ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
CENTER FOR FOOD SCIENCE AND NUTRITION

AFLATOXIN LEVELS IN TELLA AND AREKI, TRADITIONAL ALCOHOLIC DRINKS, FROM THREE ZONAL ADMINISTRATIVE TOWNS OF GOJAM, ETHIOPIA

BY:

WORKINEH BIRHANU

A THESIS SUBMITED TO THE CENTER OF FOOD SCIENCE AND NUTRITION IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTRES IN FOOD SCIENCE AND NUTRITION

ADVISORS: ASHAGRE ZEWDU (PHD)
EMANA GETU (PHD)

June, 2016

Addis Ababa University
School of Graduate Studies Center for Food Science And Nutrition

This is to certify that the thesis prepared by Workineh Birhanu, entitled: "**Aflatoxin Levels in Tella and Areki, Traditional Alcoholic Drinks, From Three Zonal Administrative Towns of Gojam, Ethiopia**" and submitted in partial fulfillment of the requirements for the Degree of Masters in food Science and Nutrition according to the regulation and standards of the University and originality of the thesis is acknowledged.

Signed by

1. Dr. Ashagrie Zewdu (PHD, Advisor) Signature_____ Date_____

2. Dr. Emana Getu (PHD, Advisor) Signature_____ Date_____

Declaration

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

.

Name: Workineh Birhanu Signature: _____ Date: _____

Table of Contents **Page No.**

Acknowledgement

I would like to express my sincere gratefulness to Dr. Ashagre Zewdu, who is my primary research advisor, for his warm mentor, commitment, genuine support from the inception of this research work proposal. And, in due course, his close mentorship extends from the laboratory activities to the thesis work progress and revision of the documents. Without his heartily support it was not possible to realize this research work. I am also thankful to Dr. Emana Getu who contributed a lot in the revision of the research proposal to the thesis work with his notable, optimistic and simplicity approach.

Moreover, I am highly indebted to all Addis Ababa University Center of Food Science and Nutrition staffs especially to Woineshet, Debebe, and Yemane for their kindness and support in facilitating a conducive laboratory atmosphere for my research. It is my special appreciation to Afework Nigussie who has been my likable companion who supported me a lot on the laboratory work. And also my classmates Tesfanesh, Tegegne, Beharu who made my academic stay in AAU very friendly and unforgettable. And I thank all respondents who participated genuinely in my KAP survey.

It is also my genuine appreciation to Bless Agri- Food Laboratory and Ethiopian Conformity Assessment Enterprise for their resourceful support.

I couldn't conduct the research on time without the remarkable efforts made by my younger brother (Dr. Yelebe Birhanu, post-doctoral in Geo-physics university of Bristol, Uk) and Melkamu Belete (PHD Student, Uni- Siegen.de, Germany) for procuring the Immuno Affinity Columns from Germany. I am in debted to Bisrat Nigussie for his contribution in safe delivery of the items.

Last but not least, my gratitude goes to BGI Ethiopia, and especially to Mr. Paul Henri Selosse, and Mr. Samy Huguet who are top managers in BGI Technical Department, for their resource support and arranging the necessary leaves during exam periods and my research work. Moreover, my deepest recognition goes to my colleagues Deggu Shiferaw and Kebena Bula for their encouragement and support to realize my studies.

List of Tables

Table Name Page No.

