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DETERMINATION OF AFLATOXIN LEVELS IN STORED MAIZE OF JIMMA TOWN, ETHIOPIA

GETNET DEMIS

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DETERMINATION OF THE LEVELS OF AFLATOXIN IN STORED MAIZE OF JIMMA TOWN, ETHIOPIA

BYGETNET DEMIS

A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES JIMMA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN CHEMISTRY (ANALYTICAL)

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ABBREIATIONS

AOAC ---------- Association of Official Analytical Chemists

APOD ---------- Afram Plains Development Organization

B1, B2 ---------- Blue 1 & 2

CEC ---------- Commission of European Community

CRI ---------- Crop Research Institute

CSIR ---------- Council for Scientific and Industrial Research

EHSO ---------- Environmental Health Service On Line

EU ---------- European Union

FAO ---------- Food and Agricultural Organization

FDA ---------- Food and Drug Administration

G ---------- Green

GAP ---------- Good Agricultural Practices

HACCP ---------- Hazard Analysis Critical Control Point

IARC ---------- International Agency for Research on Cancer

IgA ---------- Immunoglobulin A

IITA ---------- International Institute of Tropical Agriculture

M1, M 2 ----------Metabolites 1 & 2

PBS ---------- Phosphate buffered saline

PPMED ----------Policy Planning Monitoring and Evaluation Department

SPSS ---------- Statistical Package for Social Sciences

SRID ---------- Statistical Research and Information Directorate

TPI ---------- Tropical product institution

US ---------- United States

USFDA----------United States‟ Food and Drug Administration

USDA ---------- United States Development Agency

WHO ---------- World Health Organization

WFP ----------World Food Program

Abstract

In this study the levels of aflatoxins (B1, B2, G1, and G2) in maize samples stored in Jimma town, Ethiopia were analyzed by high performance liquid chromatography with Fluorescence detector. The maize samples were collected from four different maize stores in the town using standard sampling protocol. The average moisture content of the samples was also investigated. The average moisture content of the maize samples collected from store 1, store 2, store 3 and store 4 were 11.215, 11.326, 11.312 and 10.945 %, respectively and the obtained moisture contents were all below the standard value, i.e., 14%. The mean total aflatoxin levels in the collected maize samples from store 1, store 2, store 3 and store 4 were 0.617, 0.620, 0.267, and 0.315 µg/kg, respectively. The levels of aflatoxin detected in all the studied maize samples were lower than the maximum tolerance limits set by international organizations such as the US Food and Drug Administration, World Health Organization and the European Union. Thus, the maize samples stored in Jimma town are safe in terms of the studied aflatoxins.

Keywords: *Aflatoxins; Aspergillus fungi*; *stored maize of Jimma town; high performance liquid chromatography; Fluorescence detector.*

1. INTRODUCTION

1.1 Background of the study

Aflatoxins are toxic metabolites of Aspergillus fungi that can contaminate various foods and feed products. They are the most common and highly potent mycotoxins and are produced by Aspergillus species of fungi such as A. Flavus, A. parasiticus, A. nomius and, A. nigur [1]. Aflatoxins are largely associated with commodities produced in the tropics and subtropics, such as maize, rice, sorghum, barley, rye, wheat, groundnut, soyabean and cottonseed [2]. Of these products, aflatoxins highly affect maize and groundnut [3]. But, these two crops play an important role in the diets of Ethiopian people. Their infection by aflatoxicogenic fungi and hence contamination with aflatoxin is generally higher. Consequently, the exposure of human and animals to this toxin is higher and cause huge health and economic problems. A. parasiticus and A. flavus are common and widely distributed in tropical and sub-tropical parts of the world [4]. Ethiopia as a tropical country has a high risk of aflatoxin contamination. Infestation of grains with aflatoxin contamination could occur during pr- and/or post- harvest (at storage).

Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds mainly attack the liver [5]. The diseases caused by aflatoxin consumption are loosely called aflatoxicoses [6]. Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other “slow” pathological conditions [7]. The extent of damage by aflatoxin depends on the concentration of aflatoxin, way of exposure, the condition of the organism and others [8]. Once infected/injured by aflatoxin it is difficult for treatment and thus, controlling of grains from aflatoxin contamination by different management practices is appropriate remedy [9].

As has been mentioned earlier, Ethiopia is favorable for the growth of aflatoxicogenic fungi and hence aflatoxin contaminations of grains are mostly occurred. Amare A., 2010 [10] reported that, from 123 maize samples studied, 16 maize samples were contained 0.78 μg/kg aflatoxin. On the other hand, liver disease is one of the ever increasing and cause diseases in Ethiopia. Most likely, consumption of aflatoxin contaminated food is one of the expected

reasons for raising this disease [11].Animal feeds, livestock and animal products like milk could also be contaminated by aflatoxin. Damage caused by aflatoxin is usually occurred due to lack of awareness about aflatoxine effect on human health, lack of regulations, poor management and other [12].

In recent years, mycotoxins contents of maize in some African countries including Kenya [13], Nigeria [14] and Benin [15] have been reported. Some works have also reported on levels aflatoxines in pre and post-harvest maize's from four towns including Dire Dawa, Adama, Gojam and Ambo) [10]. But, despite of the fact that, maize is mainly used for food to Ethiopian and highly cultivated in Jimma zone and other south western part of the country, no report has been made on the aflatoxins content of maize produced in this area. Figure1.Shows maize which is infected by fungi, *A. flavus,* in the field.

Figure1. A. flavus infected maize

Therefore, in this study, the levels of aflatoxins (B1, B2, G1, and G2) in maize samples stored in different maize stores of Jimma town, Oromia Regional State, Ethiopia were investigated. In the study, high performance liquid chromatography with fluorescence detector was employed for separation and quantitative determination of the aflatoxins.

1.2 Statement of the problem

Aflatoxins develop in maize during pre- and/or post-harvest, making the product unwholesome for consumption. The predisposing factors of infection (development of aflotoxins) include improper drying, high relative humidity and temperature, farmers' production practices such as intercropping with aflatoxins infected grains, early and delayed harvesting and poorly constructed storage structures [12]. Maize exposed to these factors has a high probability of fungal infection (*Aspergillussp*) which may, presumably, enhance for the development of aflatoxins. It is therefore assumed that, since climatic conditions, especially rainfall, temperature and relative humidity as well as storage structures vary in the country, the infection of maize by fungi and the subsequent development of aflatoxins may also be varied [16].

