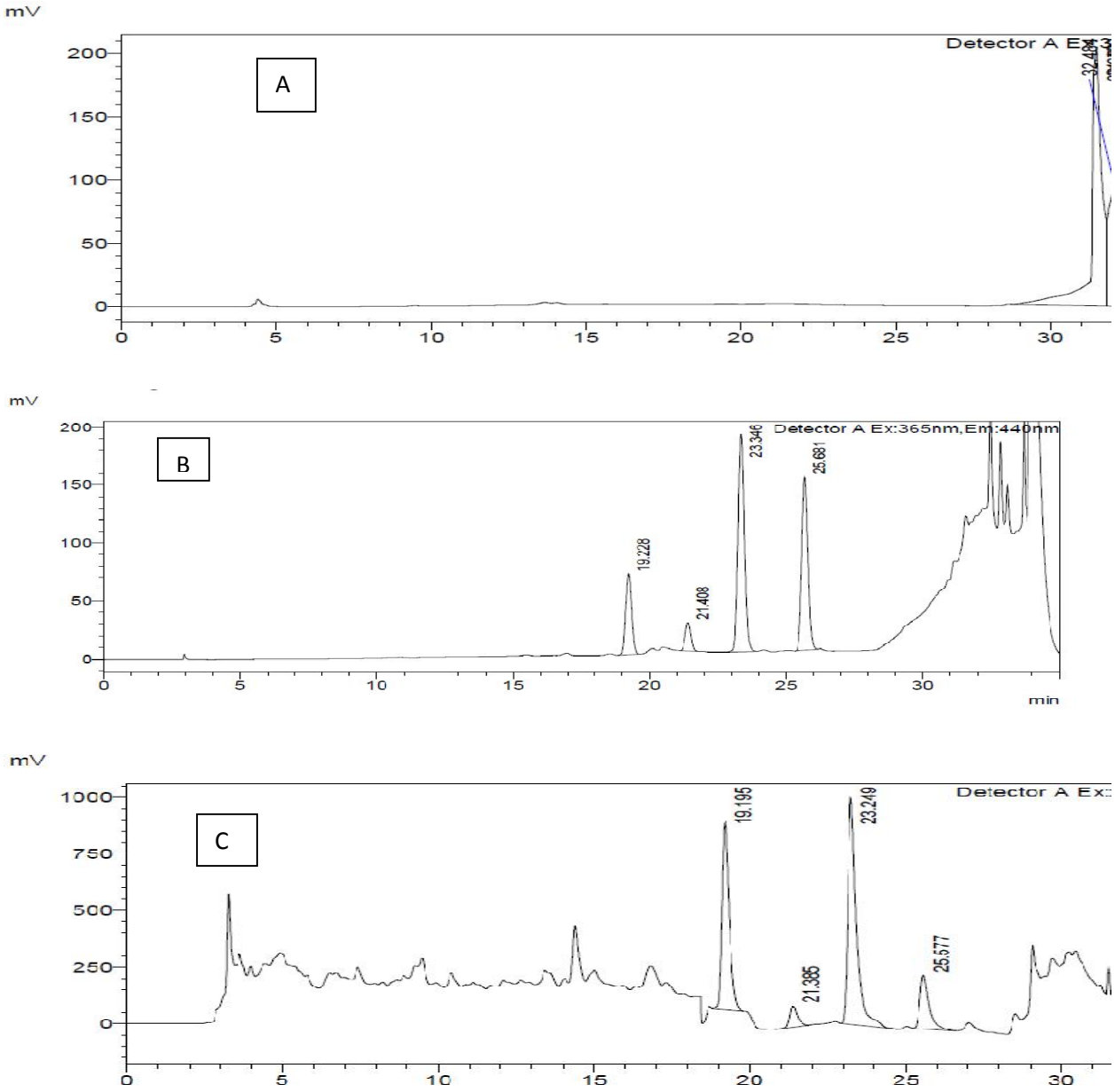


Figure 3: Chromatogram of blank sample (A), standard calibration curve (B) and naturally contaminated fungal sample (*peanut*) (C) (elution order AFG2, AFG1, AFB2 and AFB1).

In table 5, indicates, reputability and reproducibility of the method was assessed by performing the precision of experiments. Each test was performed five times and relative standard deviation for repeatability (% RSDr) and Reproducibility (% RSDr) were described as in terms of area and retention time. For reputability test lower concentration (G2=1, G1=5, B2=1and B1=10) and higher concentration (G2=8, G1=40, B2=16 and B1=80) of the aflatoxins standard injecting to HPLC and the test was performed five times for the same sample with the same day. Similarly

for Reproducibility test, but the test was performed five times for the same sample with different five day. Relative standard deviations for both retention time and peak area of five replicates were less than 5%, which showed high precision for manual injection.

Table 5. Repeatability and Reproducibility of HPLC method used for aflatoxin determination.

Characteristics	**Spike level(*µ*g/kg)**	**Area/Ret. time**	**AFG2**	**AFG1**	**AFB2**	**AFB1**
Repeatability, RSDr (%)	G2=1,G1=5 B2=1and B1=10	Area	3.26	0.75	0.82	3.39
		Retention time	0.05	0.06	0.05	0.05
	G2=8,G1=40 B2=16 and B1=80	Area	0.50	5.13	1.12	5.30
		Retention time	0.23	0.24	0.21	0.19
Reproducibility, RSDr (%)	G2=1,G1=5 B2=1and B1=10	Area	1.37	2.18	1.39	5.5
		Retention time	0.15	0.15	0.16	0.15
	G2=8,G1=40 B2=16 and B1=80	Area	0.85	5.04	1.48	5.25
		Retention time	0.04	0.03	0.03	0.03

3.6.2. Recovery, Limit of detection (LOD) and Limit of quantification (LOQ)

The method was adopted for the determination of aflatoxins B1, B2, G1, and G 2 in sorghum sample. The percentage recoveries were found to be, 82.8% for AFG2, 92.1% for AFG1, 97.5% for AFB2 and 98.3% for AFB1 as shown in Table 6. A reasonably high recovery of the most important aflatoxin components (AFB1 and AFG1), as high as 97.6%, through spiking maize sample, depicts that the method used is efficient and can be employed successfully for the reliable analysis of aflatoxins in sorghum sample. Accuracy was determined by the determination of the recoveries of aflatoxins. Standard aflatoxin solutions (diluted with mobile phase) were spiked of 40µl/ kg (B1), 4µl/ kg (B2) and (G2) and 5µl/kg (G1) with 1g of maize samples; Replicate analysis of each spiked sample was used to determine the accuracy, expressed as mean (µl/ kg) ± relative standard deviation (%).These values fall well within the range of 70 to 125%, which were acceptable according to AOAC International guidelines for method validation (Loh Saw *et al*., 2010)

Table 6. Recovery test of aflatoxin from maize sample

Aflatoxin	Aflatoxin concentration in sample (µg/kg)	AF level Added (µg/kg)	Result found (µg/kg)		Replicate % recovery		Average recovery %	RSD %
AFG2	0.000	4	3.81	3.560	95.25	89.03	92.1	4.8
AFG1	12.280	5	14.05	14.56	81.31	84.26	82.8	2.5
AFB2	0.000	4	4.051	3.750	101.3	93.75	97.5	5.4
AFB1	13.878	40	53.357	52.53	99.03	97.50	98.3	1.1

In table 7, indicates, the estimated detection limit of detection (signal to noise ratio = 3) and LOQ (signal to noise ratio = 10) in each aflatoxin (Table 7), 0.03 µg/kg for AFG2, 0.3µg/kg for AFG1, 0.014µg/kg for AFB2 and 0.15µg/kg for AFB1 was detected in a sample. The limit of quantification of each aflatoxin 0.11µg/kg for AFG2, 1.28µg/kg for AFG1, 0.05µg/kg for AFB2 and 0.50µg/kg for AFB1 was determined with acceptable precision and accuracy under the stated experimental conditions of the test. The aflatoxin contents of the samples were calculated based on the best fit equation obtained from the standard calibration curves.

Table 7. LOD, LOQ and calibration curve of HPLC for aflatoxin determination

Aflatoxins	LOD (µg/kg)	LOQ (µg/kg)	Calibration curve	r^2
AFG2	0.03	0.11	Y=59669X+55234.4	0.998
AFG1	0.3	1.28	Y=4689.08X-7929.18	0.998
AFB2	0.14	0.05	Y=181744X-34972.8	0.998
AFB1	0.15	0.05	Y=14710.2X-32648.6	0.9988

3.6.3. Linearity range

Five-point graphs were obtained with concentration of 1-16 ppb of AFG2, 5-80 of AFG1, 1- 16 ppb of AFB2 and 10 - 160 ppb of AFB1. Calibration graphs were drawn by linear regression of the least-squares method using the peak area of standard as response versus concentration as

shown in Figure 3. The correlation coefficients were >0.998, which was considered as evidence of an acceptable fit of the data to the regression line (Loh Saw *et al*., 2010).