List of Figures

Figure **Page No.**

List of abbreviations and acronyms

EFMHCA - Ethiopian Food, Medicine and Health Care Authority

ESA - Ethiopian Standards Agency

HPLC - High performance Liquid Chromatography

KAP - Knowledge, Attitude, Practice

HCC - Hepatocellular Carcinoma

DON - Deoxinivalenol

AFB1 - Aflatoxin B1

AFB2 - Aflatoxin B2

AFG1 - Aflatoxin G1

AFG2 - Aflatoxin G2

ATA - alimentary toxic aleukia disease

ZEN - Zearalenone

HBV - Hepatitis B virus hepatitis

HCV - Hepatitis C virus hepatitis

TLC - Thin layer chromatography

LC-MS/MS - Liquid Chromatography Tandem Mass Spectrometry

ELISAs - Enzyme linked immunosorbent Assays

SPE - Solid phase extraction

IAC - Immunoaffinity columns

CSA - Central Statistical Agency

DHS - Demographic Health Survey

ORA - Office of Regulatory Affairs Office of FDA

FDA - Food and Drug Administration

LOD - Limit of detection

LOQ -Limit of Quantification

ppb = parts per billion

Abstract

Aflatoxins are known mycotoxins of high public health significance. They are related to acute and chronic mycotoxicosis. This study evaluated the level of Aflatoxin in two traditional alcoholic drinks of Ethiopia (Tella and Areki) collected from three zonal administrative towns of East, West and Awi - zones of Gojam area namely in Debremarkos, Finoteselam and Enjibarra towns respectively. A total of 30 Tella and 30 Areki samples were evaluated for their Aflatoxin (B1, B2, G1, G2) level by HPLC with fluorescence detection after clean up with an Immunoaffinity column. Moreover, Knowledge, Attitude, and Practice (KAP) survey about Aflatoxin among consumers and producers of Tella and Areki were conducted. Method validation was done to address Limit of Quantification (LOQ), Limit of Detection (LOD), precision, accuracy, and recovery. Results revealed that, Aflatoxin levels detected in Areki samples were with inconclusive chromatogram peaks and the peaks possibly be "masked/modified" aflatoxins. While the mean total Aflatoxin level of the Tella samples were 12.8 ± 4.43 µg/kg, 14.4 ± 8.76 µg/kg, and 11.4 ± 3.38 µg/kg, in Debremarkos, Fintoselam and Enjibarra respectively. The level of individual Aflatoxin types detected were in the ranges of, AFG2 (< LOQ - 0.9) µg/kg, AFG1 (2.21 – 27.21) µg/kg, AFB2 (0.88 – 1.06) µg/kg, and AFB1 (< LOD - < LOQ). Except one sample, 96.67% of the Tella samples had total Aflatoxin level above the European Commission (EC) 4.0 µg/kg, total Aflatoxin limit legislation and this was contributed specifically by the G_1 type of Aflatoxin only. Nevertheless, with regard to FDA Aflatoxin action level (max20 ppb Aflatoxin limit) and Codex Alimantarius (max 10 ppb Aflatoxin limit), only 10 % of the samples in the former, and 70% of the samples in the latter had total aflatoxin levels beyond the limits. The most Carcinogenic type of AFB1 was not beyond the limits of 2.0 µg/kg EC legislation. KAP study survey on Aflatoxin, results indicated none of the study participants were never heard of Aflatoxin. Their attitude towards inclusion of mouldy cereals in their recipes didn't seem to worry them. It was observed the practices of the brewers and distillers in relation with Aflatoxin contamination, were vulnerable to Aflatoxin presence in the alcoholic drinks. Furthermore, it would be worth recommending also for further researches to integrate Mass Spectrometer with HPLC for certain identification of the peaks developed during Aflatoxin determination.

Key Words: Tella, Areki, Aflatoxin, KAP, Legislation

1. Introduction

1.1 Background

Aflatoxins are mycotoxins of high public health significance. They are known to cause both acute and chronic mycotoxicosis, which are exhibited ranging from different public health problems, like acute hemorrhagic necrosis of the liver to the chronic hepatocellular carcinoma.

There are four different types of structurally closely related Aflatoxin types designated as B1, B2, G1, G2 and another two derived aflatoxins called M1& M2 after the B1 and B2 aflatoxins respectively metabolized in the animal body and in the end transferred to Milk. Aflatoxins occur in foods and animal feeds after contamination by different Fungi species namely *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius (William* & Carl, 2000).