Therefore, determination of levels of aflatoxins in maize cultivated in different part of the country is crucial. Determination aflatoxin levels, particularly, from the areawhere there is much expectation for its presence in stored maize in relatively high humidity and temperature, as well as heavy rain fall areas such as Jimma is important. Based on this fact, this study was conducted to answer the following research questions.

- Do maize products stored in different maize stores of Jimma Town contain aflatoxin?
- Do the levels of aflatoxins in maize of Jimma Town safe for consumption?

1.3 Objectives of this study

1.3.1 General objective

- To determine the level of aflatoxin in maize samples from different stores of Jimma Town, Ethiopia.

1.3.2 Specific objectives

- To analyze the concentrations of aflatoxin (B1, B2, G1, and G2) in maize samples collected from different stores in Jimma town.
- To compare the levels of aflatoxins in the stored maize of Jimma town with the reported values.
- To compare the levels of aflatoxin in the studied samples with the national and international recommended maximum tolerance limits, for the safety consumption of products.

1.4 Significance of the study

The study could have the following significances:

- It could give an insight about the contamination status of the stored maize of Jimma town by aflatoxins.
- It could also help farmers, business people (the store owners) and other consumers on how to store maize, to protect the product from infection by aflatoxin.
- It can be used as a reference material for researchers, who are interested to perform further study on aflatoxin contents of maize or other agricultural products.

2 LITERATURE REVIEW

2.1 Aspergillus fungus

Aspergillusis a filamentous, cosmopolitan and ubiquitous fungus found in nature. The first attempt to define the genus Aspergillus was made by Micheli in 1729 [17]. Although, Aspergillus is a separate genus, it is closely related to Penicillium species in the fungal kingdom [18]. The genus Aspergillus includes over 200 species [19]. Of these, around 20 species have so far been reported as causative agents of opportunistic infections in man [19]. Aspergillus fumigates is the most commonly isolated species, followed by *Aspergillus flavus and Aspergillus niger*. *Aspergillus clavatus, Aspergillus glaucus group, Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus ustus, and Aspergillus versicolor* are among the other species less commonly isolated as opportunistic pathogens [20]. Aspergillus flavus and A. parasiticus are agriculturally important species and they are found worldwide, both in the soil and the air [21]. They rapidly colonize and produce aflatoxin, when their conidia (spores) get suitable nutrient sources and favorable environmental conditions (hot and dry conditions) [22].

2.2 Aflatoxins

Aflatoxins are toxic carcinogenic by-products of the molds Aspergillus flavus and Aspergillus parasiticus [21]. They naturally occur mixture and have been classified as class 1 human carcinogen [23]. The name aflatoxin was derived from a toxin producing fungus which caused a disease referred to as “Turkey X disease” in England in 1960 which resulted in the death of 100,000 young turkeys. The fungus was identified as Aspergillus flavus in 1961 and the toxin was named aflatoxin due to its origin (A.*flavis-Afla*) [24]. Aspergillus flavus is common and widespread in nature. It is found in the soil, decaying vegetation and grains undergoing microbial deterioration [21]. Keller, N., 1994 [25] reported that attempt had been made to isolate genes associated with aflatoxin biosynthesis through cloning of genes, to understand enzymes that regulate its biosynthesis. The Information gained on the regulation of its genes could help to develop control strategies through inhibition of these controlling genes.

2.2.1 Chemical structure of aflatoxins**.**

The chemical structures of some aflatoxins are shown in Figure. 2

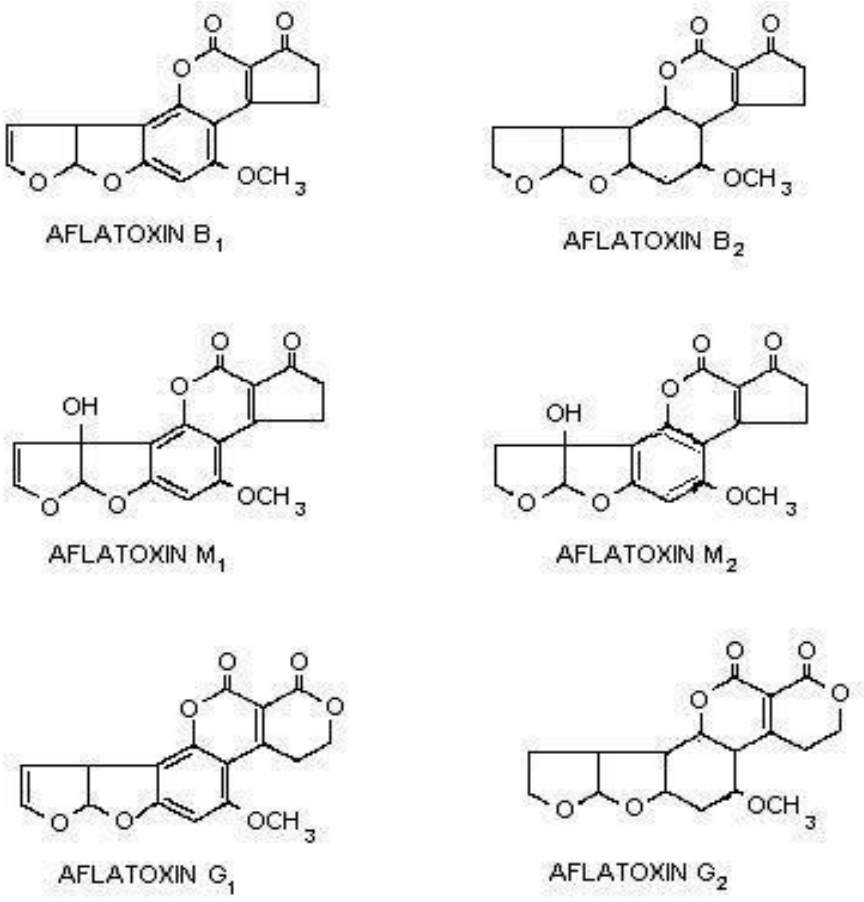

Figure 2: Chemical structure of aflatoxins

2.2.2 Properties ofaflatoxins

There are four major groups of aflatoxins: B1, B2, G1 and G2 [26]. Abbreviations, B and G, are indicative of the colors that these compounds exhibit/fluorescence under the ultraviolet light. Thus B is for blue and G is for yellow-green, as well as there is also abbreviation, M, which stands for the hydroxylated metabolic product of B [27]. Chemical formula, molecular weight and melting point of aflatoxins B1, B2, G1, G2 and M1 are presented in Table 2.

