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M.Sc. Thesis

Determination of Aflatoxins in Maize *(Zea Mays L.)* Samples from Dera and Fogera Districts of South Gondar Using LC-MS/MS

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A Thesis Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for Degree of Master of Science in Chemistry (Analytical)

Department of Chemistry
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This is to certify that the thesis prepared by Adisie Kassa Tefera entitled: "determination of Aflatoxins in maize *(zea mays L.)* Samples from Dera and Fogera districts of south Gondar using LC-MS/MS" and submitted in partial fulfillment of the requirements for the Degree of Master of Sciences in Chemistry (Analytical) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ACRONYMS

ACN	Acetonitrile
AF	Aflatoxin
AFB1	Aflatoxins B1
AFB2	Aflatoxin B2
AFG1	Aflatoxins G1
AFG2	Aflatoxins G2
A. flavus	Aspergillus flavus
A. nomius	Aspergillus nomius
A. parasiticus	Aspergillus parasiticus
DAD	Diode array detector
EATA	Ethiopian Agricultural Transformation Agency
ELISA	Enzyme-linked immunosorbent assay
EC	European Commission
FAO	Food and agriculture organization
FDA	Food and drug Administration
GDP	Gross domestic product
HPLC	High performance liquid chromatography
HPLC–FLD	High performance liquid chromatography fluorescence detector
Ha	Hectare
IARC	International agency for research on cancer
MRM	Multiple reaction monitoring
PICS	Purdue improved crop storage
S. Gondar	South Gondar
SNNP	South Nations Nationalities and Peoples
TLC	Thin-layer chromatography
UV	Ultraviolet
UPLC-MS	Ultra-performance liquid chromatography/tandem mass spectrometry
WHO	World Health Organization

ABSTRACT

Maize (*Zea mays L.)* is plant genetics and a member of the grass family, which is believed to have originated from Mexico and introduced to Ethiopia in 1600s to 1700s. Maize contains approximately 72 % starch, 10 % protein, and 4 % fat and supplying an energy density of 365 Kcal/100 g. However, the grain is vulnerable to degradation by Aflatoxins mainly produced by Aspergillus *flavus* and Aspergillus *parasiticus*, which have adverse effects on humans and livestock that ingest Aflatoxin contaminated food products and feeds. Contamination of maize grain by fungi leads to losses in quality and quantity of production. Aflatoxins are fungal secondary metabolites, which are harmful to human and animal health. The objective of this study was to determine the major Aflatoxin B1, Aflatoxin B2, Aflatoxin G1 and Aflatoxin G2 in maize sample from two districts of South Gondar Zone. The Aflatoxins were extracted from maize with ACN: H2O (84:16) and detected by LC-MS/MS. Mass spectrometry is a very sensitive technique and is widely regarded as having good selectivity. A good linearity of standard calibration was found for AFB1, AFB2, AFG1 and AFG2 at a range of 0.1–15 ppb. Regression coefficient (R^2) values were >0.999, whereas slope and intercept were in the range of 0.9776-0.9972 and 0.0147-0.1167, respectively. The average recoveries for spiked sample of the two districts were range from 65.95 to 97.60 %. The mean level of Aflatoxins in this study was below limit of detection of AFB1 (0.0253 ppb), AFB2 (0.0255 ppb), AFG1 (0.0257 ppb) and AFG2 (0.0258 ppb) limit of quantification range from (0.084-0.086 ppb) in maize samples. Further monitoring of Aflatoxins in maize from different regions of the country is justified in order to conclusively determine the actual safe/risks from Aflatoxins and possibly low Aflatoxins-risk maize production areas.

***Keywords*:** Aflatoxins, Aspergillus, Maize, LC-MS/MS

1. INTRODUCTION

1.1. Background of the study

Maize (*Zea mays L.)* is an exciting model organism in plant genetics and it is a member of the grass family. In the Western world, the term maize is used interchangeably with corn. The reason for this is that all grains were called corn under early British and American trade and the name was retained for maize because it was the most common grain in commercially. Maize (corn) which is believed to have originated in central Mexico 7,000 years ago from a wild grass; Native Americans transformed maize into a better source of food and it introduced to Ethiopia in the 1600s to 1700s. Although the origin of the word maize is also controversial, it is generally accepted that the word has its origin in Arawac tribes of the indigenous people of the Caribbean. On the basis of this common name, Linnaeus included the name as species epithet in the botanical classification Zea (*Zea mays L.*). Maize consists of approximately 72 % starch, 10 % protein, and 4 % fat, supplying an energy density of 365 Kcal/100 g. Maize provides many of vitamin B and essential minerals along with fiber, but lacks some other nutrients, such as vitamin B12, vitamin C and in general, a poor source of calcium, folate, and iron. In countries where anemia and iron deficiency are considered moderate or severe public health problems, the fortification of maize flour and cornmeal with iron and other vitamins and minerals has been used to improve micronutrient intake and prevent iron deficiency. Iron absorption, particularly the non-heme iron present in maize, can be inhibited by some components or foods in the diet, such as vegetables, tea (e.g., oxalates), coffee (e.g., polyphenols), eggs (e.g., phosvitin), and milk (e.g., calcium). Maize grown throughout the world but, the three top maize-producing countries includes United States, China, and Brazil producing approximately 563 of the 717 million metric tons/year. Maize can be processed into a variety of food and industrial products, including starch, sweeteners, oil, beverages, glue, industrial alcohol, and fuel ethanol. In the last 10 years, the use of maize for fuel production significantly increased, accounting for approximately 40 % of the maize production in the United States. It is also an important source of raw material for the industry. As the ethanol industry absorbs a larger share of the maize crop, higher prices for maize will intensify demand competition and could affect maize prices for animal and human consumption. In Africa, maize is used as both human and animal food, eaten directly as grilled cobs or as various products of maize flour. Low production costs, along with the high consumption of maize flour and cornmeal,

especially where micronutrient deficiencies are common public health problems, make this food staple an ideal food vehicle for fortification (Bekeko., 2014, EATA., 2013-2017, Ranum, *et al.*, 2014).

Maize is grown on 161,765,388 hectares with an annual production of 840,308,214 tons worldwide. When it grown in the lowlands it need at least 500 mm of precipitation well distributed throughout the season. The optimum temperature for its development in lowlands in tropical regions ranges from 30 °C to 34 °C and approximately 21 °C for the highlands. Maize crop phenology that includes the main developmental stages (tillering, heading, flowering, maturity) depends on the temperature. An increase in temperature usually results in accelerated plant growth. Maize is the third most important crop after wheat and rice cultivated in the world. It is the main staple food for hundred millions of people in developing countries especially in Sub Saharan Africa. In Ethiopia, maize grows under a wide range of environmental conditions between 500 to 2400 meters above sea level. Maize (corn) is partly used for making Injera in Ethiopia. The maize crop is third most important crop in Ethiopia after wheat and teff and accounts for largest share in total crop production (Chauhan, and Minota., 2016, Molla and Zegeye., 2014, EATA., 2013-2017, Nega, *et al.*, 2016, Lukeba, *et al.*, 2013, Chauhan, *et al.*, 2016).

As a cereal crop, maize is one of the most important food and feed commodities. Maize in its different processed forms is the staple food for large numbers of people in the developing world, providing significant amounts of nutrients, in particular calories (Cheli, *et al.*, 2009, Castells, *et al.*, 2008, Fareed, *et al.*, 2014). It is one of the most widely distributed food plants in the world and its infection by fungi can result mycotoxin contamination during the growing, harvesting, storage, transporting and processing stages (Ahsan, *et al.*, 2010). Maize is especially vulnerable to infection by mycotoxin-producing fungi in tropical and subtropical countries (Donner, *et al.*, 2009, Liu, *et al.*, 2006). Among mycotoxin, Aflatoxins are polyketide based potent liver carcinogenic, mutagenic and immuno-suppressive compounds, primarily produced by food-borne fungi, mainly *Aspergillus* species such as *flavus, parasiticus*, *niger, nomius*, *pseudotamari*a, *bombycids*, etc. These fungi can colonize a variety of products such as maize, oilseeds, groundnuts and tree-nuts, cereals, nuts, figs and spices etc., under favorable conditions, thus leading to food contamination and spoilage (Mushtaq, *et al.*, 2012, Ayejuyo, *et al.*, 2011, Traistaru, *et al.*, 2012). AFs may contaminate a wide variety of agricultural commodities, especially if they have high carbohydrate and/or fat contents. Maize and rice have been identified as very high-risk commodities for AFs contamination (Campone, *et al.*,

2011).

Ahsan, *et al*., 2010 report that mycotoxins affect a quarter of the world's food crops, including many basic food stuffs such as animal feed, crops like maize, rice and wheat. The Food and Agriculture Organization (FAO) also estimated that at least 25% of the world's cereal grains are contaminated by mycotoxins, including Aflatoxins in each year and constitute loss at post-harvest (Liu, *et al*., 2006, Molla and Zegeye., 2014, Guchi., 2015, Aiko and Mehta., 2015). The word "mycotoxin" is derived from two Latin words; "mukes" referring to "fungi" (Greek) and "toxicum" referring to "poison" (Latin). The word "Aflatoxin" is also the combination of three words "A" for the *Aspergillus* genus, "fla" for *flavus* species and toxin for poison. From group of mycotoxins, Aflatoxins are toxic secondary metabolites which are mainly produced by *Aspergillus* species, which found throughout the world and they are present both into the ground and in the air (Chauhan and Minota., 2016).

Aflatoxins (AFs), each of which is a group of closely related mycotoxins, may be produced by three of *Aspergillus* species (i.e., *A. flavus*, *A. parasiticus*, and the rare *A. nomius)* which contaminate plants and plant products. *Aspergillus flavus* produces merely the B type of Aflatoxin, while the other two species produce both Aflatoxins B (B1, B2), G (G1, G2) and the term "B" and "G" refers to the colors blue and green products fluorescence UV light on TLC plates, while the number 1 and 2 show the respective major and minor compounds (Ketney and Tifrea., 2014, Castro, *et al*., 2001, Ahsan, *et al*., 2010, Tomasevic, *et al*., 2015, Namjoo, *et al*., 2016, Herrman, *et al*., 2014, Fareed, *et al*., 2014, Gizachew, *et al*., 2016). Among all mycotoxins, Aflatoxin B1 (AFB1) is considered to be the most carcinogenic (Chauhan and Minota., 2016) and it has classified by the International Agency for Research on Cancer (IARC) in Group 1 of human carcinogen. In general, AFB1 affects not only the liver but also other organs and systems such as lung and immune system causing different health problems. It is highly toxic compound with a Lethal Dose (LD_{50} = 1-50 mg/Kg) for most species. However; its toxicity is critical (LD_{50}<1 mg/Kg) for some highly sensitive species, such as rats. Aflatoxins are fat-soluble compounds and are readily absorbed from the site of exposure, usually through the gastrointestinal tract and respiratory tract (Bakırdere, *et al*., 2012, Herrman, *et al*., 2014, Keteney and Tifea., 2014, Gizachew, *et al*., 2016). The severity of acute and chronic toxicity, which reflects the role played by epo-xidation of the 8, 9-double bond, is AFB1 > AFG1 >AFB2 > AFG2 (Adeyeye., 2016) and these four compounds are distinguished by the color of their fluorescence under long-wave ultraviolet illumination (Baydar, *et al*., 2005).

For quantification of Aflatoxins in food and feed, various analytical techniques such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS), gas liquid chromatography, liquid chromatography/tandem mass spectrometry (LC-MS/MS) and enzyme-linked immunesorbent assay (ELISA) have been developed (McDaniel, *et al.*, 2011, Mushtaq, *et al.*, 2012, Shweta, *et al.*, 2013, Sun, *et al.*, 2015). Mass spectrometry (MS) has the capability to select/separate organic molecules according to their molecular mass and permits their detection and quantization with extremely high sensitivity. In tandem, the two techniques (usually referred to as LC-MS) give a unique capability for rapid, cost-effective and quantitative measurements of organic compounds for an enormous variety of applications. Routine use of mass spectrometry began to grow in the 1950s, followed by HPLC in the 1970s. However; development of reliable interfaces to link the two techniques was not easy and it is only during the past twenty or so years that LC-MS has assumed the key role it occupies today. During that time, manufacturers have succeeded in steadily reducing the size and real cost of the instrumentation whilst software and automation have greatly lowered the learning curve for operators. As a result, LC-MS has become ubiquitous as the technique of choice for many quantitative analysis applications (Sargent., 2013). Aflatoxins are soluble in organic solvents such as; methanol, acetone, chloroform and acetonitrile. Thus, for their extraction these solvents or mixtures are used. Extraction varies according to the degree of selectivity, rapidness and convenience, and depends not only on access and conditions at which it is carried out, but also on the configuration of extraction stages (Georgievski, *et al.*, 2016).

1.2. Statement of the problem

Aflatoxin contamination is a serious food safety problem throughout the world (Abbas, *et al.*, 2005). Several issues are associated with grain moulds and their secondary metabolites (mycotoxins) in maize, which lower grain quality, cause adverse effects on human health, animal health and reproduction (Cheli, *et al.*, 2009). In India, an outbreak of hepatitis resulted for the death of 100 people due to consumption of contaminated maize. First in India, 106 people died in 1974. This event was followed 100 deaths in Nigeria in 2005 (Shephard., 2008). In Kenya, the exposure to 50 mg per day of AFB1 via ingestion of contaminated maize result the Aflatoxin-induced death (Bakırdere, *et al.*, 2012). Besides, in the year (2004), 125 people died and nearly 200 others were treated due to liver failure caused by acute Aflatoxicosis after consumption of contaminated maize (Aiko and Mehta.,

2015, Adeyeye., 2016). Unfortunately, severe Aflatoxin-poisoning events documented from different countries. In the rural communities of developing world, as in Ethiopia (western Wollo) an outbreak of gangrenous ergotism was occurred in 1978 after consumption of grain contaminated with Claviceps purpurea mycotoxin (Shephard., 2008, King., 1979). Therefore, it is important to assess the level of Aflatoxins contaminants in maize samples from storage of the maize under damp conditions, traditional storage and agriculture.

1.3. Significance of the study

Aflatoxin contamination is not merely a potential source of health hazards but it is also involved in the spoilage of agricultural commodities. Then, this calls for assessment of the occurrence and the level of Aflatoxin contamination in maize. Therefore, data generated from this study may give an insight in the level of Aflatoxin contamination in post-harvest maize. Besides, it creates awareness about the Aflatoxin problem and recommends the farmer for enhancing the production, quality and safety of the product for export commodity in the future. Finally, the results from this study may lead for the development of strategies for Aflatoxin control, prevention and development of maize value chain, above all helpful in safeguarding the public health.

1.4. Objectives

1.4.1. General objective

The general objective of this study was to determine the level of Aflatoxins in maize samples using LC-MS/MS

1.4.2. Specific objective

- To determine the level of Aflatoxins (B1, B2, G1 and G2) contamination in post-harvest maize products.
- To evaluate the results with level set by international organization.
- To compare the levels of this study with literature reported values.

1.5. Scope of the study

This study conducted in two woredas of South Gondar zone (Dera and Fogera) the assessment focused on Aflatoxins contamination of maize grains by LC-MS/MS. All the dry maize kernels were purchased from local markets of Fogera (woreta Saturday market) and Dera (anbesame Saturday market).

2. LITERATURE REVIEW

2.1. Overview of maize in Ethiopia

In Ethiopia, agriculture contributes about 52 % of the GDP and 85 % of the population is dependent directly or indirectly on agriculture. While agriculture is growing at 1.6 % per annum, the population of the country is growing at 3 % and is expected to double by year 2020 (Fufa and Hassan., 2006). Maize is one of the most important crops and is grown across 13 agro-ecological zones which together cover about 90 % of the country. It is the first crop in production accounting for 26.7 % (7.2 million tons) of 87.3 % (23.6 million tons) of the cereal production and ranks next to teff in area coverage in Ethiopia. In the drought stressed areas of Ethiopia, which covers about half (46 %) total arable land, the areas devoted to maize production occupy 38-42 % of the maize growing area, but contribute only 17 % to the total maize production (Abrha, *et al.*, 2013, Tsedaley and Adugna., 2016, Molla and Zegeye., 2014, Mitiku, *et al.*, 2015, Nega, *et al.*, 2016).