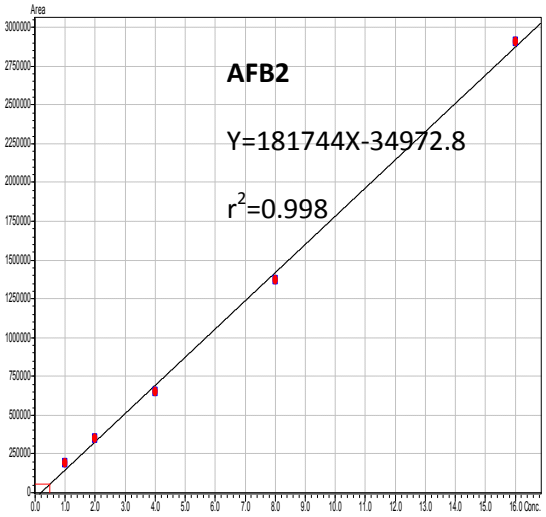


Figure A. calibration curve of aflatoxin Standard B2

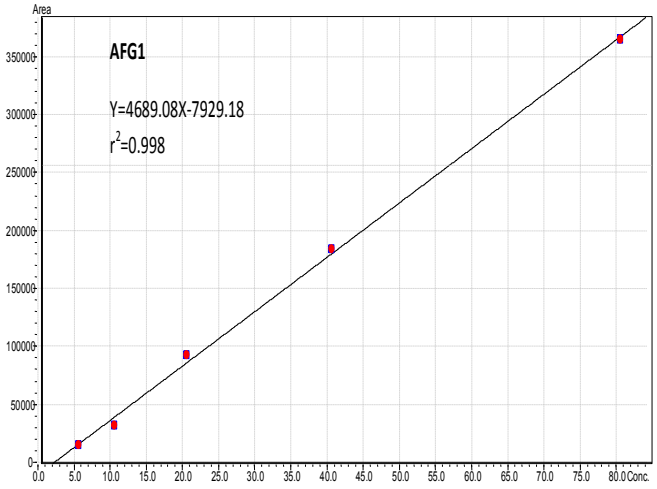


Figure B. calibration curve of aflatoxin standard G1

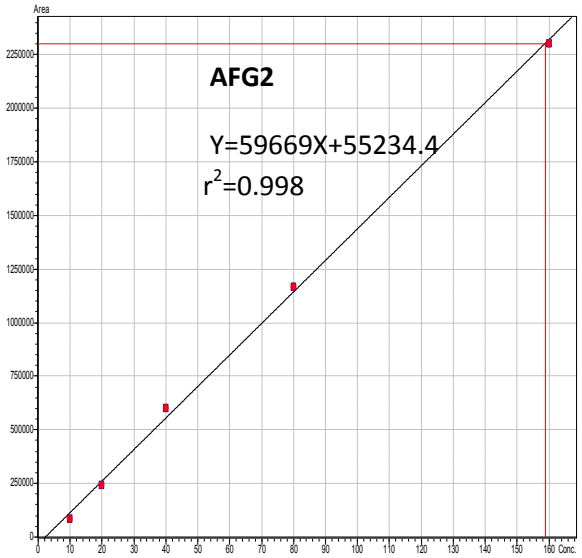


Figure C. calibration curve of aflatoxin Standard G2

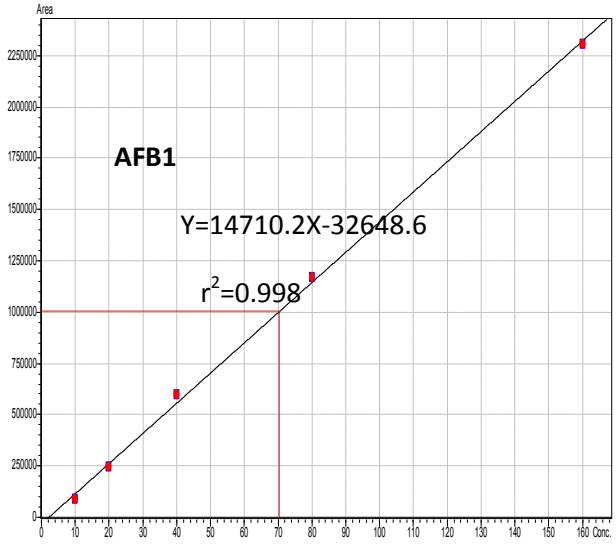


Figure D. calibration curve of aflatoxin standard B1

3.7. Determination of Moisture Content

Moisture content was determined according to AOAC (2000) using the official method 925.09. A crucible was dried in an oven at 105^{o}C for 1 hour and placed in desiccators to cool. The weight of the crucible (W1) was determined. 5gm samples was weighed in the dry crucible (W2) and dried at 105^{o}C for 3 hours and after cooling to room temperature in desiccators it was again weighed (W3). The moisture content was determined by using Eq. (2).

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

3.8. Determination of Aflatoxins

Sample Extraction and Clean-up

Extraction of aflatoxin from the sorghum samples were performed according to the QuEChERS method that was validated by Ghent University faculty of Pharmaceutical science, Laboratory of Food Analysis, Belgium, Europe (2014).

1.0 g of sorghum flour sample was accurately weighed and 5ml of water was added, vortex briefly and stand to for 30 minute. 5 ml of extraction solvent (100% ACN) was added and briefly mix was using a vortex mixer and shake for 30 minute at using shaker. Pre-weighed 2.0 ± 0.05 g of MgSO4 anhydrous salt and NaCl 0.5 ± 0.01 g were added and cap the tube immediate. Shake immediately to the tubes briefly to prevent agglomeration of the salts and vortex for 2 min, centrifuge at 4000 g for 15 min. then transfer 4 ml of the top organic layer to a new tube and evaporate under N2 at 40°C and 200µL of injection solvent (A: B 50/50) and 200µL of n-hexane were added to the residue and vortexed immediately to dissolve the residue. Then dissolved residue was transfer to a centrifuge filter and centrifuge at 10000 g for 10 min.

The final extracts were filtered through a 0.45µm PTFE membrane, which is 150 µl of the lower phase added into vials and 20µL were injected into a high performance liquid chromatography column, using a Shimadzu high pressure liquid chromatography (Kyoto, Japan) with fluorescence detector (excitation at 365 nm and emission above 440 nm).

3.8. Isolation and Identification of Fungi

Fifteen sorghum seeds per sample were surface sterilized with 10% sodium hypochlorite solution for 1 min, followed by immersion in sterile distilled water for 1 min. Surface sterilized seeds were then placed on freshly prepared potato dextrose agar (PDA) plates (ten seeds per plate) and incubated for three days at 25°C. Pure cultures of different out growing fungi were obtained by transferring fungal colonies to new PDA plates using sterile toothpicks, and incubating the plates for 5-7 days at 25°C. Pure cultures of each isolate were then stored at 4°C in test tube containing 2.5 ml of sterile distilled water for further use.

Species Identification

Isolates were identified to a species level based on morphological (phenotypic) features as described by Cotty (1994), Egel et al. (1994), Kurtzman et al. (1997), and Okuda et al. (2000). For this purpose: Isolates representing each pure culture were grown on PDA Agar 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*. while those that produce blue spores were considered as *Pencillium* spp. (Okuda et al., 2000). Isolates with dark green colonies and rough conidia were considered *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). Those, which produce conidia with smooth surface on CZDA and colonies typical of *A. flavus,* were recorded as *A. flavus*.

3.9. Data analysis

Sorghum samples were analyzed in duplicates and the data obtained were analyzed statistically to calculate the level of significance of various parameters using analysis of variance (one way-ANOVA) for storage period and paired comparison for storage system, using the SPSS software version 20.0.The results were reported as mean ± SD and percentages. Least significant difference (LSD) was utilized for mean separation and P-value <0.05 was considered to be significant.

4. Results and Discussion

4.1. Information on farmers' Awareness of mould contamination

A total of 30 farmers (4 female and 26 male) were interviewed for their knowledge regarding aflatoxin contamination in their locality. About 14 farmers (46.3%) respondents reported they were aware of mould contamination and the remaining 16 farmers (53.33%) were not. On the other hand, the 26.6% of respondents answered that the color of mould is Black and 6 farmers (20%) responded it as white. The major critical problems in sorghum production and storage identified by the farmers included insects (33.33%), rodent (33.33%) and mould contamination (16.7%). About 75% of the farmers reported they used insecticides to control insect damage to sorghum grains and about 5 (25%) of the farmers described they use other control mechanisms such as fire wood smoking. All farmers in the study area used natural drying method (sun drying) to adjust moisture of the grains prior to storage. With respect to location of the sorghum storage system, 50% of the farmers stored their grains in the field, 16.7% stored inside house and 33.3% stored out in the courtyard. Nine farmers (30%) used above ground storage system and about 10 farmers (33.3%) used underground pit storage and 11 farmers (36.7%) used both aboveground (Gotera) and pit storage system. According to the informants, sorghum can be stored for up to four years (Table 8).