Different cereals used for human food are prone to Aflatoxins contamination both in the pre-harvest and post-harvest periods. Therefore, in effect these mycotoxins inevitably transfer to food items consumed while processing of the cereals as ingredients. A decade ago and later researches in the Ethiopian context implied Aflatoxins to exist in different cereals, though it cannot be applauded these few researches to show the overall picture of the level of Aflatoxin contamination in different food items in the country (Habtamu & Kelbessa, 2001). However, researches in the traditional alcoholic beverages of different African Countries like Kenya and Malawi, showed the beverages to have contaminated with a significant amount of Aflatoxin.

Ethiopia being a cradle of mankind and a home of ancient civilization, here believed to exist an age old practice of producing traditional alcoholic beverages historically. Depending on the localities there are many kinds of traditional fermented beverages in Ethiopia, not only of animal origin, but also of plant origin. Tella, tej, areki (katikala), borde, and shamita are some of the different types of traditional beverages in Ethiopia (Belay & Awraris, 2014).

Focusing on the research objective materials of this paper, Tella and Areki were selectively considered which are known to be widely consumed traditional alcoholic drinks. Tella is an indigenous, a home processed and commercially available traditional fermented alcoholic beverage in Ethiopia, and its alcohol content reported in the range of (2-8) % (v/v) (Getachew,

2015; Mooha et al.,2015). It is prepared from various cereals like maize, sorghum, finger millet, wheat, teff, barley and herbs like Gesho. It has various vernacular names in different regions: *Tella* in Amharic, *farso* in Afan – Oromo, and *Suwa* in Tigrigna. It is, by far, the most commonly consumed traditional alcoholic beverage in Ethiopia. The consumption in the rural places like Gojam area where the proposed research was conducted, it was evident to be huge and culturally deep rooted tradition of production and consumption of Tella and Areki. It was not possible to access data on the volume of production and consumption of tella and Areki. Nevertheless, one previous research in Addis Ababa showed over 2 million hectoliters of tella per year is thought to be produced annually in households and tella vending houses as to a study in 1991 in Addis Ababa (Mogessie, 2006). Areki, is a distilled traditional liquor where the brew to be fermented and used consequently for the distillation is so similar to Tella. However, the concentration of the brew preparation is high enough in the case of Areki. The ethanol content of "dagim" Areki is reported up to 48 % (v/v) (Getachew, 2015).

As we can infer from the traditional tella processing technique, a variety of cereals which are prone to Aflatoxin contamination are used as ingredients and different microorganisms involve to spontaneously ferment tella and Areki before the distillation uncontrollably. Therefore, this brings the notion of food safety concern with regard to Aflatoxin in the minds of the scientific community of food science, public health professionals, regulatory bodies and policy makers of Ethiopia. While on the contrary other similar countries gone so far in the research to assess the level in traditional beverages nothing to access and mention was done so far in Ethiopia.

This research assessed the aflatoxin level in Tella and Areki as well as to the KAP on aflatoxin in the research community of Debremarkos, Finoteselam and Enjibarra towns, where traditional alcoholic beverages production and consumption was observed to be so common.

1.2 Statement of the problem

In the light of Aflatoxin researches in the Ethiopian context, more than a decayed ago survey on agricultural commodities revealed Aflatoxin B1, was the predominant form. Where, the incidence of samples containing Aflatoxin B1 was 30% and then accompanied by aflatoxin $G_{1,}$ 6%. The highest levels of aflatoxin B1, was observed on peanut 738 µgkg-l and on sorghum 692 µgkg-l. The highest level of aflatoxin G1 found was 201 μgkg^{-l}. Groundnut, sorghum and millet samples have been identified as high-risk commodities based on the incidence rate of aflatoxin contamination. Levels of total aflatoxin greater than 20 μgkg^{-l}, were most frequently encountered in all aflatoxin positive samples of corn, sorghum, wheat, red pepper and peanut followed by barley (17%) and teff (13%) (Habtamu, & Kelbessa, 2001).