Maize (corn) cultivated on about 1.7 million ha of land. However, maize varieties mostly grown in the highlands altitude of Ethiopia are local cultivars. The national average yield of maize under subsistence production is about 2200 kg/ha. This low yield is attributed to foliar diseases and insect pests such as stalk borer, low soil fertility and use of inferior genes (Bekeko., 2014). Maize is cultivated in all of the major agro-ecological zones in Ethiopia up to 2400 m.a.s.l. It is widely produced in southwestern, western, southern and eastern and in some north, northwestern and eastern parts of the country (Mitiku, *et al.*, 2015). Maize is mainly grown in the four big regions of the country, which are Oromia, Amhara, SNNP, and Tigray. Oromia and Amhara contribute to almost 80 % of the maize produced and ten zones found in the two regions contributed to more than half of the national maize production in 2012. Among the top maize producing zones are: (West Gojjam 5.6, East Wellega 4.3, Kaffa 3.8, East Shewa 3.1, West shewa 2.9, West Arsi 2.7, Illubabor 2.7, East Gojjam 2.2, West Wellega 2.1, and West Harerghe 2.1) million quintals, respective amount. Other regions such as Benishangul Gumuz and Gambela also grow maize and have the potential to increase their current production level in the future (EATA., 2013-2017).

In sub-Saharan Africa it is considered as the major food and income provider crop for more than 300 million households (Mitiku, *et al.*, 2015). In Ethiopia maize is a staple food, one of the main sources of calories in the major maize producing regions and also the most important crop worldwide for food,

animal feed and bio-energy production (Bekeko., 2014). Maize is so an important crop for overall food security and also used for making local beverages. Additionally, the leaves and stovers are used to feed animals and the stalks are used for construction and fuel. 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. Moreover, the crop has potential uses for industrial purposes, serving as a starch, a sweetener for soft drinks, an input for ethanol fuel production and oil extraction, etc. (EATA., 2013-2017).

Maximum quantities of maize produced are stored under poor and unsatisfactory storage conditions for considerable period of time. Traditional storage of maize in Ethiopia made from mud, bamboo strips, and pits. In addition to these storage conditions, recent technology involves storage of maize in polyethylene bags and gunny bags. Previous reports proposed that extended storage of maize under unacceptable storage conditions enhances fungal growth which promotes the production of respective mycotoxins (Chauhan, *et al*., 2016).

2.2. Transmission and life cycle of *Aspergillus flavus*

Soil serves as the primary habitat for Aspergillus *flavus* of competent saprophytic fungus that survives on dead plant tissue colonizes soil, organic debris and sometimes behaves as a weak and opportunistic pathogen. Aspergillus *flavus* and *parasiticus* are sporogenic sclerotia, conidia and mycelia capable of surviving and overwintering in plant residues as mycelium (hypha) or sclerotia that in turn serve as the source of new conidia (Abbas, *et al*., 2009, Guchi., 2015, Liu, *et al*., 2006). The life cycle of Aspergillus *parasiticus*, and Aspergillus *nominus* fungi in soil is little known (Guchi., 2015). But the life cycle of Aspergillus *flavus* can be divided into two major phases: the colonization of plant residues in soil, and the infection of crop tissues, including grain and seeds of actively growing plant tissues. At the starting of the growing season, usually in spring and sometimes at the end of winter, when sclerotia are exposed to the soil surface, they quickly germinate and form new conidial inoculums will be vectored by insects or carried by the wind to begin the colonization and infection of the freshly planted crops. During the growing season, infected plant tissues can serve as sources of secondary conidial inoculums, which colonize new non-infected plant tissues (Figure 1). Even though the reporters reveal how the initial and secondary inocula occur from plant infection, little information available about the saprotrophic activities of these fungi in soil (Abbas, *et al*., 2009).

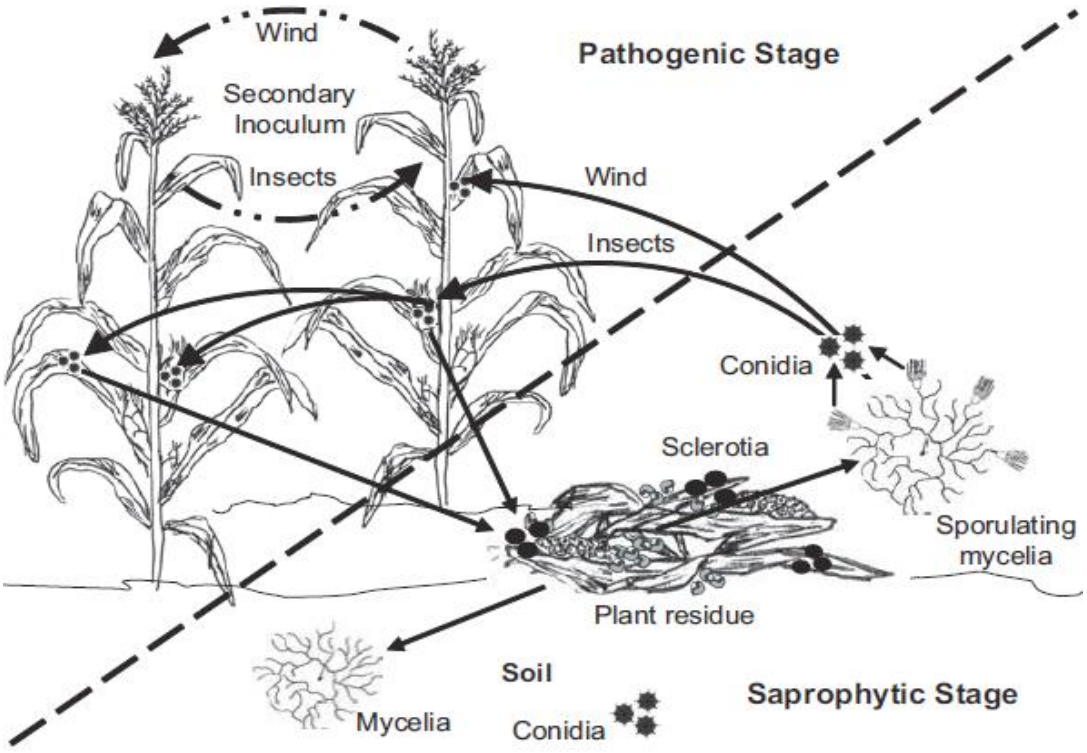


Figure 1: Life cycle of *A. flavus* in a corn cropping system saprophytic and pathogenic stages of fungal ecology (Abbas, *et al.*, 2009).

2.3. Aflatoxins

Aflatoxins (AFs) are secondary metabolites of molds belonging to the *Aspergillus* genera (Stefanovic, *et al.*, 2015). They are a group of structurally related polyketide mycotoxins that contaminate many agricultural commodities, such as almond, coffee, corn, cottonseed (*Gossypium* spp.), groundnut, pistachio, rice (*Oryza sativa* L.), soybean, sunflower, and wheat. In addition, milk and milk products can be contaminated as a result of cows being fed on Aflatoxin-contaminated feed. There are almost 20 different types of Aflatoxins identified until now, among these AFB1, AFB2, AFG1 and AFG2 are more prominent. Among the four mentioned Aflatoxin B1 is considered to be the most toxic. Aflatoxin compounds are thermo stable and pasteurization cannot destroy them. Therefore, they appear in sterilized milk as well as in fermented dairy products. Aflatoxin is the most common of the four forms of Aflatoxins, AFB1, AFB2, AFG1 and AFG2 (Guchi., 2015, Mushtaq, *et al.*, 2012, Dimitrieska, *et al.*, 2016). Research results in Turkey showed that Aflatoxins were isolated and characterized after the death of more than 100,000 turkey poults (turkey X disease) which was traced to the consumption of a mold-contaminated peanut meal. This prompted a major revolution in

mycotoxin research resulting in intensive testing for mycotoxins in any moldy products. Since then, several *Aspergilli* have been identified as capable of producing Aflatoxins. This fungus is ubiquitous in the environment, being readily isolated from plants, air, soil, and insects. Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of A. *flavus* and A. *parasiticus*; in particular, A. *flavus*is a common contaminant in agriculture. Aspergillus bombycis, Aspergillus ochraceoroseus, Aspergillus nomius, and Aspergillus pseudotamari are also Aflatoxin producing species, but they are encountered less frequently (Abbas, *et al*., 2009, Zain., 2011, Rajarajan, *et al*., 2013). Aflatoxin species have polycyclic structure; G series Aflatoxins are six-member lactones and B series Aflatoxins are pentanone derivatives and their structure is given Figure 2 (Bakırdere, *et al*., 2012, Papp, *et al*., 2002).

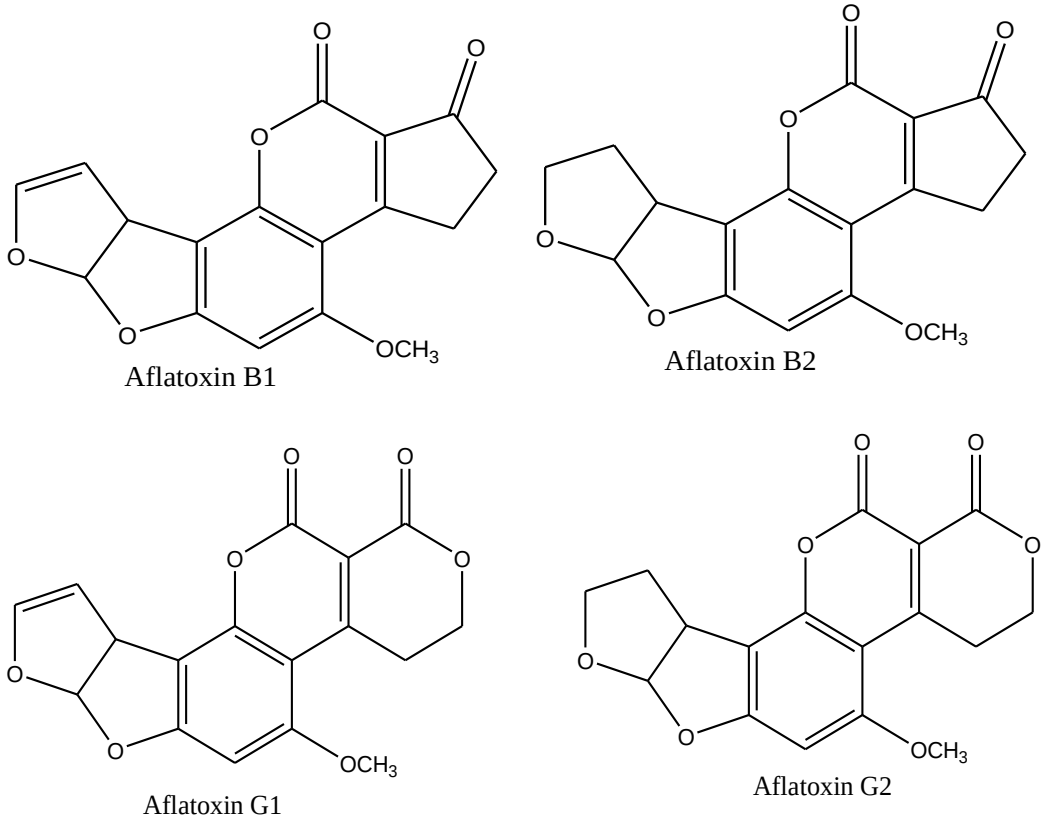


Figure 2: Chemical Structures of Aflatoxin B1, B2, G1 and G2 (Papp, *et al*., 2002, Yan, *et al*., 2016)

2.3.1. Aflatoxin contamination in maize

Maize cultivation in the world is limited by diseases which cause grain loss of about 11 % of the total production. Fungi could cause about 50-80 % of damage on farmers' maize during the storage period, if conditions favorable for their development. The maize grain undergoes quantitative and qualitative losses during storage. The losses occur mainly because of improper storage. A large number of pathogenic fungi, bacteria, viruses and insects infecting and infesting maize grain cause combined worldwide annual losses of 9.4 %. Maize is attacked by more than sixty diseases and a number of species of insect pests in the field as well as in the storage. Fungi affect the quality of grain as a result there will be, increase in fatty acid, reduction in germination, increase its mustiness, production of toxins and finally leading to spoilage of grain in many ways. Fungi are the second important cause of deterioration and loss of maize next to insects. Fungi are among the principal causes of deterioration and yield loss on farmers' maize during the storage period. Among the storage fungal pathogens *Aspergillus* are the most predominant species attacking maize seed and resulting in reduction in seed germination (Dubal, *et al.*, 2014, Tsedaley and Adugna., 2016).

A research conducted in West Gojjam, Ethiopia indicate the contamination of maize by Aflatoxin was high in pre-harvest and significantly increased from pre-harvest to post harvest. As the report indicated that from fifteen pre and fifteen post-harvest maize samples 77.7 % of pre-harvest concentration range from 3.13 to 63.66 µg/kg and 80 % of post-harvest sample concentration range from 9.02 to 139.8 µg/kg were contaminated by total Aflatoxin. The mean total Aflatoxin contamination was 18.38 µg/kg for pre-harvest and 43.36 µg/kg for post-harvest. *Aspergillus* species contaminations in pre-harvest maize 53.3 % of samples were contaminated by *A. flavus* (26.7 %), *A. parasiticus* (13.3 %) and *A. niger* group (13.3 %) and in post-harvest maize 79.9 % of samples were contaminated by *A.flavus* (46.6 %), *A. parasiticus* (20.0 %) and *A. niger* group (13.3 %). Aflatoxin B1 was detected in 66.7 % of pre-harvest maize with the mean level of 5.00 µg/kg and in 87.7 % in post-harvest maize with the mean level of 9.86 µg/kg. As they reported about 66.7 % of pre-harvest and 86.7 % of post-harvest maize samples were exceed the acceptance limit of total Aflatoxin and Aflatoxin B1 recommended by European Union maximum limit (Assaye, *et al.*, 2016).

Similarly, Chauhan, *et al.*, 2016 conducted research in Gedeo zone of Ethiopia by using biosensor approach as well as thin layer chromatography method for quantification of Aflatoxins. From a total

number of 150 different maize samples of their study, results reveal that mean concentration of Aflatoxins of all samples were observed 53 ppb and the presence of *Aspergillus* species 75 %.

2.3.2. Factors affecting Aflatoxin development in maize

Maize as an agricultural commodity is considered as one of the best substrate for the fungi to grow and produce toxicogenesis (Chauhan, *et al.*, 2016). Maize suffers from the attack of the diseases from seedlings to maturity in the field (Mitiku, *et al.*, 2015). *Aspergillus* species are ubiquitous in the environment such as in soil, air, debris and are widely distributed in tropical and sub-tropical environments. Factors such as high temperatures, high moisture, drought stress, storage duration, humidity in depot, water leaks, insect activity, faults of air conditioning during the storage period, insect damage that allows an entrance for the fungi and delayed harvesting predispose maize to infection by *Aspergillus* spp. and results contamination of Aflatoxins. Colonization of maize crops prior to harvest in the field (pre-harvest) by Aflatoxigenic fungi often resulted in spoilage and Aflatoxin accumulation in post-harvest grains during storage (Liu, *et al.*, 2006, Abbas, *et al.*, 2006, Chauhan, *et al.*, 2016, McDaniel, *et al.*, 2011, Traistaru, *et al.*, 2012, Tsedaley and Adugna., 2016).