Table 8. Scio demographic data and farmers' awareness about mould contamination

Characteristics	Response	Frequency	Percent (%)
Age	19-38	14	46.7
	38-55	13	33.33
	>55	3	10
Education	Illiterate	19	63.33
	Primary school	9	30
	Secondary school	2	6.7
Sex	Female	4	13.3
	Male	26	86.7
Mould	Yes	14	46.7
	No	16	53.3
Color	Black	8	26.6
	White	6	20
Problem	Yes	0	0
	No	30	100
Critical problem	Insect	15	50
	Rodent	10	33.33
	Mould	5	16.7
	Other	0	0
Measure taken to control	Insect sides	25	75
	Fire wood smoke	5	25
Drying technology	Sun drying	30	100
	Other technology	0	0
Cleaning and sanitation	Yes	30	100
	No	0	0
Storage Location	Field	15	50
	Inside house	5	16.7
	Courtyard	10	33.3
Mixing	Yes	0	0
	No	30	100
Storage system	Above ground(Gotera)	9	30
	Underground (pit)	10	33.3
	Both (pit and Gotera)	11	36.7
Duration of Storage	Six month	11	36.7
	One year	15	50
	Two year and above	4	13.3
	More than four year	All	100

4.2. Aflatoxin Level in Sorghum Stored at Different Storage Periods and Storage System

All 30 sorghum samples were collected and assayed in duplicate for total aflatoxin contents and the average value for each sample was calculated. The total moisture contents of sorghum samples in the present study were in the range of 8.8-11.86 % for sorghum grain stored for different storage periods (< 12 month, 1-2 year and ≥ 2 year) and storage system (aboveground and underground pit storage). An indication of the moisture content and aflatoxin detected in sorghum were shown in Table 9.

A. Aflatoxin B1 and total aflatoxin content in sorghum stored for less than 12 month

The moisture content of sorghum samples stored for less than 12 months in the present study was 9.53- 11.4% and 8.77-11.5% for aboveground and underground pit storage, respectively. Aflatoxin contamination levels detected in sorghum samples stored above ground (*Gotera*) storage system for less than 12 months were in the range of 2.15-27.12µg/kg for AFB2, 3.1-41.96µg/kg for AFG2 and 16.62-139.64µg/kg for AFG1. Sorghum samples stored underground were detected in the range of 14.17-20.38 µg/kg for AFB2, 7.46-26.16µg/kg for AFG2 and 16.98-56.39 µg/kg for AFG1 (Table 9).The mean aflatoxin B1 content of sorghum stored for less than 12 months above ground (*Gotera*) was in the range of 3.96-69.79µg/kg which was relatively lower than sorghum stored underground (5.3-82.34µg/kg). The mean total aflatoxin content of sorghum stored for <12 month above ground was in the range of 16.59-183.16µg/kg and was slightly greater than sorghum stored underground (46.48-177.33µg/kg). Therefore, the study showed that both sorghum grains stored underground pit and aboveground (*Gotera*) were highly contaminated with aflatoxins. The reason for high aflatoxin content in samples stored aboveground could be because most farmer in the study are use improper construction materials such as clay, grass, caw dung, stone, plane water, ash and sorghum stalk which encourages mould growth. Moreover, farmers repeatedly use the same storage system without properly cleaning it and without drying the sorghum grains.

B. Aflatoxin B1 and total aflatoxin content in sorghum stored for between one and two year

The moisture content of sorghum samples stored for between one and two year in the current study was 9.33- 11.86% and 9.4-12.5% for aboveground (*Gotera*) and underground pit storage, respectively. Aflatoxin contamination levels detected sorghum samples stored above ground *(Gotera)* storage system between one and two years were in the range of 2.76-52.05µg/kg for

AFB2, 4.65-51.01µg/kg for AFG2 and 18.96-101.79µg/kg for AFG1. Sorghum samples stored underground pit were detected in the range of 3.06-35.01µg/kg for AFB2, 2.04-52.84µg/kg for AFG2 and 42.30-116.13µg/kg for AFG1 (Table 9). The mean aflatoxin B1 content of sorghum stored between one and two year above ground storage (*Gotera*) was in the range of 21.75-88.90µg/kg which was relatively lower than sorghum stored for underground (3.95-153.72µg/kg). The mean total aflatoxin content of sorghum stored for between one and two year above ground storage was in the range of 44.27-210.53µg/kg and was relatively lower than sorghum stored underground (11.44-344.26µg/kg). Therefore, the study showed that the sorghum grain stored underground pit was highly infected to mould growth and leads to higher aflatoxin concentration. The reason for high aflatoxin contamination in sorghum stored underground could be because most farmers in the study area misunderstanding on mould color, infected grains with mould, separating injured grain from the health sorghum grain and moisture migration from soil to grains.

C. Aflatoxin B1 and total aflatoxin content in sorghum stored for two or more than year

The moisture content of sorghum samples stored for two or more than years in the present study were 8.9- 11.3% and 9.4-11.75% for aboveground and underground pit storage, respectively. Aflatoxin contamination levels detected sorghum samples stored aboveground for two or more years were in the range of 1.17-91.82µg/kg for AFB2, 16.95-72.65µg/kg for AFG2 and 12.92-114.10µg/kg for AFG1. Sorghum samples stored in underground pit were detected in the range of 6.89-21.35µg/kg for AFB2, 3.22-12.58µg/kg for AFG2 and9.87-129.26µg/kg for AFG1 (Table 9). The mean aflatoxin B1 content of sorghum stored for two or more than year above ground storage was in the range of 12.95-105.96µg/kg which was relatively lower than sorghum stored underground pit storage (4.59-15.57µg/kg). The mean total aflatoxin content of sorghum stored for two or more than year aboveground *(Gotera)* was in the range of 65.17-247.92µg/kg and was relatively higher than sorghum stored underground (17.68-164.3µg/kg). Therefore, the study showed that sorghum grain stored aboveground (*Gotera*) storage was highly susceptible to aflatoxin contamination. The reasons for high aflatoxin contamination levels in the sorghum sample stored aboveground (*Gotera*) could be because of most farmers in the study area use poor cultivation and harvesting, inadequate handling for example drying, insect control, cleaning, and storage practice.

Table 9.Level of aflatoxin Content in sorghum sample stored for different period and storage system

Storage system	Sample Code	B1 (µg/kg)	B2 (µg/kg)	G2 (µg/kg)	G1 (µg/kg)	Total AFs (µg/kg)	Moisture content (%)
<12 month stored sorghum							
Above ground Storage	S01	28.82	11.17	3.10	16.62	75.57	11.4
	S02	3.96	4.29	50.20	111.25	16.59	10.4
	S03	31.76	2.15	7.65	24.94	60.41	9.73
	S04	71.52	22.81	26.25	139.64	176.49	9.53
	S05	69.79	27.12	41.96	113.36	183.16	10.2
Underground Pit storage	S06	82.34	14.77	8.44	16.98	177.33	11.3
	S07	31.49	20.38	7.46	56.39	100.97	10.33
	S08	28.64	18.55	26.16	31.11	91.85	10.43
	S09	28.80	19.11	23.07	50.31	93.40	8.77
	SO10	5.30	16.54	8.47	17.24	46.48	11.53
1-2 year stored sorghum							
Aboveground Storage	SO11	21.75	2.76	21.02	101.79	44.27	11.86
	SO12	46.16	3.48	15.73	68.30	88.61	11.43
	SO13	88.90	24.43	51.01	**ND**	210.53	10.78
	SO14	33.17	52.05	26.28	18.96	175.16	9.82
	SO15	31.06	40.56	4.65	23.87	145.61	9.33
Underground Pit storage	SO16	8.86	35.01	12.65	116.13	94.28	11.05
	SO17	3.95	2.01	2.04	80.70	11.44	12.53
	SO18	64.68	17.33	18.74	42.30	152.19	9.47
	SO19	69.69	3.06	52.84	61.82	128.86	10.80
	SO20	153.72	33.45	37.89	**ND**	344.26	9.40
≥ 2 year stored sorghum							
Aboveground storage	SO21	26.27	6.58	19.40	12.92	65.17	9.40
	SO22	51.28	1.17	32.91	114.10	199.46	9.40
	SO23	12.95	30.26	16.95	78.17	138.33	8.90
	SO24	105.96	91.82	24.61	25.53	247.92	9.19
	SO25	36.75	2.85	72.65	109.20	221.45	11.13
Underground Pit storage	SO26	15.57	6.89	12.58	129.26	164.3	9.60
	SO27	9.60	21.35	15.62	51.79	98.36	8.80
	SO28	4.59	10.08	3.22	9.87	17.68	11.75
	SO29	7.25	**ND**	4.28	9.94	21.47	10.72
	SO30	**ND**	**ND**	**ND**	**ND**	**ND**	9.40
% of contamination level		**96.66**	**93.33**	**96.66**	**90**	**-----**	-----

❖ ND -means not detected

From the total 30 samples, 96.66%, 93.33%, 96.7%, and 90% were contaminated by aflatoxin B1, B2, G2 and G1, respectively. These showed total aflatoxin contamination by the level ranged from 11.44µg/kg to 344.26µg/kg and the mean total aflatoxin value of 123.85µg/kg. Aflatoxin contamination levels were detected sorghum samples in range of1.17-91.82µg/kg for AFB2, 3.22-139.64 µg/kg for AFG2 and 9.87-139.64µg/kg for AFG1 (Table 9). Enormous variation of aflatoxin contamination was observed between 23 sorghum samples and 7 samples had aflatoxin levels below detection limits (3 for AFG1, 2 for AF B2, 1 for AFB1 and AFG2). This was mainly due to the variation in fungal colonization, especially *Aspergillus flavus*, and spore density during the grain development stage. The variation of this fungus in developing sorghum grain was studied earlier by Ratnavathi, *et al* (2003).