Table 1: Chemical formula, molecular weight and melting point of aflatoxins B1, B2, G1, G2 and M1

Property	Aflatoxins				
	B1	B2	G1	G2	M1
Chemical Formula	$C_{17}H_{12}O_8$	$C_{17}H_{14}O_8$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$	$C_{17}H_{12}O_7$
Molecular weight	312	314	328	330	328
Melting point (°C)	268-269	287-289	244-249	230	299
Fluorescence	425 nm	425 nm	450 nm	425 nm	425 nm

2.2.3 Absorption, metabolism, excretion and mechanisms of action of aflatoxins

Aflatoxins are highly lipo-soluble compounds and are readily absorbed from the site of exposure usually through the gastrointestinal tract and respiratory tract into blood stream [28]. Human and animals get exposed to aflatoxins by two major routes (a) direct ingestion of aflatoxin contaminated foods or ingestion of aflatoxins carried over from feed into milk and milk products like cheese and powdered milk as well as other animal tissues mainly as AFM_1 [28] (b) by inhalation of dust particles of aflatoxins especially AFB_1 in contaminated foods in industries and factories [29]. After entering the body, the aflatoxins are absorbed across the cell membranes where they reach the blood circulation. They are distributed in blood to different tissues and to the liver, the main organ of metabolism of xenobiotics. Aflatoxins are mainly

metabolized by the liver to a reactive epoxide intermediate or hydroxylated to become the less harmful aflatoxin M1 [30]. In humans and susceptible animal species, aflatoxins especially AFB_1 are metabolized by cytochrome P450 (CYP450) microsomal enzymes to aflatoxin-8,9-epoxide, a reactive form that binds to DNA and to albumin in the blood serum, forming adducts and hence causing DNA damage [31]. Various CYP450 enzymes isoforms occur in the liver and they metabolize aflatoxin into a reactive oxygen species (aflatoxin-8,9-epoxide), which may then bind to proteins and cause acute toxicity (aflatoxicosis) or to DNA and induce liver cancer [31]. The predominant human CYP450 isoforms involved in human metabolism of AFB_1 are CYP3A4 and CYP1A2. Both enzymes catalyze the biotransformation of AFB_1 to the highly reactive exo-8, 9-epoxide of AFB1 [27]. CYP 1A2 is also capable of catalyzing the epoxidation of AFB_1 to yield a high proportion of endo-epoxide and hydroxylation of AFB1 to form aflatoxin M_1 (AFM_1), which is a poor substrate for epoxidation[32] and less potent than AFB_1 [33]. This is generally considered as the major detoxification metabolic pathway for aflatoxins. The CYP3A4 is the major CYP450 enzyme responsible for activation of AFB_1 into the epoxide form and also form AFQ_1, a less toxic detoxification metabolite. The CYP3A5 metabolizes AFB1 mainly to the exo-epoxide and some AFQ_1 [34]. However, polymorphism studies with CYP3A5 have indicated that, this enzyme iso- form is not expressed by most people especially in Africans [33]. Studies in Gambian children showed that aflatoxin cross the placenta and transported to the fetus and the new born where they can cause detrimental effects [33]. The CYP3A7 is a major CYP450 enzyme iso-form in human fetal liver and metabolizes AFB_1 to the 8, 9- epoxide that may cause fetal defects to the developing fetus [35].

The epoxidation of AFB_1 to the exo-8, 9-epoxide is a critical step in the geno toxic pathway of this carcinogen. The binding of AFB1 to DNA and DNA adduction by AFB_1 exo-8,9 epoxide has been reported to cause a functional changes of DNA conformation [36].The epoxide is highly unstable and binds with high affinity to guanine bases in DNA to form afltoxin-N7-guanine [37]. The aflatoxin-N7-guanine has been shown to be capable of forming guanine (purine) to thymine (pyrimidine) transversion mutations in DNA and hence affecting the p53 suppressor gene in the cell cycle [38]. The p53 gene is important in preventing cell cycle progression when there are DNA mutations, or signaling apoptosis. The mutations have been reported to affect some base pair locations more than others especially in the third base of codon 249 of the p53 gene in the region corresponding to the DNA binding domain of the

corresponding protein [38]and this appears to be more susceptible to aflatoxin-mediated mutations than nearby bases [39]. AFB1 induces the transversion of base G to base T in the third position of codon 249 and similar mutations have been observed in hepatocellular carcinoma (HCC) in high AFB_1 contaminated food in regions in East Asia and Africa [38].

Epoxide hydrolase and glutathione-S-transferase (GST) are both involved in hepatic detoxification of activated AFB_1, but the GST-catalyzed conjugation of glutathione to AFB_1-8,9-ep- oxides is thought to play the most important role in preventing epoxide binding to target macromolecules like DNA and various cell proteins [40]. Glutathione pathway is reported to play a vital role in the detoxification of AFB_1 [41]. The AFB_1 8, 9exo and endoepoxides are conjugated by glutathione to form AFB-mercapturate and the reaction is catalyzed by gluta-thione S-transferase (GST) [41]. The glutathione-aflatoxin conjugate is transported from the cells with an ATP-dependent multidrug-resistance protein through an accelerated process [41]. Despite a preference for conjugating the more mutagenic AFB_1exo-epoxide isomer, the relatively low capacity for GST-catalyzed detoxification of bio-activated AFB_1 in lung may be an important factor in the susceptibility of the lung to AFB_1 toxicity .The exo and endo epoxide can also be converted non-enzymatically to AFB_1-8,9-dihydrodiol which in turn can slowly undergo a base catalyzed ring opening reaction to a dialdehyde phenolate ion [32]. AFB_1dialdehyde can form Schiff bases with lysine residues in serum albumin forming aflatoxin-albumin complex [42]. Also the aflatoxin dialdehyde are reduced to a dialcohol in a NADPH-dependent catalyzed reaction by aflatoxin aldehyde reductase (AFAR) [43]. However the guanine alkylation by aflatoxin B_1 produces exo-8,9-epoxide which is the reactive form and a carcinogen to the liver and the reaction is more than 2000 times more efficient in DNA than in aqueous solution [44], (Figure 3)

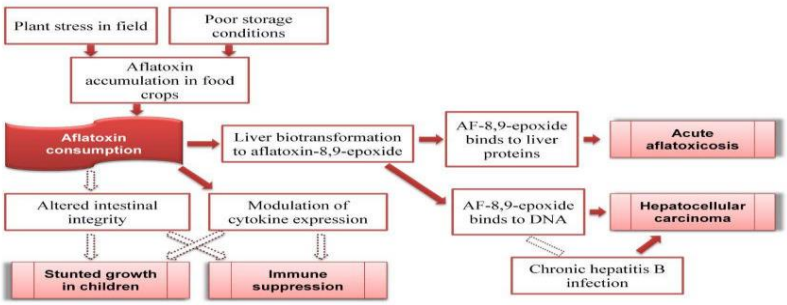

Figure 3.Aflatoxin disease pathways in humans

2.4 Overview of the maize in Ethiopia

Maize has been introduced to Ethiopia in the 1600s to 1700s [45].It grows under a wide range of environmental conditions between 500 to 2400 meters above sea level [46]. In Ethiopia, it is a leading cereal in terms of production, with 6 million tons produced in 2012 by 9 million farmers across 2 million hectares of land [46]. Over half of all Ethiopian farmers grow maize, mostly for subsistence, with 75% of all maize produced being consumed by the farming household [46, 47]. It is also the cheapest source of calorie intake in Ethiopia, providing 20.6% per capita calorie intake nationally [47].