In maize production, post-harvest practices, such as proper drying and storage, are key areas along the maize value chain to maintain grain quality, quantity and safety. Traditional storage cases deterioration of maize products and modern storage technology relies on creation of bio-generated atmospheres that hinder survival of microorganisms including fungi. As a result of fungal, insect and grain respiration within the hermetic bags, oxygen levels drop significantly while carbon dioxide levels increase. This creates an unfavorable atmosphere for survival of these organisms within the enclosed system. Triple-bagging technology is sustainable, cost effective and effectively maintains high quality maize grains for longer period of time (Chauhan, *et al.*, 2016, Georgievsk, *et al.*, 2016).

2.3.3. Effects of Aflatoxins on humans and animals health

The study of Aflatoxin contamination of foods and feeds is important because Aflatoxins are toxic and carcinogenic to humans and animals (Ayejuyo, *et al.*, 2011). Among the four classes of Aflatoxins, AFB1 is predominant in nature and functionally carcinogenic in animal models (Chauhan, *et al.*, 2016). Aflatoxin species are highly dangerous compounds for both human being and animals. Studies on animal have proved that exposure to Aflatoxin can seriously affect their growth and development (Bakırdere, *et al.*, 2012). The health issues related to Aflatoxins are equally complex and

demand more research (Mushtaq, *et al*., 2012). Contamination can be done in two major ways (a) ingestion of contaminated food with Aflatoxin or ingestion of Aflatoxins in animal feed carried in milk and milk products (Agag., 2004), (b) by inhaling dust of Aflatoxins in especially AFB1in contaminated food industries and factories (Ketney, *et al*., 2014). The main sources of Aflatoxin species for human and animals are the ingestion through the dietary channel (Bakırdere, *et al*., 2012). The ingested Aflatoxin undergoes various possible pathways depending on different parameters, like dose quantity, type of species, diet, age, and immune system of host. Exposure of biological systems to harmful levels of Aflatoxin results in the formation of epoxide, which reacts with proteins and DNA leading to DNA-adducts, thus causing liver cancer. Mycotoxins are the causes of human illness and death (Molla and Zegeye., 2014). Aflatoxins persist to some extent in food even after the inactivation of the fungi by food processing methods, such as ultra-high temperature products, due to their significant chemical stability. Infants are at much higher risks of health problems compared to adults (Mushtaq, *et al*., 2012).

Contamination by Aflatoxins can take place at any point along the food chain from the field, harvest, handling, shipment and storage (Shukla., 2016). Mycotoxins pose higher risk of causing cancer than contaminants in food such as anthropogenic contaminants, pesticides, phycotoxins and food additives (Guchi., 2015, Aiko and Mehta., 2015, Krishnan, *et al*., 2015). The diseases caused by Aflatoxins consumption are loosely called Aflatoxicosis. The acute Aflatoxicosis results death; while the chronic Aflatoxicosis results cancer, immune suppression, and other ‘‘slow’’ pathological conditions. The liver is the primary target organ and damage occurs when poultry, fish, rodents, and non-human primates are fed Aflatoxin B1. There are substantial differences in species susceptibility. Moreover, within a given species, the magnitude of the response is influenced by age, sex, weight, diet, exposure to infectious agents, and the presence of other mycotoxins and pharmacologically active substances. Thousands of studies on Aflatoxin toxicity have been conducted, mostly concerning laboratory models or agriculturally important species. A number of investigators have found that Aflatoxins are acutely toxic, carcinogenic, teratogenic and mutagenic compounds (Zain., 2011, Rajarajan, *et al*., 2013). Signs of acute Aflatoxicosis include depression, nervousness, abdominal pain, diarrhea and death (Siddique, *et al*., 2013). Intensive exposures of AFB1 at the concentration of excess two ppm are reported to cause non-specific liver problems and death within few days (Abbas, *et al*., 2009). In Sudan, Durban, South Africa and Nigeria children with *kwashiorkor* (childhood malnutrition from protein insufficiency) and high levels of Aflatoxin has been reported. In Gambia, 93 % of sampled

children (6-9 years old) tested and found to be positive for Aflatoxin albumin adducts (Guchi., 2015).

2.3.4. Strategies for Aflatoxin prevention and control

The use of agrochemicals (fungicides), timely irrigation, and alternate cropping systems have independently shown limited success in preventing Aflatoxin contamination (Abbas, *et al.*, 2009). Several preventive measures to minimize mycotoxin contamination in agricultural commodities have been attempted. These can be plant breeding and good agronomic practices and detoxification (Adeyeye., 2016)

2.3.5. Plant breeding and good agronomic practices

As researcher reported, there is a little success in providing resistant varieties of maize (corn) to minimize the problem of Aflatoxins. Scientists at United States Department of Agriculture have identified maize lines that are resistant to A. *flavus*. Gene clusters housing the genes that govern formation of Aflatoxins to elucidated and being targeted in strategies to interrupt the biosynthesis of these mycotoxins. To devise effective strategies to control fungal infection and minimize mycotoxin production in host plants, a better knowledge of genetic variability, population structure at the intra-specific level and lineages which might arise that possess significant features in terms of toxin profile or host preferences is necessary. Agronomic approaches such as avoiding water stress, minimizing insect infestation and reducing inoculums potential have been suggested and are effective when the farmers can implement such practices. In addition to this following good agricultural practices such as drying techniques, maintaining proper storage facilities and taking care not to expose the grains to moisture during transport and marketing during both pre-harvest and post-harvest conditions would minimize the problem of contamination by Aflatoxins (Zain., 2011, Adeyeye., 2016).

2.3.6. Detoxification of mycotoxins

Removal or detoxification of mycotoxins has been studied using physical, chemical or biological methods. Efficient degradation of mycotoxins is a challenge since most mycotoxins are heat-stable and form toxic degradation products. Although several detoxification methods developed, only a few have been accepted for practical use. Some of the common methods are physical, chemical and biological controls (Akiko and Mehta., 2015).

Physical control: Physical treatment includes cooking, boiling, roasting, microwave heating, extrusion, irradiation, etc. The level of mycotoxin degradation by thermal process depends on factors like temperature, moisture content and time period. In heat treatment, temperature and time period are important in determining the level of degradation. A higher level of Aflatoxin degradation achieved when heated at higher temperature (200 °C) for longer exposure time. The moisture content of a product also plays an important part in degrading Aflatoxins, which means at high moisture content, degradation more efficient. The presence of ammonia during extrusion of Aflatoxin B1 led to higher amount of degradation. Aflatoxins are photosensitive in nature; hence, various radiations such as sunlight, UV light and gamma rays have been employed for degradation studies. Sunlight efficiently used for degrading Aflatoxin B1 in olive oil, groundnut oil, etc. The cytotoxicity and mutagenecity of Aflatoxin B1 has been shown to reduce after treatment with UV in aqueous medium (Akiko and Mehta., 2015). Sanitation practices, such as mechanical/hand sorting, can reduce Aflatoxin levels by removing low-density mould-infected kernels. Dry roasting conditions and loss of Aflatoxin have positive correlations between them. Cooking and steaming for one hour under pressure reduces Aflatoxin by up to 60 %. This is because high temperature breaks the ring chemical structure of Aflatoxin (Guchi., 2015).

Chemical control: Ring opening of the Aflatoxin chemical structure occurs at 100 °C, followed by decarboxylation, leading to the loss of the methoxy group from the aromatic ring of the Aflatoxin molecule. Aflatoxin G1 and G2 are more susceptible to chemical hydrolysis than Aflatoxin B1 and B2 because of the presence of two ether linkages in the G group compared to the B group which possess a single ether linkage. Ammonia at 0.5-7 % coupled with long exposure time, ambient temperature and pressure has been successfully used to inactivate Aflatoxin in contaminated commodities, such as groundnut meal, cottonseed and maize (Guchi., 2015). Treatment with chemicals efficiently degrades Aflatoxin B1 and Acids like lactic acid and citric acid convert Aflatoxin B1 into several products such as Aflatoxins B2, B2a, D1, etc., rather than complete degradation. In a recent study, lactic acid has been shown to degrade Aflatoxin B1 into Aflatoxin B2 and B2a efficiently, with Aflatoxin B2a as the major degradation product under heat treatment. Citric acid causes the hydration of Aflatoxin B1 at the 8, 9-olefinic bond of the terminal furan ring to form Aflatoxin B2a. Treatment of Aflatoxin B1 with hydrochloric acid at elevated temperatures completely destroyed the toxin without the formation of toxic residues. Among many chemicals used for detoxification of mycotoxins, ammonia is the most efficient and it has been accepted for use by the corn production

industry. Ammonia degrades Aflatoxin B1 into Aflatoxin D1 which has reduced toxicity and mutagenic potential. Alkalis cause the hydrolysis of the lactone ring in Aflatoxin B1; however, it can revert back under acidic conditions. Boiling Aflatoxin B1 contaminated corn with NaOH decreased the level of Aflatoxin B1 by 93 %, with 18 % reversion level after treatment with acid. Sodium bisulphate and hydrogen peroxide are also used in degrading Aflatoxin B1 efficiently. Ozone has been used to degrade Aflatoxin B1 by more than 90 % in animal feed showed that ozone-treated Aflatoxins are not toxic and mutagenic. Aflatoxin B2 and G2 are also efficiently degraded by ozone (Akiko and Mehta., 2015).

Biological Control: Biological control of toxigenic *A. flavus* strains can be achieved by the application of atoxigenic *A. flavus* strains to maize, groundnut and cotton fields. Non-toxigenic strains not only reduced Aflatoxin contamination in the field but also reduced Aflatoxin contamination that occurred in storage (Guchi., 2015). Use of botanicals as anti-fungal and anti-mycotoxin agent is considered safe to humans and environmental friendly. Several fungal enzymes have been reported to degrade Aflatoxin B1. Armellariatabescens produced a multi-enzyme system which detoxified Aflatoxin B1 by opening the difuran ring. The enzyme peroxidase from A. *flavus* and A. *parasiticus* has been shown to degrade Aflatoxins B1 and G1. A horseradish peroxidase enzyme from the plant raphinus sativa has also been reported to degrade Aflatoxin B1. Various extracts from plants such aspiperine from black and long peppers; lutein and xanthrophylls from Aztec marigold; carotenoids from fruits and vegetables are reported to suppress the toxicity and mutagenicity of Aflatoxin B1 (Akiko and Mehta., 2015). Leaf extract of some medicinal plants like Bosciacoriacea and bark extract of croton megalocarpus has also high antifungal activity for controlling of Aflatoxin causing pathogen (Mitiku, *et al.*, 2015).

The essential oils of several plants have been documented to possess strong antimicrobial property. The oil of Illiciumverum, Cymbopogonmartini, Eucalyptus globulus, Cinnamon zylenium, etc., is reported to be anti-fungals. The powder and essential oil of Cymbopogoncitratus have been successfully used for inhibiting AflatoxinB1 contamination and preserving the quality of melonseed under storage. Among the number of plants, Syzygiumaromaticum (clove) has been extensively studied for its anti-microbial property. The oil of clove and its main component, eugenol, has been reported to inhibit Aspergillus growth and Aflatoxin B1 production. Removal of toxigenic fungi and mycotoxins by botanicals are usually preferred over chemical treatments (Akiko and Mehta.,

2015).

2.3.7. LC-MS/MS Method for Detection of Aflatoxins

Mass spectrometry is a very sensitive technique and is widely regarded as having good selectivity. However, in many applications it is necessary to isolate the target analyte from what could be a sample containing thousands of other different molecules. Typically mass spectrometry merely is unable to meet this need as it can only differentiate compounds by their mass-to-charge ratio (*m/z*) which is insufficient in most practical applications of the technique. For example, more than 1,500 compounds may have the same molecular mass at around 250 Dalton. Hence, an additional separation technique is needed before presenting the sample to the mass spectrometer. In this regard, liquid chromatography-mass spectrometry (LC-MS) allows in isolating and measuring the levels of analyte of interest in highly complex mixtures. Liquid Chromatography differentiates compounds by their physico-chemical properties and MS differentiates compounds by mass (specifically their mass-to-charge ratio). Because of this dual selectivity LC-MS is a powerful analytical tool for Aflatoxin determination. The mass spectrometer acts at least in principle, it provides the capability to identify the species corresponding to each chromatographic peak through its unique mass spectrum (Sargent., 2013). In chromatographic techniques the components to be separated are distributed between the mobile and stationary phases. The mobile phase is usually a fluid that penetrates through or along the stationary bed (liquid or solid). Liquid, gas, and supercritical fluids are currently used as mobile phase and chromatographic techniques derive their names from the nature of the mobile phase: liquid chromatography, gas chromatography, and supercritical fluid chromatography, respectively (Wacoo, *et al.*, 2014). Liquid chromatography coupled to triple quadrupole mass spectrometry operating in modes capable of monitoring both molecular ions and their fragments, which are formed after collision with molecules of noble gases such as Ar (LC-MS/MS in MRM), is the confirmatory technique providing structural information of the analyte and its unequivocal identification. The drawbacks of LC-MS/MS techniques are high costs of analysis and limited availability of laboratories having the technical and human resources to perform such measuring (Stefanovic, *et al.*, 2015). The entire LC-MS instrument is provided together with a brief description of the key components: the LC-MS interface/ionization source and the mass analyzer. Typical LC-MS system consists of the components shown in Figure 3.

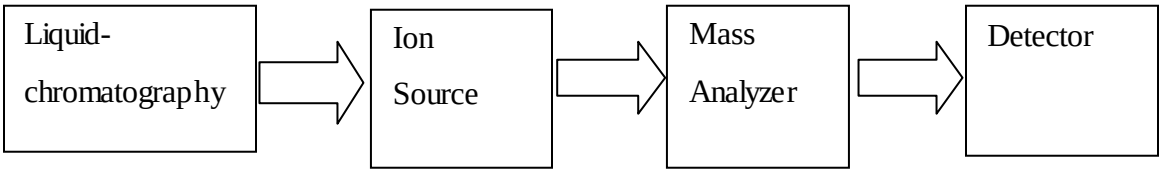


Figure 3: Basic components of LC-MS system

The ion source is used in the vaporization/ionization of the target molecules, mass analyzer is used to separate the gas phase ions by mass-to-charge ratio (*m/z*) and the detector detects the mass of separated ions and measures their relative abundance.

Triple quadrupole (QqQ) "tandem" mass analysers used for quantitation and the most widely used for quantitation of many analytes. The layout of a typical QqQ mass spectrometer is shown in Figure 4.

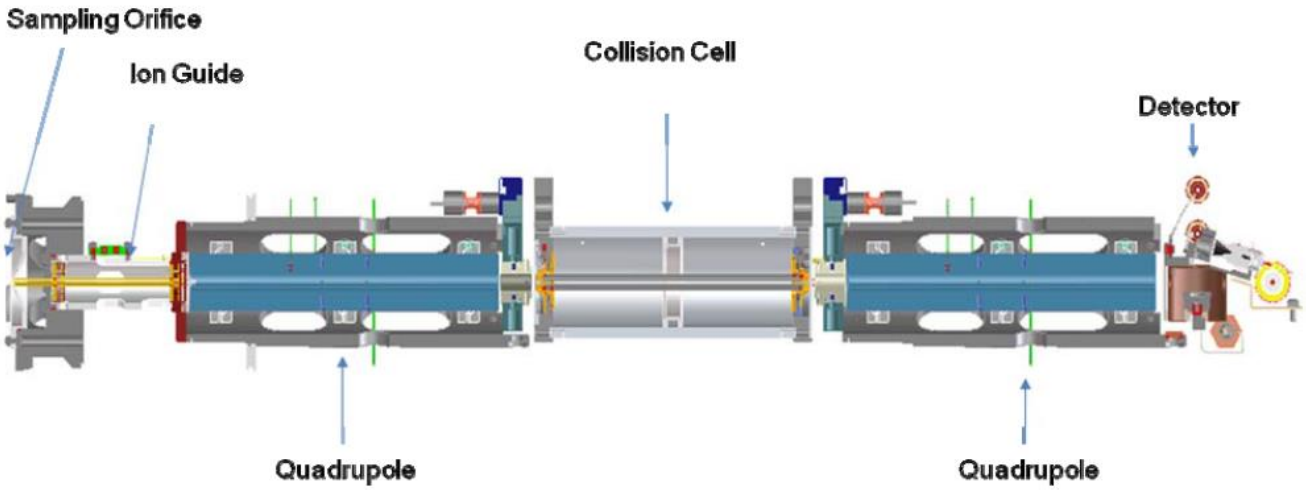


Figure 4: Schematic of a typical triple quadrupole (QqQ) mass spectrometer

Sample molecules are converted into gas phase ions in the ionization source before being accelerated into the mass analyzer through the sampling orifice and ion guide. Ions are then deflected by electrostatic fields in the quadrupoles according to their mass and their charge within the mass analyzer. The detector converts the ion energy into electrical signals, which are then transmitted to a computer for data handling including calculations required for quantification. Today, electro spray ionization (ESI) is the most common techniques in routine use for quantization of small molecules by LC-MS and schematic diagram of the source is shown in Figure 5.