About 96.66% of the total aflatoxins in the sorghum samples were attributed to AFB1 which ranged from 3.95- 153.72 µg/kg. Generally speaking, AFB1 is the most toxic aflatoxin among all types of aflatoxins and is considered to be hepatocarcinogenic and immunosuppressive as well (IARC 1993; Tchana *et al*., 2010). Therefore, the high concentration of the AFB1 in sorghum samples indicates that there could be a serious problem of aflatoxin contaminations in the study area.

The incidence of aflatoxins contamination in the examined sorghum grain samples appear to be lower than those reported for sorghum and maize (Ayalew *et al.,* 2006) and Quitet *et al*. (1993) in which the aflatoxin contamination reached up to 1000 µg/kg for sorghum and 1388 µg/kg for wheat, respectively. However, this study result showed a very high aflatoxin levels above the permissible limits. The maximum aflatoxin B1 content found in this research (153.72 µg/kg) was below the highest amount reported by Habtam and Kelbesa (692 µg/kg) (Habtamu & Kelbesa, 2001). On other hand, the presence of aflatoxin contamination (96.6%) reported in the present study was relatively higher than the previous study reported by Ayalew (2010) aflatoxin infection (88%) in maize from Ethiopia.

The presence of aflatoxin B1 detected in the present study (153.72 µg/kg) was generally higher than from the previous study by Chala *et al*., (2014) on sorghum in Ethiopia (29.5 µg/kg). However, the amount of aflatoxin B1 (153.72 µg/kg) in the sorghum samples in this research was much relatively lower than the finding reported by Alpert *et al.,* (1971) which was as high as 1000µg/kg. The concentrations of aflatoxin G1 obtained in the present study were larger

(139.64µg/kg) when compared with previous reports for sorghum grain (29.65µg/kg) by Chala *et al.* (2014).

On other hand, the percent of AFB1 level (96.66%) reported in the present study was relatively higher than the previous study reported in Brazil which was 39% for pre-fermented and 32% for post-fermented sorghum samples (Lam *et al*, 2012) with maximum values of 5.10µg/kg and 30.05µg/kg, respectively. In previous studies the exposures of aflatoxin contamination in ground peanut and red pepper in Ethiopia was reported by Geeresu (2014), Eshetu (2010), and Hbtamu (2013), respectively. They reported about 93%, 73.06% and 70% level of aflatoxin in that order. Similarly, the current study also showed aflatoxin contamination in sorghum stored under different storage system and period. This indicates that there is high problem of aflatoxin contamination in the country.

4.2.1. Effects of Storage Periods on the Level of Aflatoxin Contamination in Sorghum

Sorghum stored for two or more years had high level of aflatoxin B1 (52.19 µg/kg) followed by sorghum stored for less than 12 months (38.24 µg/kg). Although storage period had no significant ($P>0.05$) effect on the level of most of the aflatoxins, it resulted in significant difference in the level of aflatoxin B1 in sorghum. Significant difference ($P<0.05$) in the aflatoxin B1 was observed between sorghum grains stored for up to two years (52.19 µg/kg) and stored for more than two years (27.02 µg/kg). This indicates that storing sorghum for a period between one and two years result in higher contamination by aflatoxin B1. However, sorghum stored for over two years had high level of aflatoxin B2 (21.42 µg/kg) and sorghum stored for less than 12 months had high concentration of the aflatoxin G1 (57.7 µg/kg). Sorghum grains stored for above two year had high level of total aflatoxin than the remaining storage periods but with no significant difference ($P>0.05$) (Table 10). Based on the result of the present study, aflatoxin contamination can occur in grains stored for any storage period given no proper sorghum cultivation, harvesting, transporting, trashing, cleaning, insect prevention and storage practices.

Table 10. Effect of storage period (< 12 month, 1-2 year, and ≤ 2 year) on the level of aflatoxin (µg/kg) in sorghum

Duration of Storage period	Aflatoxin content (µg/kg)				
	AFG2	AFG1	AFB2	AFB1	Average total aflatoxin
< 12 months	20.28[a]	57.78[a]	15.69[a]	38.24[ab]	64.31[a]
1-2year	24.28[a]	51.39[a]	17.09[a]	52.19[a]	59.31[a]
≥ 2 year	22.22[a]	54.08[a]	21.42[a]	27.02[b]	81.64[a]

- Means within the same column followed by the same letter superscript indicate no significant difference (p>0.05).

The level of aflatoxin B1 contamination (52.19µg/kg) in sorghum storage period (< 12 month, 1-2 year, and ≤ 2 year) were lower than AFB1 in ground pepper, shiro, legumes and spices (incidences of AFB1 contamination occur 13.33% and 8.33%, and the levels of contamination range 250 to 525 µg/kg and 100 to 500 µg/kg, respectively) previously stated by Fufa and Urga, (1996).

The reason for aflatoxin content and mycotoxin development in sorghum grain stored for different period was increment due to moisture migration from the surrounding and storage condition (temperature and humidity) reported by Mashilla *et al.*(2006), Mohamed *et al*.(2013), and Habtamu and Kelbessa (2001). The level of aflatoxin increment stated by Shephard G.S., (2005), flood damage to grain (mainly sorghum) in underground storage areas resulted in visible fungal contamination and these harsh realities; it is not surprising that fungal contamination of staple food. The current study showed that the study area has a suitable condition for mycotoxin development and farmers may not used sufficiently good handling, harvesting and storage practice. This practice may lead to elevate aflatoxin production.

4.2.2. Effects of Storage System on the Level of Aflatoxin Contamination in Sorghum

Storage of grains under poor storage system and conditions often result in aflatoxin contaminations. However, in this study, storage system did not result in significant variation between the mean aflatoxin contents of sorghum grains. Grains stored above ground had high aflatoxins level of 49.67µg/kg (G2), 63.91µg/kg (G1), 21.57µg/kg (B2) and 44.0µg/kg (B1) than

grains stored in the underground pit which in that order contained 30.05µg/kg, 44.92µg/kg, 14.56µg/kg and 34.30µg/kg. Storage type also did not significantly affect the level of total aflatoxin ($p>0.05$), but the level of aflatoxin in storage type still larger than the maximum level stated by EU and FDA (Table 11).

The reason for aflatoxin level increment in the current study may be stored sorghum grains indicated by (Mohamed *et al*., 2013) factors, including soil quality, crop yields, and the biological environment of crops such as the abundance of pests and plant pathogens determine the mycotoxin level of sorghum. These factors encourage mycotoxin invasion of grains and thereby impart a food-borne risks. Basically, the ability of fungi to produce mycotoxins is largely influenced by temperature, relative humidity, insect attack, and stress conditions of the plants.

Table 11**.** Effect of storage system (above ground and underground pit) on the level of aflatoxin

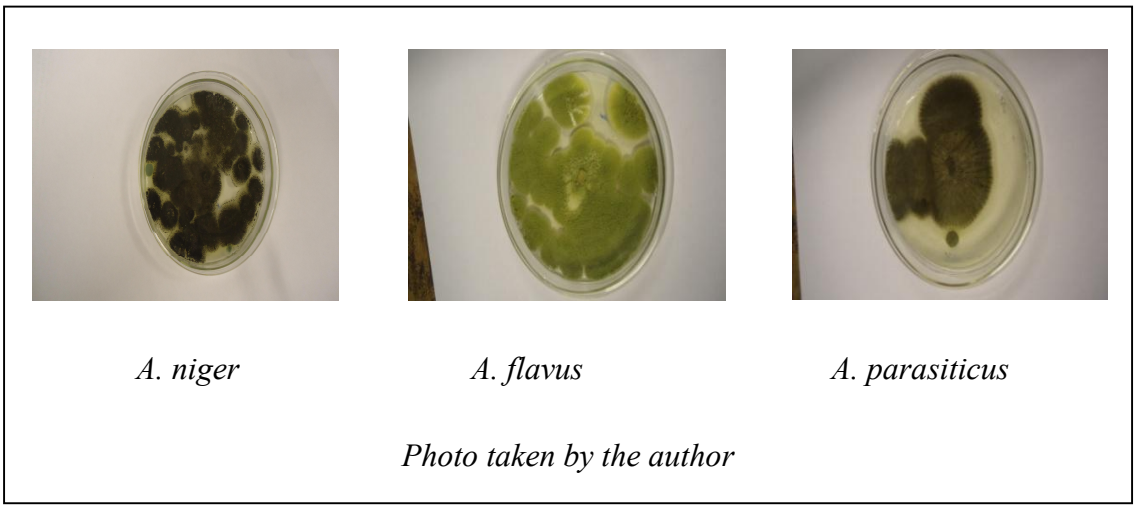
Storage type	**Average aflatoxin content(µg/kg)**			
	AFG2	**AFG1**	**AFB2**	**AFB1**
Above ground	49.67[a]	63.91[a]	21.57[a]	44.1[a]
Underground pit	30.05[a]	44.92[a]	14.56[a]	34.3[a]

- Means within the same column followed by the same letter superscript indicate no significant difference ($p>0.05$).