Maize is an important crop for overall food security. It is also used for making local beverages [48]. Additionally, the leaves and Stover's of maize are used to feed animals and the stalks are used for construction and fuel [48]. A small quantity of the grain produced is currently used in livestock and poultry feed, and this is expected to increase with the development of the livestock and poultry enterprises in the country. The green fodder from thinning and topping is an important source of animal feed and the dry fodder is used during the dry season[47]. Moreover, the crop has potentially used for industrial purposes, serving as a starch, a sweetener for soft drinks, an input for ethanol fuel production and oil extraction, etc. [48]. Ethiopia is the fourth largest maize producing country in Africa, and first in the East African region [49]. Ethiopian produces non-genetically modified (GMO) white maize, which is preferred in the neighboring markets [50].

2.5 Maize contamination by aflatoxin

Maize has been documented by several authors as an excellent substrate for mold growth and aflatoxin contamination in some West east African countries [51]. Setamou M., et al 1997[15] Reported that 42.5% and 30% of pre-harvested maize samples in Benin collected in 1994 and 1995, respectively contains aflatoxin. Bouriama Y., et al 1993[52] Also found that aflatoxin levels in stored maize in Benin were 14µg/kg for B1 and 58µg/kg for G1. Udoh JM., et al 2000 [53] reported that 33% of maize samples from different ecological zones in Nigeria were contaminated with aflatoxin. Hell K., et al 2000a[54] reported that 9.9% -32.2% of maize

samples of different eco-zones in Benin prepared for storage had aflatoxin levels more than 5 µg/kg and the levels increased to 15% and 32.2% after six months of storage. Kpodo k., 1995 [55] reported that maize samples from silos and warehouses in Ghana contained aflatoxin levels ranging from 20 -355 µg/kg; while fermented maize dough collected from major processing sites contained aflatoxin levels of 0.7 -313 µg/kg.

Insects have also been reported as playing a role in the spread of A. flavus and increase in aflatoxin contamination. Setamou M., et al 1997 [15] found that the percentage of grains infected with A. flavus and samples contaminated with aflatoxin including the mean aflatoxin content of samples increased correspondingly with increased insect damage in pre-harvest maize in Benin [56]. Invariably, Hell K., et al 2000b[56] found out that maize free of insect damage had no aflatoxin contamination, but maize with 70% of the cobs damaged by insects had 30.3% of the cobs contaminated with aflatoxin. Payne G.A., 1992[57] Indicated that pre-harvest aflatoxin production in maize depended on weather conditions during crop maturations. In addition, the risk of aflatoxin contamination before harvest is highest when there is moisture stress coupled with elevated temperatures [57].

2.6 Effects of Aflatoxin in Ethiopia

Aflatoxins are of major interest because of their impact on both human and animal health. Of the four different types of mycotoxins aflatoxin B1 is the most toxic, is a potent carcinogen and has been directly correlated to adverse health effects, such as liver cancer, in many animal species [23].

In Eastern Ethiopia, aflatoxin levels ranging from 5 -250 µg/kg were detected in groundnut samples [58]. Another study also showed that 4.1µg/kg of aflatoxin was detected from maize in Ethiopia [10]. Two major aflatoxin producing fungi namely, A. flavus and A.nigurare identified from groundnut at Northern Ethiopia [59]. Similarly Alemayehu c., et al 2014[60] also reported that all the samples of sorghum and finger millet from Ethiopia were contaminated with Aspergillus species. In late 1985 A. flavus was isolated from 70% of the maize samples. Early in 1985 [61], isolated A. flavus from 70% of the maize samples and reported that 80% of the isolates were capable of producing aflatoxin.

A survey by Dejene A., et al 2012 [59] from Northern Ethiopia indicates that there is 100% positive for Aspergillus fungi from samples of groundnut. The presence of aflatoxins in food means a risk for both animals and human beings. Another survey by Amare A., 2010 [10] from Ethiopia revealed that Aspergillus species has occurred from 94% of samples from all sample areas and aflatoxin was detected from 88% of the samples. Risk of aflatoxins exposure in Africa is very high, margin of exposure less than 10 in most countries. Aflatoxin has impact on agriculture (crop production and animal husbandry), health, trade, and economy and food security. A survey by Lou O., 2008 [62], at different locations of Ethiopia from maize indicates that around 50% of the samples were positive for AFB1 and 9.4% were positive for AFB2 at a range of 10.2-80 µg/kg.

In Ethiopia, aflatoxin B1 was detected in four major crops: barley, sorghum, teff and wheat [63]. Similarly, Habtamu F., et al 2001[4] has detected aflatoxin from maize, wheat, barley, teff, millet, sorghum, groundnut, faba bean, pea and pepper from Ethiopia, which are the major staple crops for the country. Aflatoxin analysis from Southwestern Ethiopia indicates that 3.33% of maize samples tested showed positive results. [64] In this experiment AFB1, AFG1, AFB2 and AFG2 were detected at concentrations of 41.08, 38.79, 7.56 and 5.16µg/kg, respectively. Alemayehu C., et al 2014[60] Also reported 36-100% aflatoxicogenic fungi incidence in Ethiopia from groundnut. Similarly, AFB1 contamination in maize in south Ethiopia was 22.72µg/kg [65].

WHO, 2011[66] Reported that around 4.5 billion people are chronically exposed to aflatoxin contamination. The existing food shortage in Ethiopia forces people to consume what they might have otherwise rejected, even when the food is moldy and organoleptically unacceptable. This exposes at least some of the population to a higher risk of consumption of aflatoxin contaminated food. Aflatoxin B1 is one of the most potent hepato-carcinogens known and hence levels of aflatoxins in the diet are an important consideration for human health. Aflatoxin B2 is of less toxicological significance since it is found at much lower levels than Aflatoxin B1, and is always present with Aflatoxin B1 [2]. IARC, 2002 [67] has classified aflatoxin B1 as a group 1 carcinogen agent (that means carcinogenic to humans).