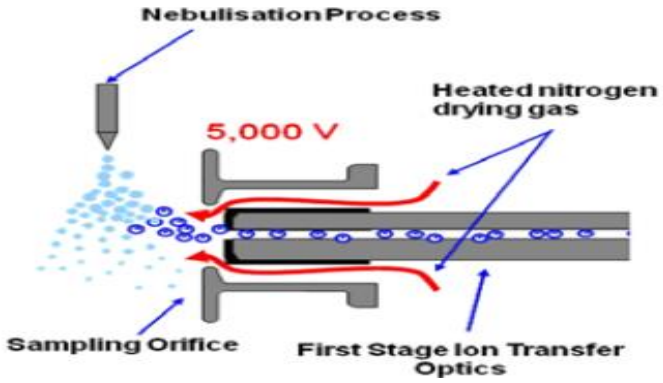


Figure 5: Schematic diagram of ESI source (Sargent., 2013)

2.3.8. Acceptance level for Aflatoxin by different Organization

Regulations for major Aflatoxins in commodities and food exist in at least 100 countries, but maximum tolerated levels differ among countries (Traistaru, *et al.*, 2012). Regulatory authorities in different countries have set tolerance limits for Aflatoxins that range from 0-50 µg/kg to control their levels in the food supply. Because Aflatoxins are unavoidable contaminants of foods, the World Health Organization (WHO) urged that their level be reduced to as low as reasonably achievable (Siruguri, *et al.*, 2012). The European Commission fixed maximum levels for Aflatoxin B1 (5.0 µg/Kg) and total (B1, B2, G1, G2) Aflatoxins (10.0 µg/Kg) in "maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in food stuffs" (Cheli, et al., 2009). The United States (US) Food and Drug Administration (FDA) established a 20 µg/Kg Aflatoxins action level for many major human foods and animal feeds (Dai, *et al.*, 2013, Abbas, *et al.*, 2006, Rahmani, *et al.*, 2010).The European Commission (EC) limits Aflatoxins (B1, B2, G1 and G2) in food for human consumption to 4 ppb and 2ppb for AFB1 (Reid, *et al.*, 2016, Campone, *et al.*, 2011, Bakırdere, *et al.*, 2012). The maximum legal limit allowed for AFB1 in infant food in the European Union is 0.1 µg/kg (Mushtaq, *et al.*, 2012). The limit prescribed by WHO for Aflatoxin B1 in various foodstuffs is 5 ppb and the total Aflatoxin level expressed by summing the concentrations of Aflatoxins B1, B2, G1 and G2 in foodstuffs cannot exceed 10 ppb. In Germany, even the Aflatoxin B1 and the total Aflatoxin level in baby-food products are regulated in values that cannot be greater than 0.02 and 0.05 ppb , respectively (Papp, *et al.*, 2002).

3. MATERIALS AND METHODS

3.1. Description of the study area

Amhara Region is located between 8°45′N and 13°45′N latitude and 35°46′E and 40°25′E longitude in North West Ethiopia. The climate of the Amhara region is affected significantly by variation in altitude, latitudinal position, prevailing winds, air pressure and circulation and its proximity to the sea. Traditionally, the climate of the region is divided into Kola (hot zone) which represents and cover 31 % of the region with altitude below 1500 meters a.s.l. and woyinadega (warm zone) which covers 44 % of the region encompasses areas between 1500-2500 m a.s.l and Dega (cold zone) which covers 25 % of the region representing areas between 2500-4620 ma.s.l. The annual average temperature of the region is between 15 °C and 21 °C. But in valleys and marginal areas the temperature exceeds 27 °C. The total area of the region is estimated at 156,960 km^2, which is divided into 11 administrative zones (Ayalew, *et al*., 2012). South Gondar is the one among the 11 zone of the Amhara regional state. This study was conducted in two woredas; Fogera and Dera their location in map of Amhara as shown in the Figure 6 by using GIS.

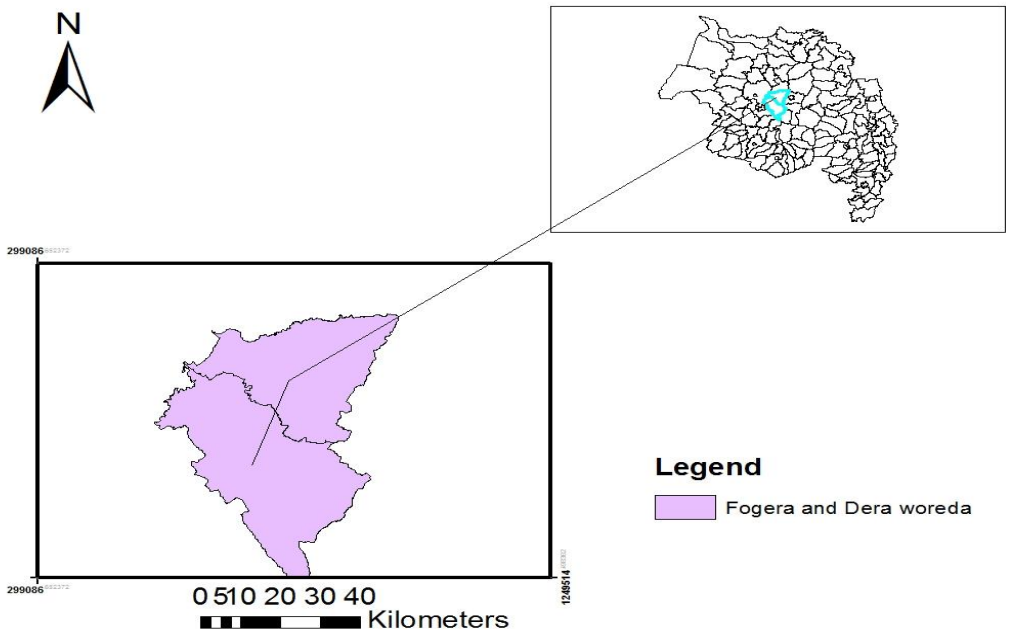


Figure 6: Location of study Area

3.2. Sample collection

The sample was collected from two districts of S. Gondar zone by random sampling in two local markets of those districts and the study period was conducted from March to June, 2017.

3.3. Apparatus, chemicals and reagents

Chemical and reagents

All chemicals and reagents used in this study were analytical grades and used without any further purification. The reagent; acetonitrile (ACN), methanol (MeOH) (HPLC grade and purity ≥ 99.9 %), acetone (purity> 99.8%), de-ionized water (conductivity < 0.06 μS/cm) and formic acid (purity > 99 %) were used.

Apparatus

Volumetric flasks, micropipette, glass amber bottle, falcon tubes screwed type, grinding device (KOHINOOR®), analytical balance (METTLER TOLEDO), beaker, glass syringes, syringe filters (0.2 and 0.45 μm), auto-sampler vials, filtration apparatus, vortex (Karl Hecht KG D97647 sand heim, Jermeny), shaker, centrifuge, rotatory evaporator, ultrasonic bath, eclipsed plus C-18 column (4.6×15mm), 3.5 μm) and Agilent 6460A triple quadrupole LC-MS/MS were the apparatuses and instruments used in the study.

3.4. Procedure

Preparation of mobile phase: 250 mL ACN (25 %), 150 mL MeOH (15 %), 1 mL formic acid (0.1 %) and 599 mL de-ionized water (60 %) were mixed and sonicated in HPLC reservoir for 30 min. Then, the mixture was filtered using 0.45 μm filter paper in the filtration apparatus. Similarly, equal volume of mixture was prepared without formic acid for standard solution preparations.

Preparation of working standard solutions

Accurately weighed 10 mg of each Aflatoxin (AFB1, AFB2, AFG1 and AFG2) were transferred into four different 100 mL volumetric flasks and dissolve using mobile phase for the preparation of 100 ppm individual AFs. With serious dilution various concentrations (10 ppm and 1 ppm) of individual

standard Aflatoxins were prepared. From 1 ppm standard solutions of individual Aflatoxins mixed 50 ppb solution were prepared and finally 0.1, 0.5, 1, 2, 5, 8, 10 and 15 ppb were prepared from 50 ppb by mobile phase without formic acid for calibration curve.

3.5. Sample preparation and extraction

Before analysis, the collected maize samples were mixed for homogenization and then, ground with grinding device (KOHINOOR®). The grinder was cleaned by acetone before and after grinding in order to prevent Aflatoxin cross-contamination. A 5 g of each maize flour sample was prepared for sample extraction. Direct analysis of samples using LC-MS/MS is possible, but it is usually important to clean up the samples to remove the worst interferences and concentrate the sample as the analyte present at very low concentrations. 15 mL of ACN:H_2O (84:16) was used as extraction solvent and each were blend in vortex mixer (Karl Hecht KG D97647 sand heim, Jermeny) at 800 rmp for 1 min and then shacked for 60 min on auto mechanical shaker. Centrifugations were performed for 10 min at 3000 rpm and then quantitatively the supernatant transferred to 50 mL round bottom flask. The extractions were performed two times with the extraction solvent in order to enhance the extract of the analyte from the sample. Then, the extracts were evaporated using rotary evaporator at (40 ^{0}C, 772 mbar) and then reconstituted using 10 mL of mobile phase (60 % H_2O: 25 % ACN: 15 % MeOH). The solution was filtrated using 0.45 µm syringe filter paper followed by 0.2 µm syringe filter paper and the filtrates were transferred into an auto sampler vial for LC-MS/MS analysis without further pre-treatment.

3.6. Recovery

Recovery is 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 percentage of recovery can be calculated as follows:

$$\text{Recovery (\%)} = \frac{A-B}{C} \times 100 \qquad (1)$$

Where, A is the concentration of the spiked sample, B is the concentration of non-spiked sample and C is the concentration standard added.

3.7. LC-MS/MS measuring conditions

The detection and quantification of Aflatoxins were performed with LC-MS/MS method by injecting 1 mL of the sample on a reversed-phase, eclipsed plus C-18 column (4.6×15 mm), 3.5 µm particle size) used as the stationary phase. The column was eluted using a gradient flow (0.5 mL/min) of the mobile phases (60 % H_2O: 25 % ACN: 15 % MeOH containing 0.1 % formic acid) and the injection volume was 10 µL used for in LC-MS/MS analysis. The parameter of ESI operation conditions was gas flow rate 0.5 mL/min, nebulizer 40 psi and positive ion source (mass spectrometry detection was carried out on positive ionization mode, because this mode gives sharp and sensitive signals). The temperatures of column, sheath gas, dry gas were 35, 350 and 350 ^{o}C, respectively. The LC-MS/MS parameters for analytes were cell accelerator voltage (7 V), ionization mode (+ve), fragmentor (130 V), Dwel (100 ms). The LC system was coupled to a triple-quadrupole mass spectrometer equipped with electro spray ionization (ESI) probe. The two most abundant product ions per analyte were chosen for quantitative and confirmation purposes. Two product ions were monitored for each Aflatoxins and LC-MS/MS quantitative analysis was carried out using multiple reaction monitoring (MRM) mode. The use of precursor and product ions in MS analysis allowed for sensitive detection and confirmation of all four Aflatoxins. The parameters for MRM transitions including, precursor ions, primary (quantifier) and secondary (qualifier) product ions and respective collision energies of four Aflatoxins are shown in Table 1.

Table 1: LC-MS/MS parameters for MRM transition of the four Aflatoxins

Compound	Precursor ion (m/z)	Primary Product ion (m/z)	Secondary Product ion (m/z)	Collision Energy of Primary/ Secondary (eV)
B1	313.1 $[M+H]^+$	285.1	241.1	20/35
B2	315.1 $[M+H]^+$	287.1	259.1	25/25
G1	329.1 $[M+H]^+$	311.1	243.1	20/25
G2	333.1 $[M+H]^+$	313.1	245.1	25/30

4. RESULTS AND DISCUSSION

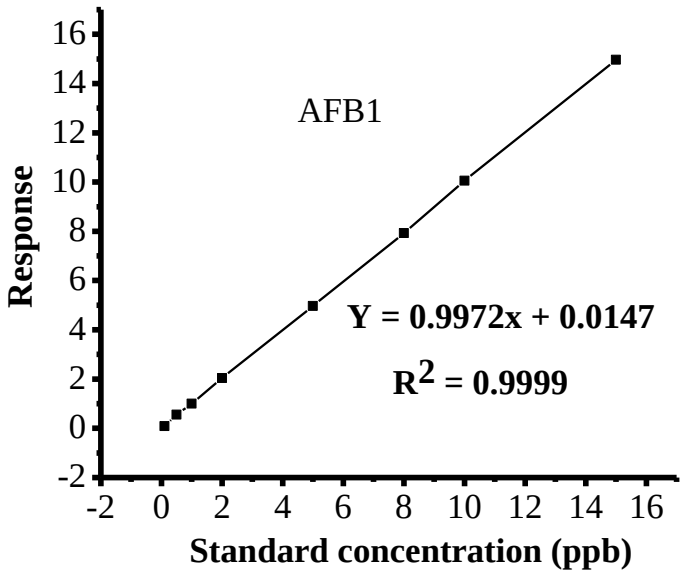
4.1. Calibration curve

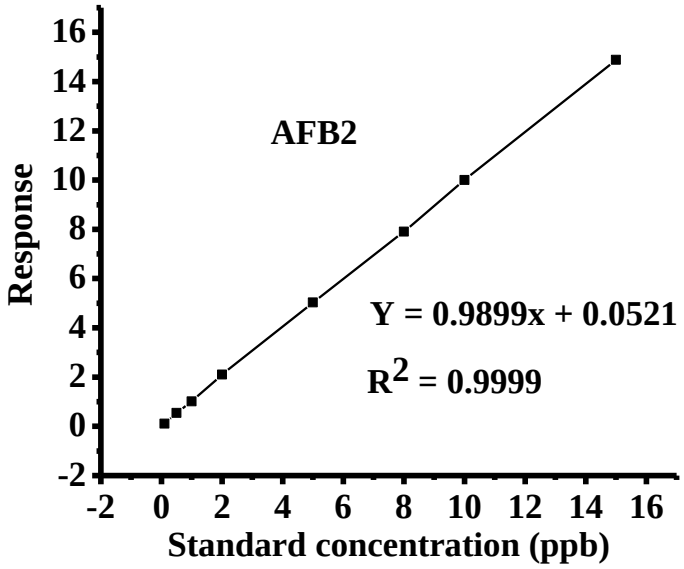
Linearity of LC-MS system was done by injecting different concentrations of standard. The system was calibrated by using the working solutions of Aflatoxins in the range of 0.1-15 ppb in a mobile phase of ACN: H_2O: MeOH. The series of working standard solutions of Aflatoxin were prepared from 50 ppb and the response (integrated peak area) is given in Table 2.