4.3. Isolation and Identification of *Aspergillus Species* from Sorghum Sample

Aspergillus spp. isolation representing each pure culture was grown on PDA Agar at 25°C for 5-7 days. The first species isolated from the sorghum samples was *A. niger*. The major distinction of separating *A. niger* from the other species of *Aspergillus* is the production of carbon black or dark brown spores of *Biseriate phialides* were indicated as plate 7. *A. flavus* was the second species identified in this study. Colonies of this fungus were characterized by yellow to dark, yellowish-green pigments, consisting of a dense felt of conidiophores or mature vesicles bearing phialides over their entire surface. In general, *A. flavus* was known as a velvety, yellow to green or the old colony was brown mould with a goldish to red-brown on the reverse. *A. parasiticus*

was the third species identified from sorghum samples tested in the current study. Colonies representing this species produced dark green and rough conidia on PDA at 25 and 37°C after 5-7 days of incubation. The three identified *Aspergillus species were* indicated in plate7 below.



A. niger *A. flavus* *A. parasiticus*

Photo taken by the author

Plate 7. Morphological (*phenotypic*) feature of *Aspergillus ssp.*

In this study all sorghum samples come from regions with temperatures ranging from 17 to 30°C.which supports the growth of *Aspergillus species*. Under tropical condition, stored products are more susceptible to *Aspergillus species* than other fungi as many *Aspergillus species* are favored by the combination of low water activity (aw) and relatively high storage temperature (Habtamu and Kelbesa, 2001). Although, cereal grains belong to corn, rice, barley; wheat and sorghum are found susceptible to aflatoxin accumulation by aflatoxigenic fungus.

In table 12 indicated that the occurrence of *Aspergillus spp*. in sorghum grain stored at different storage period and storage system; 56.7%, 16.7% and 23.33% of *A. flavus*, *A. parasiticus* and *A.niger* was found, respectively from the total 30 samples.

Table 12. *Aspergillus spp.* identified from sorghum sample using PDA agar

Aspergillus spp.	**Number of sample**	**% of Aspergillus spp. found**
Aspergillus flavus	17	56.66
Aspergillus parasiticus	5	16.66
Aspergillus niger	7	23.33
Total	**29**	**96.65**

The incidence of *A. flavus* (56.7%) in the present study was bigger than the occurrences of *A. flavus* in maize samples from Ethiopia were indicated by Ayalew (2010). However, the invention of *Aspergillus fulvous* (56.7%) in sorghum sample was lower than the occurrence of *A. flavous (72.7%)* in sorghum grain reported from India (Sreenivasa, 2010). Therefore; the occurrence of aflatoxin content in sorghum sample is associated with *A. flavu*s and *A. parasiticus.* These indicate aflatoxin contaminations in sorghum sample collected from the study area is highly significant.

However, the current results showed that sorghum was more profoundly colonized by aflatoxin-producing *Aspergillus spp*., with overall aflatoxin levels being correspondingly higher. This study also shows that sorghum grain contamination by *Aspergillus* and production of aflatoxin are highly influenced by the weather conditions prevailing during the grain development stage, i.e. seed set to physiological maturity stage (Ratnavathi, *et al*., 2003). But this may be caused by the variations in cultivars, storage periods, and storage system and over all handling practices used.

In table 13 showed that the *A.flavous* (13.33% for < 12 months 23.33% for 1-2year and 20% for ≥ 2year) and *A. parasiticus* (6.67% for < 12 months 6.67% for 1-2year and 3.33 % for ≥ 2 year) were occurred sorghum stored at different periods. *A. Flavus* and *A .parasiticus* stored at 1-2year was slightly larger than sorghum grains stored with ≥ 2 year and <12 month. This indicated that aflatoxin contamination in sorghum grain was highly disposed from storage periods. The presence of *Aspergillus niger* (3.3%) in sorghum stored between one and two year had lower

than both 10 % of *Aspergillus flavus* and *Aspergillus parasiticus. Sorghum stored with two or more year had relatively lower Aspergillus parasiticus (3.33%) than both 6.67% of Aspergillus parasiticus* at sorghum stored in less than 12 month and between one and two year.

Table 13. The occurrence of *aspergillus spp.* in different storage periods

Duration of Storage period	***Aspergillus species (%)***		
	A.flavous	***A. parasiticus***	***A.niger***
< 12 months	13.33	6.67	10.0
1-2year	23.33	6.67	3.33
≥ 2 year	20.00	3.33	10.0

4.4. Evaluation of Aflatoxins Results against Different International Standards

The maximum tolerable limits of aflatoxins in foodstuffs are laid by European Union legislation are specified for aflatoxin B1 (5µg/kg) and maximum total aflatoxin (10µg/kg) (EFSA, 2013; Herzallah, 2009). Another regulation, the US Food and Drug Administration (FDA) have established maximum acceptable level of 20 ppb for aflatoxin in maize, sorghum and other cereals for human consumption, with the level in milk being even lower (0.5 ppb) (Grybauskas et al., 2000). Likewise, FAO underline the maximum tolerated levels limit of 20µg/kg for mycotoxins in foodstuffs, Maize, kidney beans, rice, sorghum, groundnuts and groundnut butter. Based on the result of the current study, the mean average of aflatoxin concentration in range of AFB1 (3.95-153.72 µg/kg) and total aflatoxin (11.44-344.26 µg/kg) is higher than the EU regulation limit and FDA maximum tolerable limit.

The aflatoxin content measured in all the sorghum samples showed that natural aflatoxin was higher and 96.66 % of samples were contaminated by toxin as compared to highly susceptible crops like maize and groundnut (Anna, 2012). However, the result also above the safety limit (20µg/kg) recommended by the Codex Alimentarius Committee.

In East African standard specification (CD-ARS 462:2012(E) for sorghum, it is stated that the sorghum grains shall comply with those maximum mycotoxin limits established by the Codex Alimentarius Commission. In particular, total aflatoxin levels in sorghum grains for human consumption shall not exceed 10µg/kg with AFB1 not exceeding 5µg/kg when tested according to ISO 16050. However, the present study showed that the total aflatoxin concentration and AFB1 were surpassed the specified tolerable levels by the above mentioned regulations.

5. Conclusions and Recommendations

5.1. Conclusion

From this study, about 56.7% of *Aspergillus flavus*, 16.7% of *aspergillus niger* and 23.3% of *Aspergillus parasiticus* were found in sorghum samples. This showed that the sorghum was more susceptible to *aspergillus spp.* infection. The presence of *Aspergillus flavus and Aspergillus parasiticus* in the studied sorghum samples indicated that there could be a greater likelihood of aflatoxin contamination in the area.

The mean total aflatoxin concentration determined in sorghum samples stored at different period and storage system was as high as 344.26μg/kg. About 96.66%, 93.33%, 96.7%, and 90% of the samples were contaminated by aflatoxin B1, B2, G2 and G1, respectively. The most potent carcinogenic agent aflatoxin B1 were contributed to 96.66% of the total aflatoxins in the sorghum samples was found.

Although storage period had no significant ($P>0.05$) effect on the level of AFB2, AFG1, and AFG2, it resulted in significant difference in the level of aflatoxin B1 in sorghum. Based on the findings of this study, one can conclude that storing sorghum for a period between one and two years result in higher contamination by aflatoxin B1 and that aflatoxin contamination can occur in grains stored for any storage period given no proper sorghum grains handling practices. Sorghum grains stored aboveground had relatively high aflatoxin concentrations. Therefore, underground storage might be preferred over aboveground storage system.

The results obtained in this study showed that the magnitude of AFB1 contamination varied among sorghum samples stored at different periods. The levels of aflatoxins in the sorghum grains stored at different system and period's was found to be higher than the permissible limits set by the European Union, Codex, FDA regulation and East African Standard of sorghum for human consumption. Therefore, the overall occurrence of aflatoxin in sorghum stored at different storage system and period was above tolerable limit. This can be more hazardous to individuals who are more sensitive and prone to toxic effects of such highly carcinogenic food contaminants. Sorghum stored at different storage system (above ground or underground) in the study area was

more contaminate with aflatoxin. Therefore, the storage system and its construction materials have to be selected properly to reduce aflatoxin contamination.

5.2. Recommendations

The suggested recommendations based on the present finding are the following:-

- The Agricultural sectors and their regulatory bodies should put value chain strategies to identify critical control points for minimizing mycotoxin production.
- The presence of aflatoxins in levels higher than the tolerable limits in this research calls for further research pending investigation of mode of aflatoxin contamination.
- Consumers should be aware of aflatoxin contamination in sorghum grain.
- Creating unfavorable conditions for fungal growth and use of improved storage methods (providing polythene coating bag) will help in minimizing sorghum contamination due to infestation by insects and mould growth.
- Future priority and research in the area of sorghum should lie in identifying the most stages in the value chain where greater aflatoxin contaminations occur.
- The health impact of aflatoxin due to sorghum grains consumption on human or animal health needs to be investigated.
- Storing sorghum underground is relatively better than storing aboveground.