A survey from Addis Ababa and its surrounding cities indicates that all milk samples were containing ranging from 0.028µg/L- 4.98µg/L. From this study 93% of the sample milk in the area exceeds the EU maximum tolerable limit of aflatoxin in milk, which is 0.05mg/L [68]. The finding indicates that there is high exposure of aflatoxin contamination in the area. The presence of AFM1 in milk poses risk on health of humans, especially children, as it can have immunosuppressive, mutagenic, teratogenic, and carcinogenic effects [69]. A report by PACA, 2012 [70] also indicates that aflatoxin contamination of key staple crops- maize, groundnut and sorghum occur above safe level which is higher than the EU and USA standard for many African countries [71].

The presence of high concentration of aflatoxin in food and feed products of Ethiopia may be due to conductive climatic conditions, traditional crop production practices, inadequate harvesting, drying and storage practices, policy and institutional capacity, lack of awareness and high reliance on one or two crops for food [12]. EC, 2010, [71] has reported Eastern and Southern Africa region are highly contaminated with aflatoxin occasionally above the internationally recommended maximum limit.

Aflatoxins also cause a variety of adverse effects in different domestic animals [72]. In animals, aflatoxins impair growth and are immunosuppressive. Among livestock, they are particularly toxic to chickens. Effects on chickens include liver damage, impaired productivity and reproductive efficiency, decreased egg production in hens, inferior egg-shell quality, and inferior carcass quality and, most important from a human perspective, increased susceptibility to disease [7]. In livestock, consumption of very high levels of aflatoxins causes acute toxicity and death, while chronic consumption of lower levels can cause liver damage, gastrointestinal dysfunction, and decrease in appetite, reproductive function, growth, average daily gain, body weight and production [73]. The effects of aflatoxicosis are similar in ruminants and non-ruminants. A dose of 0.2 mg/kg body weight can cause a decrease in weight gains.

3 MATERIALS AND METHODS

3.1 Description of the study site

The study was carried out in the capital of Jimma zone, Jimma Town, Oromia Regional State, Ethiopia, which is located at about 346.1 km southwest of the country. Jimma zone lies between latitudes 7°15´ N and 8°45´ S, and longitudes 36° 00´ E and 37°40´ E. The elevation of the zone ranges from 880 to 3360 m. a. s. l. The area experienced an annual average rainfall of 1000 mm for 8 to 10 months [74]. The main rainy season extends from May to September and small rainy season may also occur from February to April. The temperature of the zone varies between 8 to 28°C with an annual average of 20°C. It has a sub-humid, warm to hot climate. The study was conducted on maize harvested2017/2018 production year. Figure 4 shows map of Jimma Town.

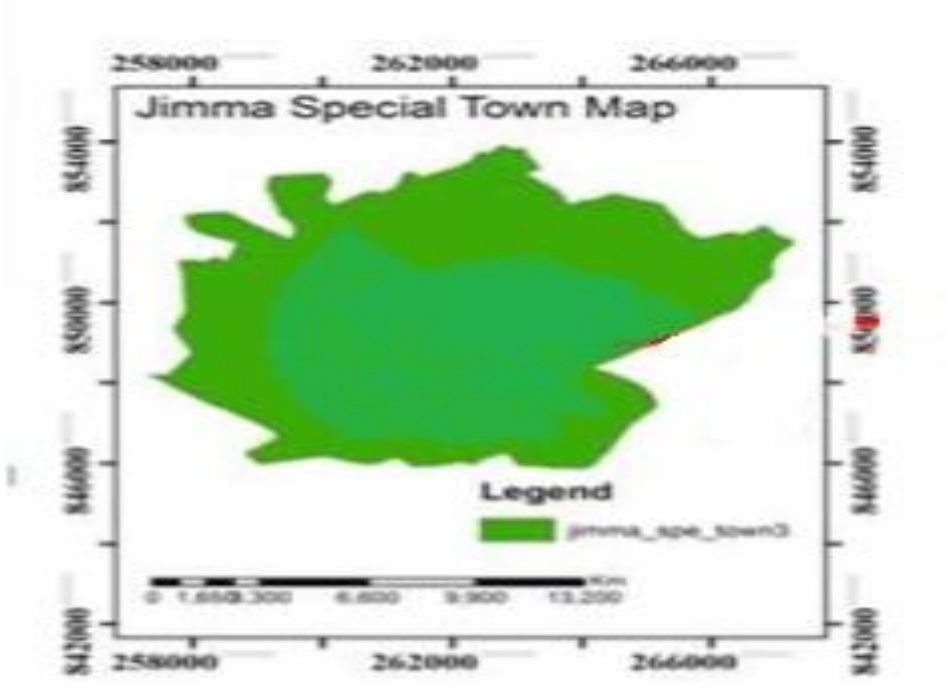

Figure 4: Jimma town map

3.2 Chemicals and reagents

Chemicals and reagents used in this study were analytical and HPLC grades. Analytical grade standards of Aflatoxin standards **(**B1, B2, G1, G2), and Trifluoroacetic acid (TFA) as well as HPLC grade methanol (99.8%) were obtained from Sigma-Aldrich (St. Louis MO, USA). HPLC grades acetonirile and n-hexane were obtained from (France) and from BDH Chemicals Ltd (Poole, England), respectively. Sodium chloride (99.5%and phosphate buffered saline (PBS) were supplied by Loba chemie pvt. Ltd., (Jehangir villa, Mumbai, India).

3.3 Instruments and Apparatus

High performance liquid chromatography (Agilent technologies USA, 1260 infinity) with fluorescence detector, ZORBAX Eclipse plus C18, Analytical column (4.6 x 150mm, 5 μm), Adjustable Pipettes (SOCOREX; Acura), Micro filter,, Centrifuge(Thermo Scientific Reacti-Them III # 18824 ,USA), Analytical balance (model mettle Toledo, Columbus, USA), Homogenizer (Kenwood , chef), Mechanical miller (Thomas- wiley , model 4 , USA), Sonicatore (ultra wave), Immunoaffinity column (LC Tech, GmbH, Germany) were employed during the study.