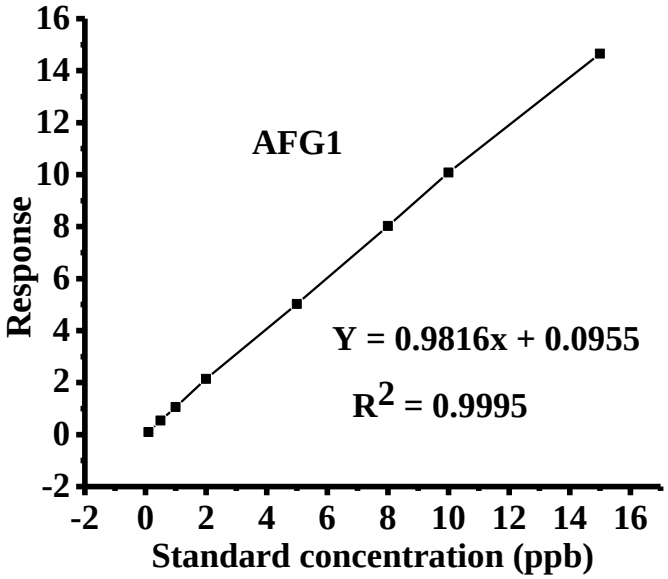
Table 2 : The series working standard solutions of Aflatoxin and responses

Standard (ppb)	Responses			
	AFB1	AFB2	AFG1	AFG2
0.1	0.0862	0.1092	0.0929	0.1091
0.5	0.5562	0.5477	0.5341	0.5812
1	0.9976	1.0160	1.0562	1.0137
2	2.0411	2.0993	2.1367	2.1239
5	4.9692	5.0309	5.0227	5.1698
8	7.9285	7.9089	8.0218	7.9576
10	10.0544	10.0034	10.0793	10.0072
15	14.9667	14.8847	14.6563	14.6375

The calibration curve, chromatogram, mass spectra and resultant mass peak were performed with origin 8 and Agilent mass hunter workstation software-data Acquisition for 6460 Series of triple quadrupole. The calibration standard of each concentration was constructed using the peak-area integration of the AFs versus the concentration of the standard. The calibration curve for Aflatoxins (B1, B2, G1 and G2) in LC-MS/MS method as shown in Figure 7 and their linear equations are given in Table 3. The analyzed working solution gives excellent values of regression coefficient for Aflatoxins. Regression coefficient (R^2) values were > 0.999 which was considered as evidence of an acceptable fit of the data to the regression line.


AFB1
Response
Standard concentration (ppb)
Y = 0.9972x + 0.0147
R2 = 0.9999


AFB2
Response
Standard concentration (ppb)
Y = 0.9899x + 0.0521
R2 = 0.9999



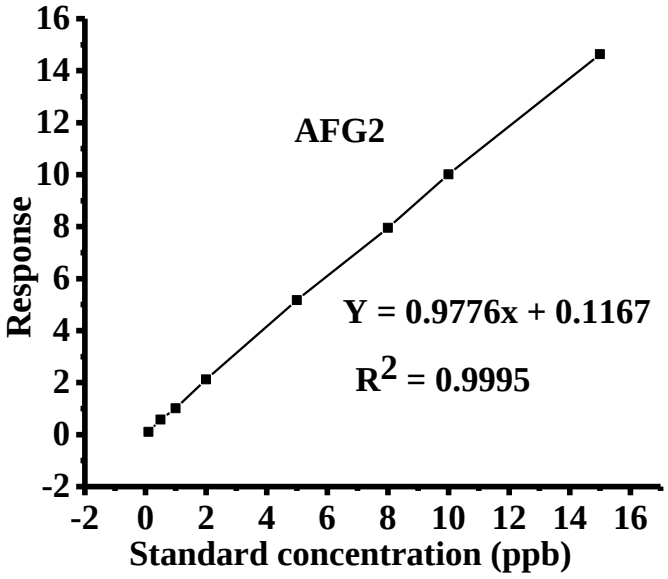


Figure 7: The calibration curve for standard Aflatoxins in LC-MS/MS method

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using equation (2):

$$LOD = \frac{3Sb}{m} \quad \text{and} \quad LOQ = \frac{10Sb}{m} \qquad (2)$$

Where m is the calibration sensitivity, Sb is the standard deviation of the blank.

The LOD and LOQ of all Aflatoxin were found in the range of 0.0253-0.0258 and 0.084-0.086 ppb, respectively.

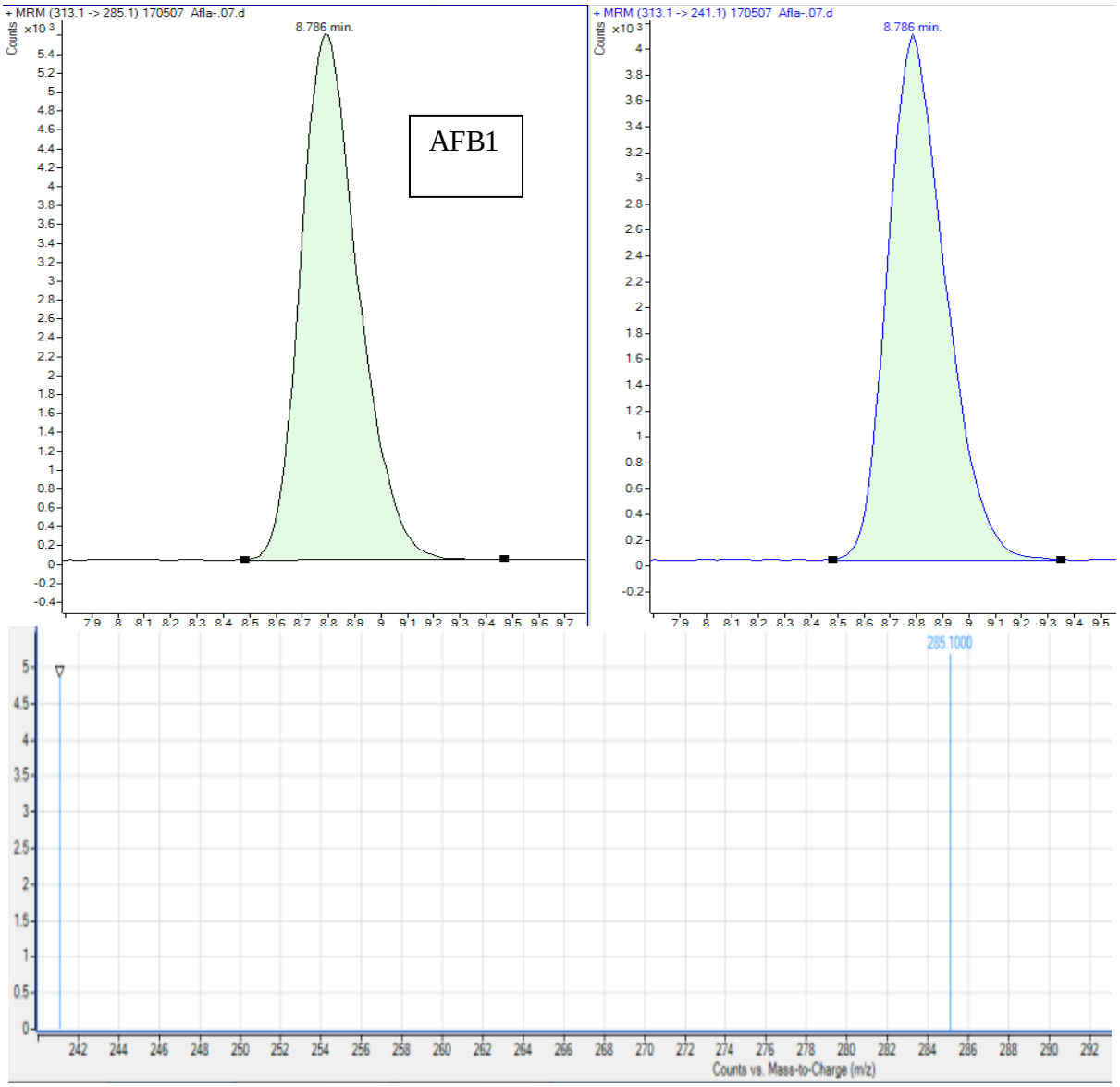
Table 3: Parameters of linear regression measured for Aflatoxins in LC-MS, LOD and LOQ

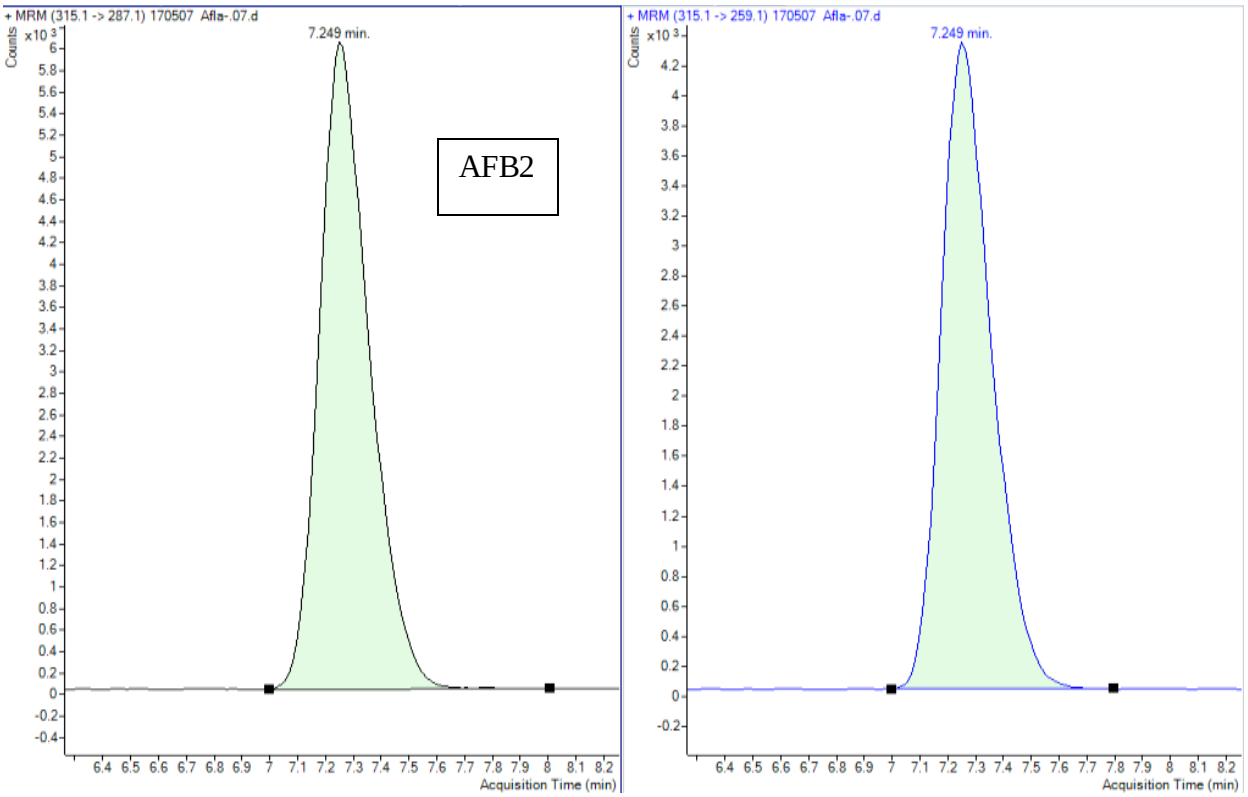
Aflatoxins	Linear equations	Slope	Intercept	R^2	LOD^n	LOQ^n
AFB1	$y = 0.9972x + 0.0147$	0.9972	0.0147	0.9999	0.0253	0.084
AFB2	$y = 0.9899x + 0.0521$	0.9899	0.0521	0.9999	0.0255	0.085
AFG1	$y = 0.9816x + 0.0955$	0.9816	0.0954	0.9995	0.0257	0.086
AFG2	$y = 0.9776x + 0.1167$	0.9776	0.1167	0.9995	0.0258	0.086

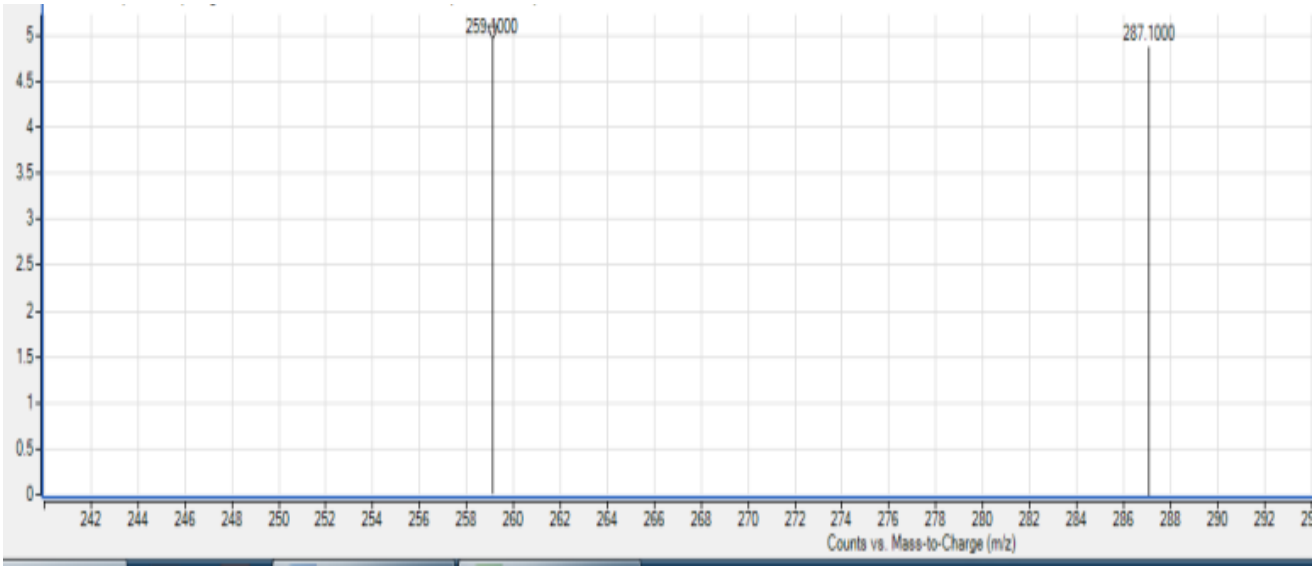
n=5 measurements

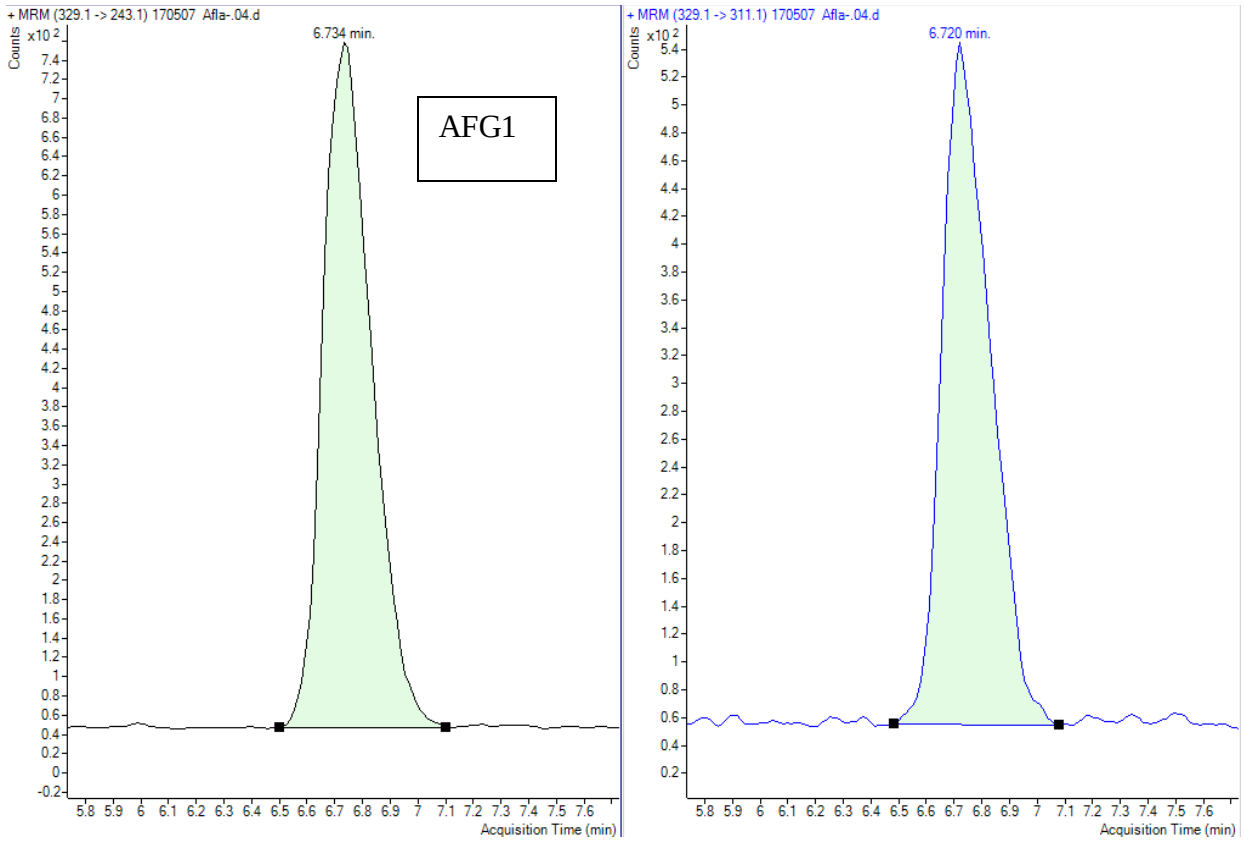
Standard Aflatoxin resultant mass peak and MS spectra analysis (MRM mode) of retention times used for recognize for AFs in samples analysis. The ion fragments were evaluated by their acquisition time to the most abundant m/z and the ion with the uppermost intensity was selected as the basic ion for quantization. From the chromatogram of standard Aflatoxins (B1, B2, G1 and G2) acquisition time were 8.786, 7.224, 6.727 and 5.652 min, respectively (Figure 8). These results agree with the acquisition time reported by Siddique, *et al.*, 2013. The mass spectral analysis of standard Aflatoxins of run time and m/z were 7.8-8.1 and 313.0 for AFB1, 7.1-7.5 and 315.0 for AFB2, 6.5-6.8 and 329.0 for AFG1 and 5.6-6.0 and 331 .0 for AFG2, respectively.