Reference

Abate, D. and Gashe, B. (1985). Prevalence of Aspergillus flavus in Ethiopia cereal grain: A preliminary survey. *Ethiopian Medical journal, 23: 143-148.*

Abu Tefera (2013). Report Contains Assessments of Commodity and Trade Issues Made by USDA Staff. Official U.S. Government Policy, Global agricultural information network.

Ajoy K., Choudhary and Priyanka K. (2010). Management of mycotoxin contamination in pre harvest and post harvest crops: present status and future prospects. *Journal of phytol 2/7:37-52.*

Akiyama, H., Goda, Y., Tanaka, T., and Toyoda, M. (2001). Determination of aflatoxins B1, B2, G1 and G2 in spices using a multifunctional column clean-up. *Journal of Chromatogr. A 932:153–157.*

Alpert, M. E., Hutt, M. S. R., Wogan, G. N., & Davidson, C. S. (1971). Association between aflatoxin content of food and hepatoma frequency, Uganda. *Cancer, 28:253-260*

Anna Chiara, M. (2011). Aflatoxin: there measure and analyses. Department of food and feed science, university of Teramo Italy.

Audilakshmi, S., Das, I. K., Ghorade, R.B., Mane, P.N., *e al.* (2011). Genetic improvement of sorghum for grain mould resistance: performance of sorghum recombinant inbred lines for grain mould reactions across environments. *Journal of Crop Protection, 30: 753-758.*

Ayalew, A. (2010). Mycotoxins and surface and internal fungi of maize from Ethiopia. Nutrition and Development, *African Journal of Food Agriculture, Vol. 10, No. 9:4109-4123*

Ayalew, A., Fehrmann H., Lepschy,J., Beck, and Abate, D. (2006). Natural occurrence of mycotoxin in staple cereals from Ethiopia. *Journal of Mycopathologia, 162 (1): 57-63.*

Bagley, C. V. (1997). MYCOTOXINS. Animal Health Fact Sheet. Extension Veterinarian Utah State University, Logan UT 84322-5600

Bandyopadhyay, R., Williams, J.H., and Ojiambo, P.S. (2007). Social and political challenges in the regulation of mycotoxin contamination. *Journal of Phytopathology 97: 148.*

Benti, T. and J. Ranson. (1993). The need and objective of the first national maize workshop. Proceedings of the first national maize workshop of Ethiopia. 5-7 *May 1992, IAR/CIMMYT, Addis Abeba:pp 1- 5.*

Besrat, A. and P. Gebre. (1981). A preliminary study of aflatoxin content of selected Ethiopian foods. *Journal* of *Ethiopian Medical,*19: 47-52.

Bhat, R.,V. (1988). Mould deterioration of agricultural commodities during transit: problems faced by developing countries. *Journal of food Microbial,* 7: 219-225

Bhattacharyya, A., K. (1986). Protein-energy malnutrition (Kwashiorkor-Marasmus syndrome): terminology, classification and evolution World Review of Nutrition and Dietetics, *47: 80-133.*

Black, R., E., et al. (2013). Maternal and child under nutrition and overweight in low-income and middle-income countries, *The Lancet*, *382 (9890): 427–451.*

Brandfass, C., and Karlovsky, P. (2008). Up scaled CTAB-based DNA extraction and realtime PCR assays for Fusarium culmorum and F. graminearum DNA in plant material with reduced sampling error. *International Journal of Molecular Sciences*, *9, 2306-2321.*

Chala, A., Mohammed, A., Ayalew, A., Skinnes, H. (2012). Natural occurrence of mycotoxin in ground nut (*Arachis Hyogaea L.)* from eastrn Ethiopia. *Food control*, 30: 602-605.

Chala, A., Taye W., Ayalew, A., Krska, R., et al., (2014). Multi-mycotoxin analysis of sorghum (*Sorghum bicolor L. Moench*) and finger millet (*Eleusine coracana L. Garten*) from Ethiopia, *journal of Food Control 45; 29-35*

Chandra, H., Bahuguna, J., and Singh, A., (2013). Detection of Aflatoxin in *Zea mays L.* from Indian Markets by Competitive ELISA. *Journal of Biosciences*; *Vol. 1(1):62-68.*

Christensen, C.M., Mirocha, C., J. and Meronuck, R., A. (1977)."Moulds, Mycotoxins and Mycotoxicoses". *Agricultural Experiment Station Miscellaneous Report 142. University of Minnesota, St. Paul, MN*

Clement, A., Lennox, J.A., Bassey E. A., Asitok, A., Ikpoh S., *et al.* (2013). Isolation of aflatoxin producing species of *Aspergillus* from foodstuffs sold in calabar markets, Cross River State, Nigeria. *Journal of Microbial. Biotech. Res., 3 (1):8-13*.

Codex Alimentarius Commission. (1989). Report of the Twentieth Session of the Codex Committee on Food Additives and Contaminants, Alinorm 89/12. *Food and Agriculture Organization, Rome.*

Codex Alimentarius Commission. (2001). Joint FAO/WHO Food standards program, Codex committee on food Additives and contaminants. Thirty-third session Codex, Hague, Netherlands.

Cole, R., J. and Cox, R., H. (1981). Handbook of Toxic fungi metabolites. *Academic Press New York, London,* p.937.

Cotty, P., J. (1994). Influence of field application of a toxigenic strain of Aspergillus flavus on the populations of A. flavus infecting cotton bolls and on the aflatoxin content of cottonseed. *Phytopathology 84: 940-945.*

CSA. (2007). National census conducted by the Central Statistical Agency of Ethiopia (CSA) rural area.

CSA. (2010). Agricultural Sampling Survey Report on area and production of crops: *http://www.csa.gov.et Accessed 17.12.10.*

Deiner, U.L., R. J., Cole, T., H Sanders, G., A. Payne, et al. (1987). Epidemiology of aflatoxin formation by Aspergillus flavus. *Ann. Rev. Phytopathology*, *25:240-270.*

Devegowda, G., Raju, M.V., L.N. and Swamy, H.V., L.N. (1998). Mycotoxins: Novel Solutions for Their Counteraction. Feedstuffs, *70, 12-15.*

EFSA. (2013). *Scientific Report of EFSA*. Aflatoxins (B1, B2, G1, G2) in cereals and cereal-derived food products. *Parma, Italy, EFSA; En-406; www.efsa.europa.eu/publications.*

Egel, D., S., Cotty, P.J, Elias, S. (1994). Relationship among isolates *of Aspergillus sect. flavi* that vary in aflatoxin production. *Phytopathology. 84: 906-912.*

Fadia, F. H., Muna, H.J., and Abdul Kareem, J. H. (2014). Isolation and Identification of Fungal Propagation in Stored Maize and detection of aflatoxin B1 Using TLC and ELISA Technique. *Iraqi. Journal of Science*, *55(2):634-642.*

FAOSTAT. (2005). FAO statistical data base for Agriculture

FDA. (2011). A Guide for Grain Elevators, Feed Manufacturers, Grain Processors and Exporters. *National Grain and Feed Association 1250 Eye St., N.W., Suite 1003, Washington, D.C., 20005-3922*

FEHD. (2001). Aflatoxin in Food. Risk Assessment Studies, Chemical Hazards Evaluation. Report *No.5:.4-17*

Fufa, H. and Urga, K. (1996). Screening of aflatoxins in Shiro and ground red pepper in Addis Ababa, *Ethiop. Med. J., 34, 243–249*

Geresu Bechere. (2014). Aflatoxin contamination and sanmollenlea in local and imported raw, roasted and peanut butter collected from Addis Ababa , *Master thesis of food science and nutrition*

Geyid, A. and Maru A.(1987). A survey of aflatoxin content in maize, sorghum and teff samples. *Journal of Ethiopian Health Development. Vl.2, pp.59-70.*

GHENT university faculty of parasitical department of food laboratory laboratory. (2014). Determination of aflatoxin in Maize sorghum and peanut, *MYTOX, Belgium*

Gilman, G.A. (1968). Storage problems in Ethiopia with special reference to deterioration by fungi, *Reports of Tropical Products Institute, R48.21: pp 52*

Gourama, H.; Bullerman, L.B. (1995). *Aspergillus flavus*: aflatoxigenic fungi of concern in foods and feed. *Journal of Food Protection,* 58:1395-1404.