3.4 Sample collection

Four maize stores having almost similar features like; cement ground, good air ventilation, which store only maize products were purposively selected from the main local market area (i.e., Merkato) of Jimma town. The names of the considered stores are, Abduka Maize Store (ST1), Nigatu Maize Store and Distributors (ST2), Eyshalu Maize Stores and Distributors (ST3) and Sitina maize stores (ST4). They are well known as the main maize stores and distributors for the surrounding community. They also obtain maize products from different Jimma zone districts. After identifying the stores, sampling plan was designed based on the reported procedure [76]. The sampling plan was not similar for different stores, depending on the number of maize containing sacks in the store (lot). The number of sacks to be sampled was calculated by the following formula [76].

$$\mathbf{NS} = \mathbf{4}\sqrt{SL}$$

Where: NS and SL are minimum number of sacks to be sampled and number of sacks of the lot (store), respectively.

Then, the sample size was determined by following the standard sample size determination protocol [75, 77]. According to the standard, 0.2 kg incremental sample was taken from each sack until it produces a bulk sample of 5.0 kg. By using the formula the amount of sack to be sample was calculated and marked by a systematic sampling. From every marked sack an incremental sample (0.2 kg) was collected from the top, middle and bottom of the sack using a sharpen steal spoon in to clean polyethylene bags with hermetic sealing. Then, after labeling it was transported to the laboratory for analysis of aflatoxin.

3.5 Sample Preparation

The collected incremental maize samples were homogenized and a representative sample of 1 kg was taken and milled to a desired particle size (1mm) by mortar and pestle. The ground maize was homogenized for 2 minutes using homogenizer and then, 20g was taken and submitted for aflatoxin analysis. The rest of the ground maize sample was stored at -15^{o}C.

3.6 Extraction and clean-up

The standard extraction and clean-up procedure for analysis of aflatoxin was utilized AOAC, 2005[78]. 20 g ground maize sample and 5 g NaCl were weighed and mixed in blending jar, containing100 mL of methanol: distilled water (80:20, *v/v*) solution. The mixture was blended at high speed for about10 min. The solution was then filtered with whattman filter paper. Then, after mixing 10 mL of the filtrate and 40 mL phosphate buffered saline (PBS) and sonicated for 2 – 3 min. Then, 25 mL of the resulting solution was transferred to the glass syringe barrel to elute through the prepared immune affinity column at 2-3 mL/min flow rate. Following this, 10 mL distilled water was added to wash the column. After eluting the residual water, 2 mL methanol was added and waited for 25 min to completely elute aflatoxins. Then, 500 µL of the extract was transferred into vial and dried by purging nitrogen gas. Finally, the residue was reconstituted via 200 µL hexane and 200 µL trifluoro aceticacid (TFA) and incubated for 10 min, before injecting 50 µL into HPLC system.

3.7 Chromatographic conditions

Chromatographic separation was performed in isocratic mode using ternary mobile phase consisting of deionized water (solvent A), Methanol (solvent B) and Acetonitrile (solvent C) at 65:25:15, *v/v*), column temperature 30 ºC and 1.5 mL/min flow rate. Figure 5 shows the chromatograms of the target aflatoxins under these chromatography operating conditions.

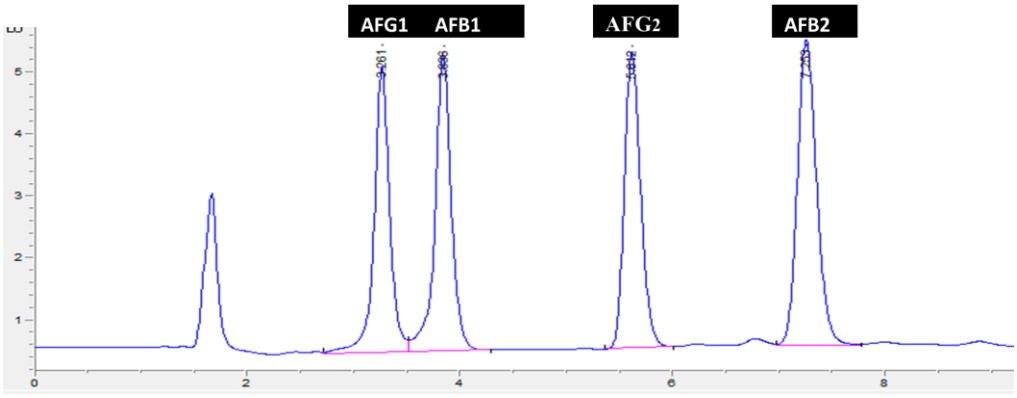

Figure 5.Chromatograms of the target aflatoxins.

3.8 Determination of moisture content

Moisture contents of the maize samples were determined according to Ethiopian standard [79]. The dishes used for the moisture determination were oven dried at 105ºC for 1 h and 30 min in desiccators. Then, after measuring the mass of the dish (W1), maize sample was added to the dish the mass was recorded (W2). Then, the sample was thoroughly mixed and oven dried at130ºC for 4 h. After drying was completed, the mass was measured (W3), and finally moisture content was calculated as follows:

%Moisture content $=((W2-W3)/(W2-W1))\times 100\%$

Where: W1 is Weight of a crucible in gram, W2 is weight of crucible plus weight of sample before dry and W3 is weight of crucible plus sample after drying

3.9 Validation of the method

Validating analytical methods includes performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analyte in a given sample is reliable for the intended use.

Validation of the analytical method was carried out according to the criteria proposed by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [80]. The analytical methodology was validated in terms of linearity; percent recovery, limits of detection (LOD) and quantification (LOQ). Linearity and linearity range were evaluated by constructing calibration curves using peak areas versus standard concentrations from 0.5 to 15 μg/kg.

The limits of quantification (LOQ) and detection (LOD) were calculated mathematically by the relationship between the standard deviation (*sd*) of the calibration curve and its slope (*S*) of the last three lowest calibration points using the multiplier suggested by the ICH standard [80]. The LOD and LOQ were calculated as:

$$SD = SE\sqrt{n}$$

$$LOD = 3.3(SD/S)$$

And $LOQ = 10(SD/S)$

Where: SD is standard deviation of the intercept; **SE** is standard error; and **n** is number of calibration points.

Recovery study (%R) was also performed by spiking maize sack which is aflatoxin free and similar species with the sample was taken from Ethiopian conformity assessment enterprise laboratory and the accuracy of the method was measured from analytical recovery with known concentrations of aflatoxin standards. Precision **(**repeatability) of the employed method was evaluated by the preparation of four different concentrations solution made from the standard solution in the working interval of the method; each one was prepared by the same analyst and read seven times in a short time period (1 day).