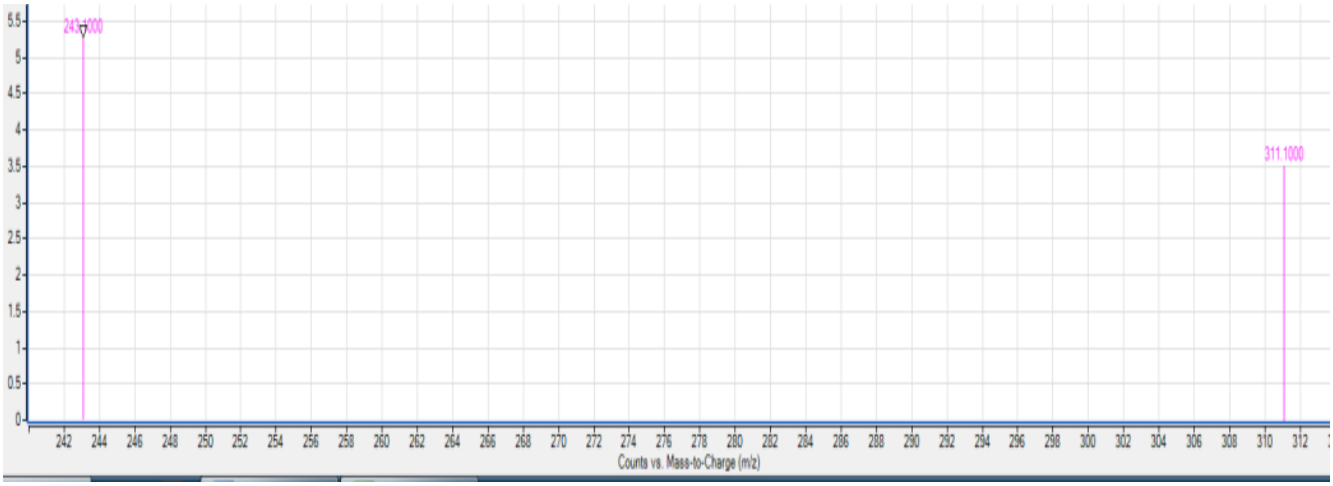
The results of LC-MS/MS chromatogram of standard Aflatoxin of retention time, peak response; peak sharpness as well as broadening described the sensitivity and separation efficiency of the method for Aflatoxins determination. Chromatogram of standard Aflatoxins, mass spectral and acquisition time of precursor $[M+H]^+$ to primary product ions & secondary product ion (m/z) were given Figure 8.


+ MRM (313.1 -> 285.1) 170507 Afla-.07.d
Counts
x10 3
8.786 min.
AFB1
+ MRM (313.1 -> 241.1) 170507 Afla-.07.d
Counts
x10 3
8.786 min.
285.1000
Counts vs. Mass-to-Charge (m/z)


+ MRM (315.1 -> 287.1) 170507 Afla-.07.d
7.249 min.
Counts
x10 3
AFB2
Acquisition Time (min)
+ MRM (315.1 -> 259.1) 170507 Afla-.07.d
7.249 min.
Counts
x10 3
Acquisition Time (min)


259.1000
287.1000
Counts vs. Mass-to-Charge (m/z)


+ MRM (329.1 -> 243.1) 170507 Afla-.04.d
6.734 min.
AFG1
Counts
Acquisition Time (min)
+ MRM (329.1 -> 311.1) 170507 Afla-.04.d
6.720 min.
Counts
Acquisition Time (min)


243.1000
311.1000
Counts vs. Mass-to-Charge (m/z)

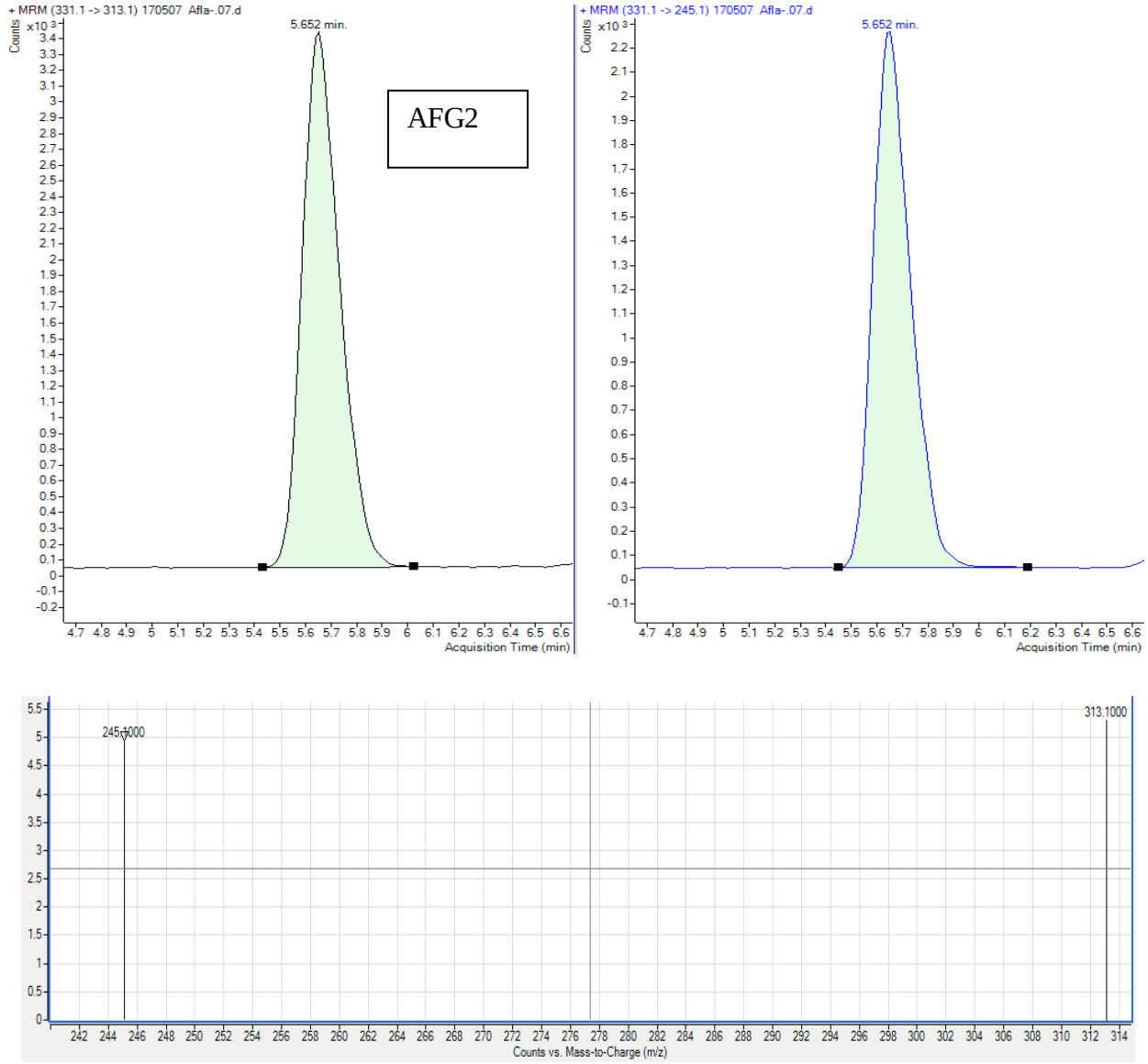


Figure 8: Chromatogram of standard Aflatoxins, mass spectra analysis and acquisition time

4.2. Levels of Aflatoxin in maize

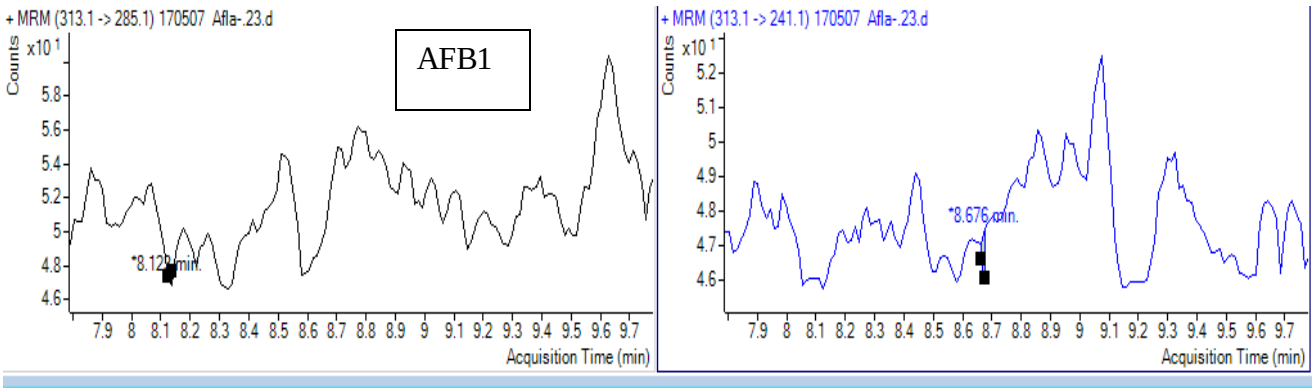
The present study was designed to assess the level of Aflatoxins contamination in maize grain by LC-MS/MS method. The concentration of each Aflatoxin was obtained by using the peak-area integration of chromatogram of maize sample. As shown in Table 6, the levels of all Aflatoxins were below LOD.

Table 4: LC-MS/MS results of Aflatoxins in maize sample from two districts of S. Gondar zone

Aflatoxins	Dera	Fogera
	Mean± SD (ppb)[n]	Mean± SD (ppb) [n]
AFB1	N.D	N.D
AFB2	N.D	N.D
AFG1	N.D	N.D
AFG2	N.D	N.D

n= 4 measurements, N.D = means not detected

In this study resultant mass peak of AFB1, AFB2, AFG1 and AFG2 were observed below the detection limit of 0.0253, 0.0255, 0.0257 and 0.0258 ppb. The LC-MS/MS chromatogram of maize sample in Fogera and Dera districts, counts vs acquisition time (min) and resultant mass peak of AFB1, AFB2, AFG1 and AFG2 are as shown Figure 9 and 10. As can be in the Figures no Aflatoxins were detected in maize samples. The acquisition times of all Aflatoxins in maize sample were range approximately between (5 to 9 min).



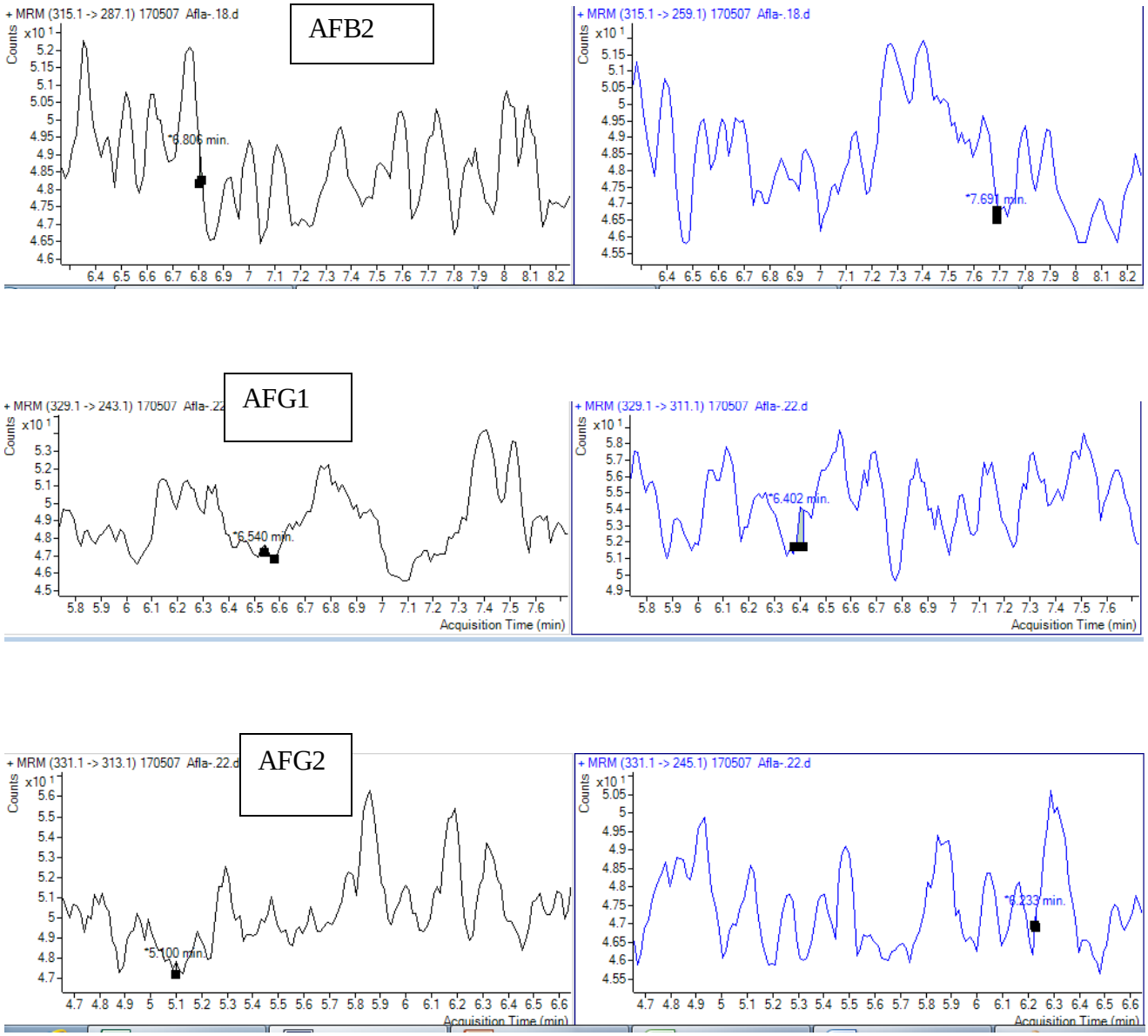


Figure 9: LC-MS/MS Chromatogram of maize sample and counts *vs* acquisition time (min) of Fogera

The chromatogram and resultant mass peak of AFB1, AFB2, AFG1 and AFG2 with transition of molecular fragment acquisition times for Aflatoxins in Fogera were 8.128 & 8.676, 6.806 & 7.691, 6.540 & 6.402, 5.1 & 6.233 min, respectively.