Grybauskas, A. P., Thomison, P. R. and Cassel, E. K. (2000). Aflatoxins. *Maryland Cooperative Extension Fact Sheet 444: Pp88*

Guo, B., Widstrom N., *et al.* (2000). "Control of pre harvest aflatoxin contamination in corn: Fungus-plant-insect interactions and control strategies." *Recent Research Developments in Agricultural and Food Chemistry*, *4:165-176.*

Habtamu Gashaw. (2014). the occurrences of aflatoxin contamination in Red pepper in relation to storage conditions, *Master thesis of food science and nutrition, Addis Ababa university*

Habtamu, F. and Kelbessa, U. (2001). Survey of Aflatoxin Contamination in Ethiopia. *Ethiopian journal of Health science, 11 (1):17-25.*

Harish, C., Jyotsana B., and Ajay, S. (2013). Detection of Aflatoxin in Zea mays L. from Indian Markets by Competitive ELISA. *Octa. J. Biosci. 1(1):62-68.*

Hendrickse, R. G., Coulter,J.B., Lamplugh,S.M., MacFarlane, S. B., Williams,T.E., et al. (1983). Aflatoxins and kwashiorkor. Epidemiology and clinical studies in Sudanese children and findings in the autopsy liver samples from Nigeria and South Africa Bulletin of the Exotic Pathology Society, *76:559-566.*

Herzallah, S. (2009). Determination of aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors. *Journal of Food Chemistry, 114(3):1141–1146.*

Hesseltine, C. W. (1974). Natural occurrence of mycotoxin in cereals. *Mycopathologia, 53(1-4), 141-153.*

Hesseltine, C. W. (1974). Natural occurrence of mycotoxin in cereals. *Mycopathologia, 53(1-4), 141-153.*

HKSAR. (2001). Aflatoxin in Food .Risk assessment studies, chemical hazards evaluation, Food and environmental Hygiene Department. *Report No.5.*

Ilunga kamika. (2012). Determination of aflatoxin in peanut (*Arachis Hypogaea L.)* collected from Kinshasa, democratic republic of Kongo and Pretoria, south Africa : a comparative study.

International Agency for Research on Cancer (IARC). (1993). Aflatoxins, *56:pp245*.

Iyanda, A.A., Anetor, J. I., Oparinde, D. P. and Adeniyi, F.A.A. (2014). Aflatoxin contamination of foodstuffs: Its health implications in Sub-Sararan Africa: Nigeria: *Annals of Experimental Biology 2 (3):63-73*

Jian, Y.; Jolly, P.E.; Ellis, W.O.; Wang, J.-S.; PhillipsT.D. *eat al.* (2005**).** Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanians. *Int. Immunol*, *17: 807-814.*

Jolly, P., Jiang, y., Ellis, W. o., Wang, J. S. *et al.* (2008). Modulation of the human immune system by aflatoxin. Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade. *Oxford shire, UK: Cab international, 41–52*

Kaaya, A.N., Harris C., and Eigel W., (2006). Peanut aflatoxin levels on farms and in markets of Uganda. *Peanut Science, 33:68 -75.*

Kabak, B., Ozbey F. (2012). *Food Control, 28: 354-361*

Khalil, M.M. H., Gomaa, A.M, and Sebaei, A. S. (2013).Reliable HPLC Determination of Aflatoxin M1 in Eggs .*Journal of Analytical Methods in Chemistry*: 1-5.

Kiran, D. R., Narayana K.J.P., and Vijayalakshmi M. (2005). Aflatoxin B1 Production in Chillies (*Capsicum Annuum L.*) kept in Cold Stores, *African Journal of Biotechnology*, *4 (8): 791-795.*

Klich, M.A. (2002). Identification of common *Aspergillus* species. Central bureauvoor Shimmel cultures. Utrecht.,*Netherlands. p.116.*

Komala, V.V., Ratnavathi, C.V., Vijay Kumar, B.S., Das I.K. (2012). Inhibition of aflatoxin B1 production by an antifungal component, eugenol in stored sorghum grains. *Food Control 26: 139-146: www.elsevier.com/locate/foodcont*

Krska R., Schubert-Ullrich P., Molinelli A., Sulyok M., *et al* (2008). Mycotoxin analysis: Food Additives & Contaminants: *Part a, 25(2): 152–163*

Kumar, M., Bandyopadhyay, R., Akinbade, S.A. and Kumar, P.L. (2008). Production of polyclonal antibodies to aflatoxin B1 and development of an indirect ELISA for estimation of aflatoxin contamination in crops. *Nigeria, Lekki, Lagos. Page 7.*

Kumeda, Y., and Asao, T. (2001). Hetroduplex panel Analysis, a Novel Method for Genetic Identification of Aspergillus section Flavi strains. *Applied and environmental microbiology*, 67*(9):4084-4090.*

Kurtzman, C.P., Horn B.W., Heseltine, C.W. (1997). Aspergillus nomius, a new aflatoxin-production species related to *A. flavus* and *A. tamari*. *Ant. Van Lee. 53: 147-158.*

Lacy, S.M., Cleveland, D.A., Soleri, D., (2006). Farmer choice of sorghum varieties in southern Mali. Hum. *Ecol. 34, 331–353.*

Lal, S. (1986). Role of moisture and mycoflora in storage. In warehouse management of bag storage of grains. FAO. Rome.

Loh Saw, H., Nur Iryani Mohd, Y. & How Mun, L. (2010). Determination of Aflatoxins B1 and B2 in peanuts and Corn based products (Penentuan Afltoksin B1 Dan B2 Dalam Kacang Tanah Dan Produk Jagung). *Sains Malaysiana 39(5): 731–735*

Machida, M. and Gomi, K. (2010). An Overview of the Genus Aspergillus, National Institute of Advanced Industrial Science and Technology, Japan; *Tohoku University, Sendai,p.1-17*

Mashilla Dejene. (2004).Grain Storage Methods and Their Effects on Sorghum Grain Quality in Hararghe, Ethiopia, Department of Ecology and Crop Production Science. Doctoral thesis Swedish University of Agricultural Sciences Uppsala

Mashilla, D., Yuen, J., Sigvald, R.(2006). Effects of storage methods, storage time and different agro ecological zones on chemical components of stored sorghum grain in Hararge, Ethiopia. *Journal of Stored Products Research 42 : 445 – 456*

Masresha, F., Okori P., Samuel, G., Mneney, E., Kassahun, T. (2010). Delivering New Sorghum and Finger Millet Innovations for Food Security and Improving Livelihoods. Eastern Africa, *International Livestock Research Institute (ILRI).*

Matthijs Kool and Frank van Steenbergen. (2014). Sorghum Production under Spate Irrigation Systems, Pakistan

Mekbib, F. (2009). Farmers' Breeding of Sorghum in the Centre of diversity. Socio-Ecotype Differentiation, Varietal Mixture And Selection Efficiency, Ethiopia, Haramaya University, *54 : 25-37*

Mohamed, A. M., Al-Othman, M. R., Abeer, R., Abd El-Aziz, M. (2013). Mycotoxigenic Fungi Contaminating Corn And Sorghum Grains in Saudi Arabia, Agricultural Research Center, Giza, Egypt *Journal of Bot., 45(5): 1831-1839.*

Nissen, N.N., and Martin, P. (2002). Hepatocellular carcinoma: the high risk patent. *Journal of clinical gastroenterology,35(50):79-85.*

Okuda, T., Klich, M.A., Seifert, K.A., Ando, K. (2000). Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* Classification. *In: Samson RA, Pitt JI Editors. Hardwood Academic Publishers: Reading, UK. pp 83-100.*

Partenership for Aflatoxin control in Africa. (PACA). (2012). Aflatoxin impacts and potential solutions in agriculture, trade, and health. a background paper for the PACA strategy development- Stakeholder consultation Workshop.

Patten, R. C. (1981). Aflatoxins and disease. *Am J Trop Med Hyg*. 30: 422 - 425.

Pestka, J. J., & Smolinski, A. T. (2005). Deoxynivalenol: toxicology and potential effects on humans. *Journal of Toxicology and Environmental Health B, 8, 39-69.*

Peterson, S. W., Ito, Y., Horn, B. W. and L. Goto. (2001). *Aspergillus* bombycis, a new aflatoxigenic species and genetic variation in its sibling species, A. nomius. *Mycologia, 93:639-703.*

Priyanka, S. R., Venkata Ramana, M., Balakrishna, K., Murali, H. S., Batra, H. V. (2012). A Novel Non Radioactive PCR-DNA probe for the detection of Aflatoxin Producing *Aspergillus Species* from major food crops grown in India: *Journal of Advances in Microbiology, vl(2):577-586*

Quitet, SM, Shehata, A.M.E, Mesellam, A.S. (1983). Occurrence of aflatoxins in some Egyptian food crops collected from two coastal regions. *Journal of Food Chemistry; 10: 149*.

Rahul, P., and Jha, S.N. (2014). Basic of the genus Aspergillus. *International journal of research in botany, 4(2):26-30.*

Raihanatu, M. B., Modu, S., Falmata, A. S., Shettima, Y. A. and Heman, M. (2011). Effect of processing (sprouting and fermentation) of five local varieties of sorghum on some biochemical parameters. *Vol. (23), No. 2: 91-96.*

Rajarajan, P. N., Rajasekaran, K.M., Asha Devi, N.K. (2013). Aflatoxin Contamination in Agricultural Commodities., *Indian J.Pharm.Biol.Res*, *1(4)., pp.148-151*.