4 RESULTS AND DISCUSSION

4.1 Determination of moisture content

Moisture content is one of the determinant factors for the development of aflatoxins in maize and other grain products [16]. The moisture content was determined by using the standard [78]. The obtained moisture contents were ranging from 10.95 - 11.33% and the specific store average moisture contents were 11.22 ± 0.253%, 11.33 ± 2.89%, 11.31 ± 0.07% and 10.95 ±0.24%, for ST1, ST2, ST3 and ST4, respectively. The moisture contents of all the studied maize were below 14%, the maximum moisture content limit set to store maize in Ethiopia [78], indicating that the stored maize were completely dried.

Generally, the allowable storage time for grain products like maize depends on their seeds temperature and moisture contents. The increase of kernel temperature by 10 °C reduces the storage time by about 50%. Similarly, grains should be dried to lose their moisture contents before storing for future use. Generally, high moisture contents and favorable temperatures enhance the growth of microorganisms and germination of stored seeds [81].

4.2 Validation of HPLC–FLD method

4.2.1 Constriction of calibration curves

External calibration curves were constructed using five concentration levels including 0.5, 1, 5, 10 and 15 µg/kg. The given calibration curves were obtained by drawing peak areas as a function concentrations of the standards and demonstrated good linearity in the concentrations ranges used, with coefficient of determinations, r^2, greater than 0.999 (Figure 6 i - iv), and which indicates that there is a good correlation of linearity through all the concentrations used and a homo static distribution of replicates at all levels that were applied in the calibration curve assembly.

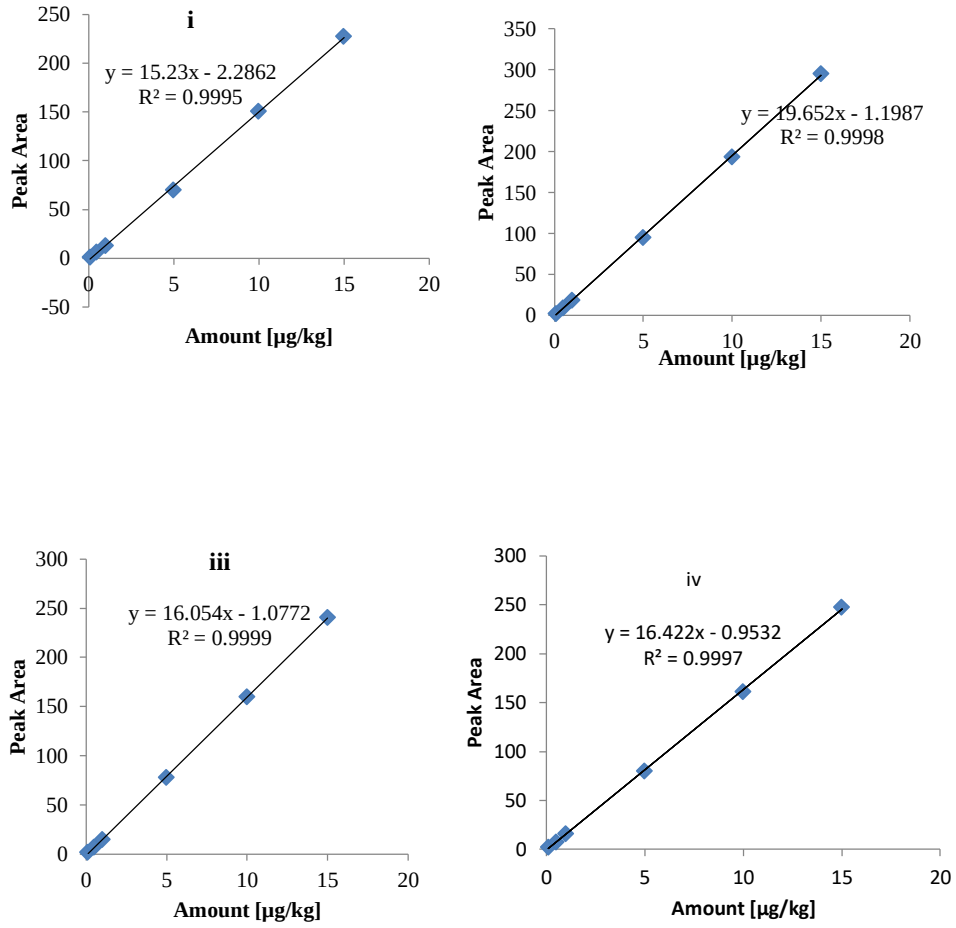

Figure 6. Calibration curves of aflatoxin (i) AFB1; (ii) AFB2; (iii) AFG1; and (iv) AFG2

One-way ANOVA output also revealed that the slope of calibration curves were significantly different from zero (at $P< 0.05$) indicating the linearity and sensitivity of the methods. The standard errors obtained from linear regression analysis (ANOVA) of aflatoxin B1, B2, G1 and G2 points of calibration curves and other analytical performances of the utilized are presented in Table 2.

Table 2 Analytical performance characteristics of the method for determination of aflatoxin B1, B2, G1 and G2.

Aflatoxins	Linear range (µg/kg)	R^2	Standard error	LOD (µg/kg)	LOQ (µg/kg)
B1	0.5 - 15	0.999	0.1282	0.049	0.148
G1	0.5 - 15	0.999	0.0899	0.031	0.099
G2	0.5 - 15	0.999	0.0543	0.019	0.059
B2	0.5 - 15	0.999	0.1179	0.035	0.107

4.2.2 Recovery and Precision studies

To validate the reliability of the method recovery and precision in terms of repeatability were assessed. Repeatability of the peak area and retention time of the target analytes were investigated by preparing four different concentrations of standard solutions by the same analyst and running each concentration level seven times in the same day. The findings are presented as RSD in Table 3.

Table 3. Repeatability the HPLC method for the determination of AFG1, AFB1, AFG2 and AFB2

		Repeatability (n = 7)			
		Peak Areas		**Retention time**	
Aflatoxin	**Conc.(µg/kg)**	**Mean**	**%RSD**	**Mean**	**%RSD**
B1	0.05	4.034	1.674	3.815	0.123
	0.1	7.287	1.177	3.816	0.176
	0.5	27.243	0.139	3.813	0.088
	10	583.043	0.381	3.803	0.307
B2	0.05	4.300	0.716	7.221	0.264
	0.1	9.074	2.283	7.089	0.368
	0.5	36.514	0.797	7.173	0.041
	10	713.743	0.338	7.194	0.127
G1	0.05	1.200	1.828	3.238	0.107

	0.1	2.551	2.296	3.241	0.096
	0.5	11.020	0.655	3.241	0.091
	10	223.286	1.407	3.232	0.322
G2	0.05	1.895	1.714	5.576	0.108
	0.1	4.469	1.689	5.576	0.079
	0.5	15.105	0.378	5.586	0.201
	10	321.257	0.573	5.601	0.111

The RSD% results of peak area and retention time were found 0.12 - 2.29% and 0.04-0.36%. According to the regulation of the European Commission (EC) No. 401/2006, [82] the recommended RSD% values for each concentration must be equal or lower that those derived from the Horwitz equation, which in turn is based on the analyte concentration, regardless of the matrix and analysis method used. These values were below the reference values established by the Commission Decision 2002/657/CE [83] and agree with the results achieved by Wen et al., [84] who obtained RSD% values in the range of 0.7-2.7%. The finding also agrees with the results achieved by Marcel, 2015 [85], who obtained RSD% values ranging from 0.2-4.4%. The precision results were lower than 2.29%, indicating a good Precision. Recovery study was carried out to evaluate the accuracy of the method. The obtained recovery values are presented in Table 4.