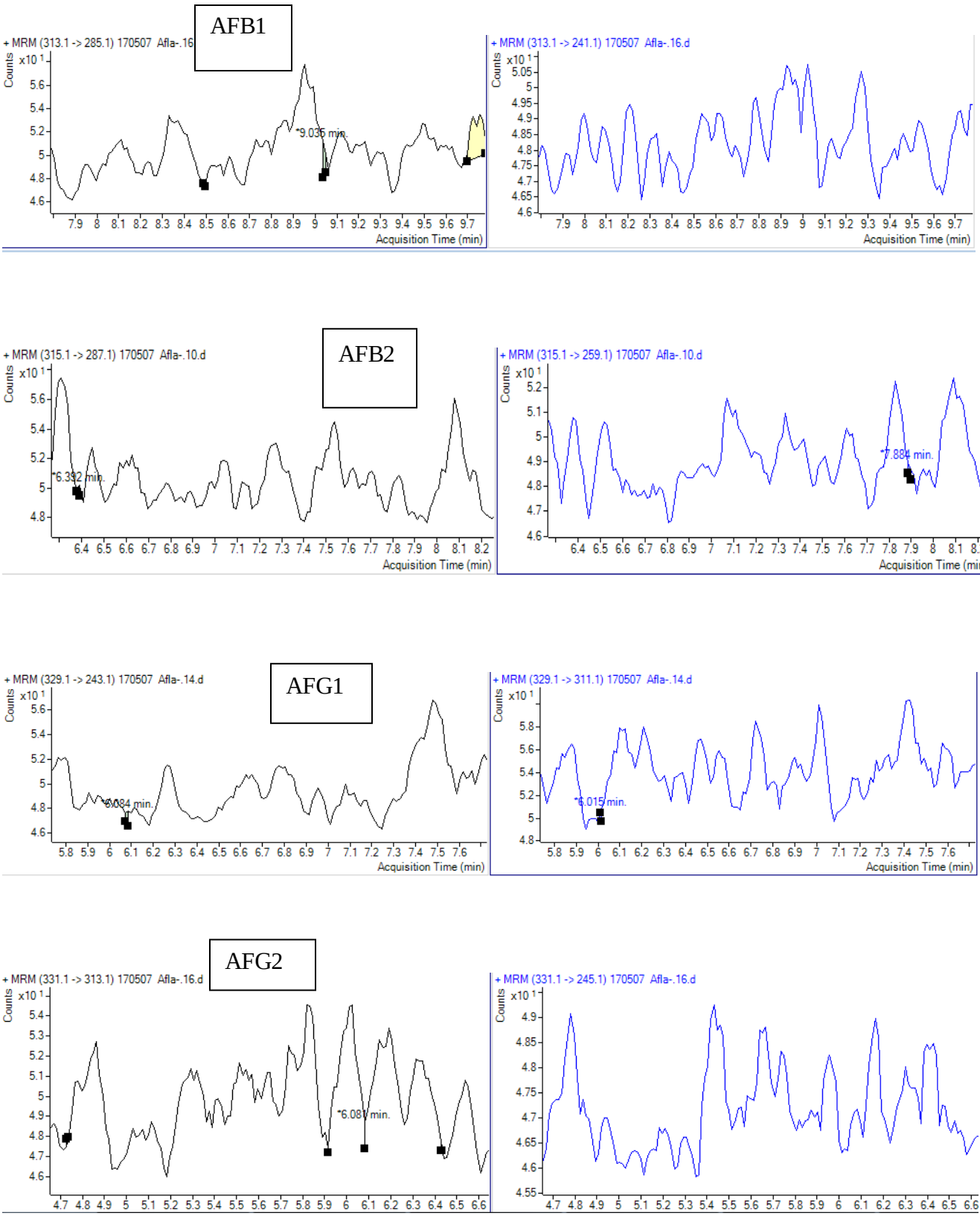


Figure 10: LC-MS/MS Chromatogram of maize sample and Counts *vs* Acquisition time (min) of Dera

The chromatogram and resultant mass peak of AFB1, AFB2, AFG1 andAFG2 with transition of molecular fragment acquisition times for Aflatoxins in Dera were 9.035, 6.392 & 7.884, 6.084 & 6.015, 6.081 min, respectively. The AFs in samples can be recognized by comparing their retention times with the standards and from the two districts results show comparable acquisition time (retention times) with standard chromatogram with suspected range of AFs acquisition time. But transition of molecular fragment acquisition times were varies it may be due to resultant mass peak below LOD.

4.3. Evaluation of matrix effect (recovery)

To study the matrix effects (accuracy) of the method, recovery experiments were carried out by spiking of 400 µL and 500 µL Aflatoxin standards into maize samples of Dera and Fogera, respectively. Then after, the Aflatoxins extracted with the same procedure as were done in non-spiked samples which described in section 3.5. The percentage recovery of Aflatoxins for triplicate measurements were found in the range of 65.95–97.60 % (Table 5 and 6)

Table 5: Recovery of Aflatoxins in Dera Maize samples

AFs	Aflatoxin conc. in spiked sample (ppb)	Un spiked sample (ppb)	Recovery(%)± SD	RSD (%)
B1	1.952	0.000	97.60±0.002	0.102
G1	1.591	0.000	79.55±0.011	0.691
B2	1.319	0.000	65.95±0.002	0.152
G2	1.458	0.000	72.90±0.029	1.989

Table 6: Recovery of Aflatoxins in Fogera Maize samples

AFs	Aflatoxin conc. in spiked sample (ppb)	Un-spiked sample (ppb)	Recovery(%)± SD	RSD (%)
B1	2.436	0.000	97.44±0.081	3.325
B2	2.380	0.000	95.20±0.16	6.722
G1	2.402	0.000	96.08±0.017	0.708
G2	2.425	0.000	97.00±0.048	1.979

The recoveries obtained in this study except 65.95 were within the range of guidelines described by Association Officials of Analytical Chemistry in the range of 70 to 125% (Loh saw, *et al.*, 2012).

4.4. Comparison of the results of the current study with other studies

Even though all the mass peaks below LOD the resultant mass peaks of this study were comparable with the results reported by Siddique, *et al.*, 2013. The reports showed that the medicinal plant Portulaca *oleraceae* (*P. oleraceae)* was contaminated with AFB1 (1.675 µg/kg) and AFB2 (1.335 µg/kg). The levels in Mucuna *pruriens* (*M. pruriens)* were below the detection limit for AFB1 (0.04 g/kg) and AFB2 (0.05 µg/kg) and were not detected in Delphinium *denudatum* (*D. denudatum)*. AFG1 (No resultant mass peak were observed) in *M. pruriens* and *P. oleraceae,* the chromatogram of (*P. oleraceae*, *M. pruriens* and *D. denudatum* respectively) and their study results as shown Figure 11. The black colored (the upper peak) chromatogram is for quantifier ion; while blue colored (the lower peak) chromatogram shows the qualifier ion for the MRM transition for LC-MS/MS of Aflatoxins. Ratio in the chromatogram is quantifier to qualifier ion ratio for the compound, (which determines confirmation of a compound). The letter A, B, C and D represents resultant mass peak of B1, B2, G1 and G2 in three medicinal plants of their study, respectively.

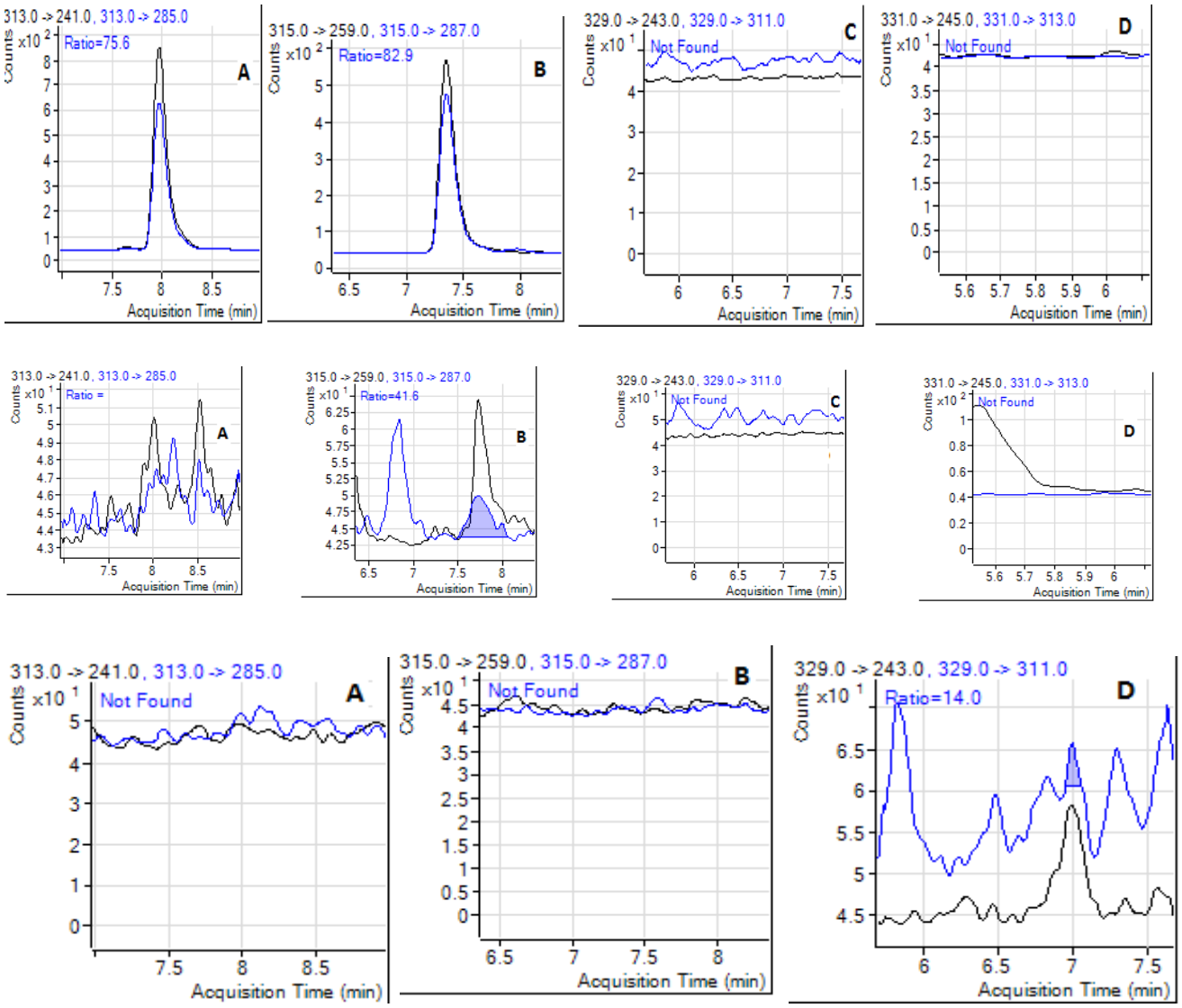


Figure 11: HPLC-MS/MS Chromatogram of *P. oleraceae*, and *M. pururiens D. denudatum*. Counts *vs* Acquisition time (min): sources (Siddique, *et al.*, 2013)

In South Africa research was also conducted on forty-two ground maize samples for determination of mycotoxins by Micro-HPLC-MS/MS and the LOD and LOQ were 0.025 and 0.05, respectively. Besides, the recoveries were in the range 86 to 120 % (Hickert, *et al.*, 2015). These results are comparable with the results of this study.

Another research also conducted on maize sample collected from Gedeo zone, Ethiopia using immuno-chromatographic assay and thin layer chromatography. Results of thin layer chromatography demonstrate that the mean Aflatoxin concentration for all 150 samples tested was 52.1 ppb. Among 150 samples tested, 56 % (84 samples) contained more than 50 ppb Aflatoxin. While, 28 % (42

samples) showed Aflatoxin concentration in the range of 40–50 ppb and 16 % (24 samples) have Aflatoxin concentration in the range of 20–40 ppb. Whereas, results of immuno-chromatographic assay revealed that the mean Aflatoxins concentration for all samples was observed as 53 ppb. Out of total 150 numbers of samples 53 % (80 samples) contained more than 50 ppb concentration of Aflatoxins, while 38 % (57 samples) have Aflatoxins level in the range of 40–50 ppb. In the remaining 9 % (13 samples), Aflatoxins concentration was found to be in the range of 20–40 ppb (Chauhan, *et al*., 2016).

In addition, Assaye, *et al*., 2016) and Khayoon, *et al*., 2010 were also determined the levels of Aflatoxins in livestock feeds and human food in pre-harvest and post-harvest maize samples. The results of the studies describe as AFB1 (5 ppb), AFB2 (1.17 ppb), AFG1 (10.1 ppb) and AFG2 (2.1 ppb) and AFB1 (9.86 ppb), AFB2 (7.2 ppb), AFG1 (18.11 ppb), AFG1 (8.14 ppb) pre-harvest and post-harvest maize samples, respectively. Finally, Ayalew, *et al*., 2006 also reported Aflatoxin in wheat bean with a level of AFG1 (8.6 ppb) and in maize AFB1 (6.31 ppb), AFB2 (0.13 ppb) and AFG2 (0.07 ppb). But, in this study the level of Aflatoxin contamination was below the LOD.

5. CONCLUSION

Maize (*Zea mays L.)* is a member of the grass family, which is believed to have originated in Mexico and introduced to Ethiopia in the 1600s to 1700s. It is very important agricultural crop and contains approximately 72 % starch, 10 % protein, and 4 % fat. The Food and Agriculture Organization (FAO) estimated that at least 25 % of the world's cereal grains are contaminated by mycotoxins, including Aflatoxins in each year and constitute loss at post-harvest. Aflatoxin contamination is the cause of disease in humans, animals, and has aggravated loss in quality and quantity of corn. Due to their effect on human health, Aflatoxins (AFs) have received substantial attention among the various mycotoxins. Aflatoxins are mainly produced through a polyketide pathway by several species and unnamed strains of Aspergillus species, which includes Aspergillus *flavus*, Aspergillus *parasiticus* etc. A method for simultaneous analysis of Aflatoxins (B1, B2, G1 and G2) toxin was developed using an LC-MS/MS method. The analyzed working solution gives excellent values of regression coefficient (R^2) values were >0.999 for Aflatoxins. In the concentration range of 0.1-15 ppb, this is considered as an evidence for linear relationship between the response and concentration. The LOD and LOQ were in the range of 0.0253-0.0258 and 0.084-0.086 ppb, respectively. The levels in maize samples from both study areas were found below the detection limit. The recovery of Aflatoxins B1, B2, G1 and G2 were in the range of 65.95 to 97.60 %. This indicated that the method was efficiently used for the determination of Aflatoxins in maize samples.

6. REFERENCES

Abbas, H. K., Cartwright, R. D., Xie, W. and Shier, W. T., 2006. Aflatoxin and fumonisin contamination of corn (maize, zea mays) hybrids in Arkansas. *Crop protection*, 25, 1-9.

Abbas, H. K., Weaver, M. A., Zablotowicz, R. M., Horn, B. W. and Shier, W. T., 2005. Relationships between aflatoxin production and sclerotia formation among isolates of aspergillus section flavi from the Mississippi Delta. *European journal of plant pathology*, 112, 283-287.

Abbas, H. K., Wilkinson, J. R., Zablotowicz, R. M., Accinelli, C., Abel, C. A., Bruns, H. A. and Weaver, M. A., 2009. Ecology of aspergillus flavus, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. *Toxin reviews*, 28, 142-153.