Raper. K.B., Fennell, D.I. (1965). The genus Aspergillus. Williams and Wilkins. Baltimore, Maryland. *pp. 686.*

Rapid Alert System for Food and Feed (RASFF). (2011). Determination of aflatoxin level and monitoring the effectiveness of the controls in place to limit consumer exposure these toxins, available from: *RASFF portal:https://webgate.ec.europa.eu/rasffwindow/portal/*

Ratnavathi, C. V., & Sashidhar, R. B. (2003). Substrate suitability of different genotypes of sorghum in relation to Aspergillus infection and aflatoxin production. *Journal of Agricultural Food Chemistry, 51, 3482-3492.*

Rehima Mussema. (2006). Analysis of red pepper marketing: The case of Alaba and Siltie in SNNNPR of Ethiopia. Haramaya University.

Russell, R. and Paterson, M. (2007). Aflatoxins contamination in chilli samples from Pakistan

Saleemullah, I.A., Khalil, I.A., Hamidullah, S. (2006). Aflatoxin contents of stored and artificially inoculated cereals and nuts. *Food Chem*., *98:699-703.*

Serna-Saldivar, S. and Rooney, L. (1995). Structure and chemistry of sorghum and millets. In: Dendy DAV, ed. Sorghum and millets, chemistry and technology. *St Paul, MN: American Association of Cereal Chemists. 69–124.*

Shashidhar, R.B., Ramakrishna, Y. & Bhat, R.V. (1992). Moulds and mycotoxins in sorghum stored in traditional containers in India. *Journal of Stored Products Research 28, 257-260.*

Shephard, G. S. (2005). Aflatoxin and Food Safety: Recent African Perspectives

Tigabu E., Andargie M., and Tesfaye K., (2012). Response of sorghum (*Sorghum bicolor* (L.) Moench) genotypes to NaCl levels at early growth stages. African Journal of Agricultural Research Vol. 7(43): 5711 -5718.

Annex-1 Consent Form and Questionnaire, English Version

Addis Ababa University college of Natural science

Center for food science and Nutrition

Questionnaire for assessment of the Aspergillus species and Aflatoxin levels in sorghum bicolor (L.) stored at different storage system and periods in kewet Woreda Northern Showa, Ethiopia

1. Date of interview_____/_____/_____

2. Questionnaire identification number _____

3. Interviewer name_____ signature_____

Checked by supervisor. Signature _____date_____

Introduction

"My name is…….I am working as a data collector for a research being conducted by AAU, college of Natural science Center for food science and Nutrition. We are interviewing and testing ***Aspergillus species and Aflatoxin levels at various stored sorghum bicolor (L.) in Kewet Woreda Districts, Northern Shewa Ethiopia.*** Contamination is a potential source of health hazards and the spoilage of agricultural commodities**.** Your storage has been selected for this study. This finding of the study will be used as a basis for better planning of prevents possible aflatoxin contamination.

Verbal consent and confidentiality

Your answers are completely confidential. Your name will not be written on this form, and will never be used in connection with any of the information you tell me. You don't have to answer any questions that you don't want to answer, and you may end this interview at any time you want to. However, your honest answers to these questions and giving blood sample will help us better understand the Aspergillus flavus and Aflatoxin levels in various stored sorghum bicolor (L.) in Northern Showa Ethiopia**.** We would greatly appreciate your help in responding to this

survey. The study will be taken one time after stored only 1 kg of sample will be taken .Would you be willing to participate?"……

(Signature of interviewer certifying that informed consent has been given verbally by respondent).

4. Age_____, Sex_____, Educational level (**A**, illiterate, **B**, primary school, **C**, secondary school)

No	**Questions**	**Coding categories**	**Skip to**
101	Have You ever Heard about Mould?	Yes…….....1 No……...2	103
102	How do You discribe the type of color	_____	
103	Do you have storage problems?	Yes……1 No……..2	106
104	Which storage problem is the most important?	Insects……1 Rodents……..2 Birds……...…….3 Mould…….4 Others_____	
105	What did you do to solve this problem?	No treatment……..1 Storage insecticides…….……...…….2 Smoke……..3 Other (specify)_____	
106	What Drying method do you use?	Sun …….…….…...….1 Drying machine…….…….....…..2 Other, specify_____	

107	Do you clean the store house before storage?	Yes……..……1 No……...2	
108	Where is your storage structure located?	Field……..……..,……1 In the house…….…..…….2 Courtyard…….…….3	
109	Do you store other products in the store, together with sorghum?	No…….,,,……..1 Yes……..……2 list?_____	
110	What storage method do you use?	_____	
111	For how long do You store the sorghum grain?	_____	

Annex 2: Consent Form and Questionnaire, Amharic Version

በአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና ስነ-ምግብ ትምህርት ክፍል

በአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ ሳይንስና እና ስነ-ምግብ ትምህርት ክፍል የአፍላቶክሲን ብክላ በማሽላ ምርት ክምችት ላይ በተመለከተ ለሚደረግ ጥናት የተዘጋጀ መጠይቅ

1.ቃለመጠይቅ የተካሄደበት ቀን _____/_____/_____

2.መለያቁጥር _____

3.የጠያቂውስም_____ፊርማ_____

የሀላፊው ማረጋገጫ. ፊርማ _____ቀን_____/____/___

መግቢያ

ሰላምታ። ስሜ _____ይባላል።እኔ ለአዲስ አበባ ዩኒቨርስቲ ሳይንስ ፋካልቲ የምግብ እና ስነምግብ ትምህርት ክፍል ለሚደረግ ጥናት መረጃ ሰብሳቢ ነኝ። እኛ እዚህ አካባቢ በሚገኙ የማሽላ ምርት ላይ የአፍላቶክሲን ብክላ በማሽላ ክምችት ላይ ለሚደረግ ጥናት ቃለ መጠይቅና በማሽላ እየሰበሰብን እንገኛለን። አፍላቶክሲን ማልት ፈንግስ በተባለ በተለይም አስፓርጅን በተባለ የሚፈጠር መርዛማ ነገር ሲሆን በሰወች ጤና ላይ ጉዳት የሚያመጣ ሲሆን በተጨማሪም በምርታማነት ላይጉዳትያመጣል።

የሚሰጡት መረጃ ሚስጥርነቱ ሙሉ ለሙሉ የተጠበቀ ነዉ። መጠይቁ ላይ የርሰዎን ስም የሚገልፅ ማንኛዉም አይነት ነገር አይጠቀስም ወይም አይያያዝም፣ በመጠይቁ ወቅት የማይፈልጉትን ማንኛዉንም አይነትጥያቄ መተዉ ወይም በማንኛዉም ሰዓት መጠይቁን ማቋረጥ ይችላሉ። ሆኖም ግን እርሰዎ የሚሰጡን እዉነተኛ መረጃ ወደፊት አፍላቶክሲን በማሽላ የምርት ክመችት ላይ በተመለከተ ለሚያደርጉ ጥናቶች ጠቃሚ ይሆናል። ለዚህ ጥናት ለሚያደርጉልን ትብብርም ስጋናችን ከልብ የመነጨ ነዉ።ስለዚህ በመጠይቁ ለመሳተፍ ፈቃደኛ ነዎት?

_____

(የጥናቱ ተሳታፊ ሙሉ ፈቃደኝነት ያረጋገጠዉ የጥናቱ መረጃሰብሳ ቢፊርማ)

4. መረጃውን የሰጠው እድሜ.....ዕታ .....የት/ደረጃ.....

ተቁ	ጥያቄ	መልሶች	ይለፍ
101	ስለሻጋታ ሰምተው ያውቃሉ?	አዎ……1 አላውቅም……..2	201
102	እባክወ ቀለሙ ምን ዐይነት እንደሆነ ይግፁልን ;	----------	
103	ምርተዎን ለርጅም ግዜ ስያስቀምጡ ችግር ገጥሞወት ያዉቃል	አዎ……..1 አልነበረም……2	204
104	አዎካሉ ምን አይነት ?	ነፍሳት……1 አይጦች……...2 ወፎች……...……...……3 ሻጋታ……..……4 ሌላ _____	
105	እነዚህን ለማጥፋት ምንተጠቀሙ?	ምንም……1 ተባይማጥፍያ……..……..…..2 ሞዶስ……..……3 ሌላ _____	
106	ምርተዎን ለማድረቅ ምን ይጠቀማሉ	ፀሀይ ……..…..1 የማድረቅያመሳርያ…….....2 ሌላ _____	

107	ምርተዎን ለርጅም ግዜ ከማስቀመጠዎ በፊት ቦታዉን ያፀዳለ?	አዎ.....1 አላፀዳም.....2	
108	ምርተዎን ለርጅም ግዜ የት ያስቀምጣለ?	ሜዳላይ.....,.1 ቤትዉስጥ.....2 ከቤትአጠገብ.....3	
109	ከማሽላ ጋር ሌላ ምርት ቀላቅለዉ ያስቀምጣለ?	አላቀምጥም.....1 አዎ.....2 ይዘርዝሩልን _____	
110	የማሽላ ምርተዎን በምን ያስቀምጣለ?	_____	
111	የማሽላ ምርተዎን ለምን ያህል ግዜ ያስቀምጣለ?	_____	