Table 4: Recovery test of Aflatoxin from maize sample

Aflatoxin	Aflatoxins added (µg/kg)	Result found (µg/kg)		Replicate % recovery		*%R	%RSD
		1	2	1	2		
AFG1	3	2.99	3.12	99.79	106.31	103	4.47
AFG2	3	2.94	3.12	98.20	106.29	102	5.59
AFB1	3	2.88	3.12	96.15	103.93	100	5.49
AFB2	3	3.01	3.31	100.35	110.18	105	6.61

The average recovery of replicate analysis of spiked samples was ranging from 100 – 105%, (Table 4). The recoveries obtained were within the range of 70 to 125%, which were acceptable according to AOAC International guidelines for method validation [86].

4.3 Levels of Aflatoxin in maize samples

The concentration levels of aflatoxin (AFB1, AFB2, AFG1 and AFG2) in studied maize samples are shown in Table 5.

Table 5 Concentrations (µg/kg, n = 3) of aflatoxin in the stored maize

Sample	Aflatoxines	Conc	SD
Store 1 (ST1)	AFB1	0.27	0.01
	AFB2	0.20	0.03
	AFG1	ND	-
	AFG2	0.18	0.01
	Total AF	**0.65**	**-**
Store 2 (ST2)	AFB1	0.26	0.01
	AFB2	0.15	0.01
	AFG1	0.24	0.01
	AFG2	ND	-
	Total AF	**0.66**	**-**
Store 3 (ST3)	AFB1	ND	-
	AFB2	0.14	0.01
	AFG1	ND	-
	AFG2	0.13	0.01
	Total AF	**0.27**	**-**
Store 4 (ST4)	AFB1	ND	-
	AFB2	0.17	0.02
	AFG1	ND	-
	AFG2	0.15	0.03
	Total AF	**0.32**	**-**

ND: indicates not detected

As can be seen average aflatoxin concentrations of the studied samples were ranging from ND - 0.27 µg/kg (for AFB1); 0.14 – 0.20 µg/kg (for AFB2); and ND - 0.18 µg/kg (for AFG2). AFG1 was detected only in maize samples from ST2, at relatively high concentration level, i.e., 0.24 µg/kg. Among the studied aflatoxins, AFB2 was detected in all maize samples. The average concentrations of each aflatoxins in maize samples collected from ST1 was 0.27 ± 0.01 µg/kg (for AFB1), 0.20 ± 0.03 µg/kg (AFB2) and 0.16 ± 0.01 µg/kg (AFTG2), but no AFG1 was not detected. Similarly, the average concentrations of aflatoxin detected in the sample collected from ST2 were 0.26 ± 0.01 µg/kg (AFB1), 0.15 ± 0.01 µg/kg (for AFB2) and 0.24± 0.01 µg/kg (for AFG1) but no AFG2 was not detected in the sample maize of this store. From maize samples of ST3 and ST4, only AFB2 and AFBG2 were detected at relatively lower concentration levels, i.e., 0.14 ± 0.01 µg/kg and 0.13 ± 0.01 µg/kg, as well as 0.17 ± 0.02 µg/kg and 0.15 ± 0.01 µg/kg, respectively. The studied samples were showed that the total aflatoxin levels ranging from (0.27 – 0.66 µg/kg) and specifically, 0.65, 0.66, 0.27 and 0. 32 µg/kg for maize sample of ST1, ST2, ST3 and ST4, respectively.

Consumption of food and feed containing high concentrations of aflatoxin has recognized potential health risks to animals and humans posed. Thus, national and international regulatory bodies have set the MRL for individual and total aflatoxins in food and feed consumed by human beings [87 - 89]. For instance, the United States‟ Food and Drug Administration (USFDA) [87] and WHO [88] established the MRL of total aflatoxin in all cereals to be 20 µg/kg. Similarly, EU also set the MRL 2 µg/kg AFB1 and 4 µg/kg total aflatoxin in all cereals [89]. Based on this guideline, the concentrations of individual aflatoxin and total aflatoxins observed in the present study are far below the MRL set by USFDA/WHO and EU, indicating the safeness of the maize of the area in terms of the studied aflatoxins. The detected concentrations of the studied aflatoxins were also lower than the aflatoxin levels reported in cereals (barley, wheat, maize, millet, sorghum and teff) collected from different local markets of Ethiopia [4].

5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Aflatoxins are toxic metabolites of Aspergillus fungi that can contaminate various foods and agricultural crops. Consumption of aflatoxin-contaminated foods is a common problem in both humans and animals. This study was conducted on maize samples that were collected from four stores of jimma town. The result has shown that the levels of total aflatoxin contamination in those samples collected from ST1, ST2, ST3 and ST4 were 0.617 μg/kg, 0.620 μg/kg, 0.267 μg/kg and 0.315 μg/kg, respectively. All the obtained results were by far below the maximum permissible limit set by the US Food and Drug Administration (FDA) and the World Health Organization (WHO) maximum limit of that the total aflatoxin 20 μg/kg and the European Union (EU) also set maximum limit of 2 μg/kg for aflatoxin B1 and 4 μg/kg for aflatoxin total. Thus it can be concluded that all the samples were safe for human and animal consumptions.

5.2 Recommendations

The current study investigated the levels of aflatoxin of maize samples stored in Jimma town. Based on the obtained findings, the research would like to forward the following recommendations. To get detailed information about the aflatoxin levels of maize of Jimma town and its area

- More research should be done on maize stored for different storage time,
- Additional studies should be conducted based on seasonal variations (spring, autumn, winter and summer seasons).
- Store owners should Choice storage structures that ensure good drying of maize are recommended.

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