Abrha, S. W., Zeleke, H. Z. and Gissa, D. W., 2013. Line x tester analysis of maize inbred lines for grain yield and yield related traits. *Asian journal of plant science and research*, 3, 12-19.

Adeyeye, S. A., 2016. Fungal mycotoxins in foods: a review. *Cogent food and agriculture*, 2, 1-11.

Ahsan, S., Bhatti, I. A., Asi, M. R., Bhatti, H. N. and Sheikh, M. A., 2010. Occurrence of aflatoxins in maize grains from central areas of Punjab, Pakistan. *International journal of agriculture and biology*, 12, 571-575.

Aiko, V. and Mehta, A., 2015. Occurrence, detection and detoxification of mycotoxins. *Journal of biosciences*, 40, 943-954.

Assaye, M. A., Gemeda, N., Weledesemayat, G. T., 2016. Aspergillus species and aflatoxin contamination of pre and post-harvest maize grain in west Gojam, Ethiopia. *Journals of food science and nutriration*, 2, 13-18.

Ayalew, A., Fehrmann, H., Lepschy, J., Beck, R. and Abate, D., 2006. Natural occurrence of mycotoxins in staple cereals from Ethiopia. *Mycopathologia*, 162, 57-63.

Ayalew, D., Tesfaye, K., Mamo, G., Yitaferu, B. and Bayu, W., 2012. Outlook of future climate in northwestern Ethiopia. *Agricultural sciences*, 3, 608-612.

Ayejuyo, O. O., Olowu, R. A., Agbaje, T. O., Atamenwan, M. and Osundiya, M.O., 2011. Enzyme-linked immunosorbent assay (ELISA) of aflatoxin B1 in groundnut and cereal grains in Lagos, Nigeria. *Research journal of chemical sciences*, 1, 213-219.

Bakırdere, S., Bora, S., Bakırdere, E. G., Aydın, F., Arslan, Y., Komesli, O. T., Aydın, I. and Yıldırım, E., 2012. Aflatoxin species: their health effects and determination methods in different foodstuffs. *Central European journal of chemistry*, 10, 675-685.

Baydar, T., Engin, A. B., Girgin, G., Aydin, S. and Sahin, G., 2005. Aflatoxin and ochratoxin in various types of commonly consumed retail ground samples in Ankara, Turkey. *Annals of agricultural and environmental medicine*, 12, 193-197.

Bekeko, Z., 2014. Effect of enriched farmyard manure and inorganic fertilizers on grain yield and harvest index of hybrid maize (bh-140) at Chiro, eastern Ethiopia. *African journal of agricultural research*, 9, 663-669.

Campone, L., Piccinelli, A. L., Celano, R. and Rastrelli, L., 2011. Application of dispersive liquid–liquid microextraction for the determination of aflatoxins B 1, B 2, G 1 and G 2 in cereal products. *Journal of chromatography A*, 1218, 7648-7654.

Castells, M., Marín, S., Sanchis, V. and Ramos, A. J., 2008. Distribution of fumonisins and aflatoxins in corn fractions during industrial cornflake processing. *International journal of food microbiology*, 123, 81-87.

Castro, L. D. and Vargas, E. A., 2001. Determining aflatoxins B1, B2, G1 and G2 in maize using florisil clean up with thin layer chromatography and visual and densitometric quantification. *Food science and technology (campinas)*, 21, 115-122.

Chauhan, N. M., Washe, A. P. and Minota, T., 2016. Fungal infection and aflatoxin contamination in maize collected from Gedeo zone, Ethiopia. *Springerplus*, 5, 753-759.

Cheli, F., Campagnoli, A., Pinotti, L., Savoini, G. and Dellorto, V., 2009. Electronic nose for determination of aflatoxins in maize. *Biotechnologie, agronomie, societeet environnement*, 13, 39-43.

Dai, S., Lee, K. M., Li, W., Balthrop, J. and Herrman, T., 2013. Aflatoxin risk management in Texas: test kit approval for maize. *Journal of regulatory science*, 1, 15-22.

Dimitrieska-Stojković, E., Stojanovska-Dimzoska, B., Ilievska, G., Uzunov, R., Stojković, G., Hajrulai-Musliu, Z. and Jankuloski, D., 2016. Assessment of aflatoxin contamination in raw milk and feed in Macedonia during 2013. *Food control*, *59*, 201-206.

Donner, M., Atehnkeng, J., Sikora, R. A., Bandyopadhyay, R. and Cotty, P. J., 2009. Distribution of aspergillus section flavi in soils of maize fields in three agro ecological zones of Nigeria. *Soil biology and biochemistry*, 41, 37-44.

Dubale, B., Solomon, A., Geremew, B., Sethumadhava, R. G. and Waktole, S., 2014. Mycoflora of grain maize (zea mays L.) stored in traditional storage containers (Gombisa and sacks) in selected woredas of Jimma zone, Ethiopia. *African journal of food, agriculture, nutrition and development*, 14-19.

Eissa, A. A., Hassan, A. S. and El Rahman, T. A. A., 2014. Determination of total aflatoxins and carbamate pesticide residues in some bee honey samples using QuEChERS method and high performance liquid chromatography. *Food and public health*, *4*, 209-213.

Ethiopian transformation agency, maize sector development strategy (Working Document 2013-2017) Unpublished.

Fareed, G., Anjum, M. A. and Ahmed, N., 2014. Determination of aflatoxin and ochratoxin in poultry feed ingredients and finished feed in humid semi-tropical environment. *Journal of advanced veterinary and animal research*, 1, 201-207.

Fufa, B. and Hassan, R. M., 2006. Determinants of fertilizer use on maize in eastern Ethiopia: a weighted endogenous sampling analysis of the extent and intensity of adoption. *Agrekon*, 45, 38-49.

Georgievski, B., Kostik, V., Stojanovska-Geprgievska, L., Kochubovski, M. and Memeti, S., 2016. Qualitative and quantitative analysis of aflatoxins in raw peanuts (arachishypogaea L.). *Journal of environmental protection and ecology (JEPE)*, 17, 961-969.

Gizachew, D., Szonyi, B., Tegegne, A., Hanson, J. and Grace, D., 2016. Aflatoxin contamination of milk and dairy feeds in the greater Addis Ababa milk shed, Ethiopia. *Food control*, 59, 773-779.

Guchi, E., 2015. Aflatoxin contamination in groundnut (arachishypogaea L.) caused by aspergillus species in Ethiopia. *Journal of applied & environmental microbiology*, 3, 11-19.

Herrman, T. J., Lee, K. M., Jones, B. and McCormick, C., 2014. Aflatoxin sampling and testing proficiency in the Texas grain industry. *International journal of regulatory science*, 2, 7-13.

Hickert, S., Gerding, J., Ncube, E., Hübner, F., Flett, B., Cramer, B. and Humpf, H. U., 2015. A new approach using micro HPLC-MS/MS for multi-mycotoxin analysis in maize samples. *Mycotoxin research*, 31, 109-115.

Ketney, O., Ovidiu, T. and Tifrea, A., 2014. Structural diversity and biochemical and microbiological characteristics of aflatoxins. *Acta universitatis cibiniensis. Series e: food technology*, 18, 3-18.

Khayoon, W. S., Saad, B., Lee, T. P. and Salleh, B., 2012. High performance liquid chromatographic determination of aflatoxins in chilli, peanut and rice using silica based monolithic column. *Food chemistry*, 133, 489-496.

Khayoon, W. S., Saad, B., Yan, C. B., Hashim, N. H., Ali, A. S. M., Salleh, M. I. and Salleh, B., 2010. Determination of aflatoxins in animal feeds by HPLC with multifunctional column clean-up. *Food chemistry*, 118, 882-886.

King, B., 1979. Outbreak of ergotism in Wollo, Ethiopia. *The lancet*, 313, 1411. Cross reference

Krishnan, B., Ghadevaru, S., Manimehalai, Athmaselvi N., K. A. &, Padmavati, R., 2015. Determination of Aflatoxin B1 in corn flour using high performance liquid chromatography. *International journals of advanced biological research*, 5, 172-176

Liu, Z., Gao, J. and Yu, J., 2006. Aflatoxins in stored maize and rice grains in Liaoning Province, China. *Journal of stored products research*, 42, 468-479.

Loh saw, H., Nur iryani, M. Y., and How mun, L. (2012). Determination of aflatoxins B1 and B2 in peanuts and corn based products. *Sains malaysiana*. 39, 731–735

Lukeba, J. C. L., Vumilia, R. K., Nkongolo, K. C., Mwabila, M. L. and Tsumbu, M., 2013. Growth and leaf area index simulation in maize (zea mays L.) under small-scale farm conditions in a sub-Saharan African region. *American journal of plant sciences*, 4, 575-581.

Mahmoudi, R., Norian, R., Katiraee, F. and Alamoti, M. R. P., 2013. Total aflatoxin contamination of maize produced in different regions of Qazvin-Iran. *International food research journal*, 20, 2901-2904.

Maina, A. W., Wagacha, J. M., Mwaura, F. B., Muthomi, J. W. and Woloshuk, C. P., 2016. Postharvest practices of maize farmers in kaiti district, Kenya and the impact of hermetic storage on populations of aspergillus spp. and aflatoxin contamination. *Journal of food research*, 5, 53-59.

McDaniel, A., Holmes, W. E., Armbrust, K. L., Sparks, D. L. and Brown, A. E., 2011. Effect of matrix clean-up for aflatoxin analysis in corn and dried distillers grains. *Natural resources*, 2, 250-257.

Mitiku, M., Eshete, Y. and Tadesse, A., 2015. Evaluation of maize variety for ear rot (fusariumgraminearum) in south omo zone of Ethiopia. *Academia journal of agricultural research*, 3, 195-197.

Molla, A. and Zegeye, A., 2014. Effect of extrusion conditions on aflatoxin content of corn–peanut flakes. *Zede journal*, 32, 47-56.

Mushtaq, M., Sultana, B., Anwar, F., Khan, M. Z. and Ashrafuzzaman, M., 2012. Occurrence of aflatoxins in selected processed foods from Pakistan. *International journal of molecular sciences*, 13, 8324-8337.

Namjoo, M., Salamat, F., Rajabli, N., Hajihoseeini, R., Niknejad, F., Kohsar, F. and Joshaghani, H., 2016. Quantitative determination of aflatoxin by high performance liquid chromatography in wheat silos in Golestan province, north of Iran. *Iranian journal of public health*, 45, 905-911.

Nega A, Lemessa F, Berecha G 2016. Distribution and importance of maize grey leaf spot *cercospora zeae-maydis* (Tehon and Daniels) in South and Southwest Ethiopia. *Journal of plant pathol microbiol* 7, 362-369.

Nega, A., Lemessa, F. and Berecha, G., 2016. Morphological characterization of cercospora zeae-maydis (Tehon and Daniels) isolates in Southern and Southwestern Ethiopia. *Scientia*, 15, 348-355.cross refer

Papp, E., Klara, H., Záray, G. and Mincsovics, E., 2002. Liquid chromatographic determination of aflatoxins. *Microchemical journal*, 73, 39-46.

Rahmani, A., Jinap, S. and Soleimany, F., 2010. Validation of the procedure for the simultaneous determination of aflatoxins ochratoxin A and zearalenone in cereals using HPLC-FLD. *Food additives & contaminants: part a*, 27, 1683-1693.

Rajarajan, P. N., Rajasekaran, K. M. and Devi, N. K., 2013. Isolation and quantification of aflatoxin from aspergillus flavus infected stored peanuts 2, 87-94.

Ranum, P., Peña-Rosas, J. P. and Garcia-Casal, M. N., 2014. Global maize production, utilization, and consumption. *Annals of the new York academy of sciences*, 1312, 105-112.

Reid, C.X., Sparks, D.L., Williams, W.P. and Brown, A.E., 2016. Single corn kernel aflatoxin B 1 extraction and analysis method. *Natural resources*, 7, 405-409.

Sargent, M., 2013. *Guide to achieving reliable quantitative LC-MS measurements: RSC analytical methods committee*. ISBN 978-0-948926-27-3.

Shephard, G. S., 2008. Impact of mycotoxins on human health in developing countries. *Food additives and contaminants*, 25, 146-151.

Shenkut, A., 2015. Performance assessment irrigation schemes according to comparative indicators: a case study of Shina-Hamusit and Selamko, Ethiopia. *International journal of scientific and research publications*, 2, 451-465.

Shukla, S., 2016. Estimation of aflatoxins in peanut or maize by enzyme linked immunosorbent assay. *Bangladesh journal of pharmacology*, 11, 628-631.

Shweta, S., Madhavan, S., Paranidharan, V. and Velazhahan, R., 2013. Detection of aspergillusflavus in maize kernels by conventional and real-time PCR assays. *International food research journal*, 20, 3329-3335.

Siruguri, V., Kumar, P. U., Raghu, P., Rao, M. V. V., Sesikeran, B., Toteja, G. S., Gupta, P., Rao, S., Satyanarayana, K., Katoch, V. M. and Bharaj, T. S., 2012. Aflatoxin contamination in stored rice variety PAU 201 collected from Punjab, India. *Indian journal of medical research*, 136, 89-95.

Siddique, N. A., Mujeeb, M., Ahmad, S., Panda, B. P. and Makhmoor, M., 2013. Determination of aflatoxins in medicinal plants by high-performance liquid chromatography–tandem mass spectrometry. *Journal of pharmacy & pharmaceutical sciences*, 16, 321-330.

Stefanovic, S., Spiric, D., Petronijevic, R., Trailovic, J. N., Milicevic, D., Nikolic, D. and Jankovic, S., 2015. Comparison of two analytical methods (ELISA and LC-MS/MS) for determination of aflatoxin B1 in corn and aflatoxin M1 in milk. *Procedia food science*, 5, 270-273.

Sun, W., Han, Z., Aerts, J., Nie, D., Jin, M., Shi, W., Zhao, Z., De Saeger, S., Zhao, Y. and Wu, A., 2015. A reliable liquid chromatography–tandem mass spectrometry method for simultaneous determination of multiple mycotoxins in fresh fish and dried sea foods. *Journal of chromatography a*, 1387, 42-48.

Tomašević, I., Petrović, J., Jovetić, M., Raičević, S., Milojević, M. and Miočinović, J., 2015. Two year survey on the occurrence and seasonal variation of aflatoxin M1 in milk and milk products in Serbia. *Food control*, 56, 64-70.

Traistaru, E. and Moldovan, R. C., 2012. Comparative study regarding the application of HPLC and ELISA methods for aflatoxins detection in peanuts. *Journal of agroalimentary proc technol*, 18, 81-85.

Tsedaley B, and Adugna G (2016) Detection of fungi infecting maize (*zea mays* L.) seeds in different storages around Jimma, southwestern Ethiopia. *Journal of plant pathol microbiol* 7, 338-346.

Wacoo, A. P., Wendiro, D., Vuzi, P. C. and Hawumba, J. F., 2014. Methods for detection of aflatoxins in agricultural food crops. *Journal of applied chemistry*, 4, 232-236.

Yan, W. A. N. G., Dong, Y. J., Li, Z. M., Deng, L. G., Guo, C.Y., Zhang, S. Q., Li, D. P. and Zhao, S. C., 2016. Fast determination of multi-mycotoxins in corn by dispersive solid-phase extraction coupled with ultra-performance liquid chromatography with tandem quadrupole time-of-flight mass spectrometry. *Journal of integrative agriculture*, 15, 1656-1666.

Yao, H., Hruska, Z. and Di Mavungu, J. D., 2015. Developments in detection and determination of aflatoxins. *World mycotoxin journal*, 8, 181-191.

Zain, M. E., 2011. Impact of mycotoxins on humans and animals. *Journal of Saudi chemical society*, 15, 129-144.

7. ANNEXES

Annex 1: Some maize kernels sample pictures taken from photo during investigation

Annex 2: Aflatoxin analysis on LC-MS/MS pictures taken from photo