In another survey in Ethiopia for natural occurrence of toxigenic Fungi Species and Aflatoxin in freshly harvested groundnut Kernels in Tigray, Northern Ethiopia, showed all samples were found 100 percent positive for *Aspergillus* species. And the detected aflatoxin concentrations were ranging from 0.1 to 397.8 ppb (Dereje, 2012).

Despite, the here and there few researches in Ethiopia in relevance to aflatoxin, the investigation on fungi associated with grain, and also researches on the extent of the mycotoxins they cause can be concluded as very limited (Ephrem, 2015; Phelix, 2004)

Focusing on some of African traditional beverages, Aflatoxins on traditional opaque beer (sweet beverage called thobwa) in southern Malawi, revealed the average aflatoxin content in the beer was 22.32 µg/l (Limbikani et al., 2014). Also in another maize based traditional beer in Malawi on 9 samples, except one all beers contained aflatoxins at a mean concentration of 90 ± 95 µg/kg (Limbikani et al., 2011).

Busaa, a socio-cultural significant Kenyan traditional brew, aflatoxin level analysis on 61 samples tested, 93 % samples are positive and have mean of 5.2 ± 0.2 µg/kg of aflatoxin level (Mary, 2014). In another study in Africa, Nigeria of Cocoa powder based beverages showed 16.01 µg/kg aflatoxin level (Mary A., et al 2013).

The implications of previous researches on the cereals aflatoxin levels are the clues that it is mandatory to the study of level of contamination in the traditional alcoholic beverages in the Ethiopian context, which are prepared from the Aflatoxin prone cereals. Moreover, as stated earlier at least in the African context different countries have assessed the Aflatoxin level in their traditional beverages while the case is not so in Ethiopia. It is an observational inference of the researcher, the great majority of rural population, low income urban dwellers as well as the culture to consume traditional beverages in different feasts like wedding and holidays and different social gatherings even by high income communities is a deep rooted cultural practice in Ethiopia. There is a high prevalence of alcohol consumption culture in the research area. According to Ethiopia Demographic and Health Survey, 2011, 84.2 % of men and 78.3 % women aged 15- 49 drink alcoholic beverages and liquors (DHS, 2011). Despite this fact, there are no known studies to show the level of Aflatoxins contamination of alcoholic traditional beverages.

Therefore, the researcher believes in Ethiopia where, there is no clear mycotoxin exposure regulation and legislations, absence of organized population based cancer registry (Okobia, 2003) which makes aflatoxins induced health burden statistics remain to be unclear. Moreover, consumption of aflatoxin prone-cereals based traditional alcoholic beverages is worth researching in the process of aflatoxin exposure reduction strategies.

1.3 Significance of the study

This piece of research was focuses on:

- For surveying the aflatoxins level of contamination in selected traditional alcoholic beverages of Ethiopia. Thereby, rendering baseline data for future similar researches.

- It was believed also, to sensitize and bring the agenda of aflatoxins in general by different stakeholders like the agricultural sector, Ethiopian Food, Medicine and Health Care Authority (EFMHCA), Ministry of Health, Ethiopian Standards Agency (ESA), Ethiopian Public Health Association, Policy Makers, Food Scientists etc.

- Consequently, the stake holders would be benefited from the research findings to play their role in aflatoxin mitigation strategies. They may device awareness and advocacy endeavors, in effect to harmonize alcoholic traditional beverages consumption in the Ethiopian context in relation to Aflatoxin.

1.4 Objectives

1.4.1 General Objective:

- To determine the level of Aflatoxin contamination in Tella and Areki using ImmunoAffinity Column Clean up technique by sensitive HPLC with fluorescence detection.

1.4.2 Specific Objectives:

- To determine the types of different aflatoxin types (B1, B2, G1, G2) in Tella and Areki
- To Compare the Aflatoxin level between Tella and Areki.
- To assess the KAP of aflatoxin on traditional beverages preparation and consumption.

- To compare the level of Aflatoxin with international standards and similar traditional alcoholic beverages of African studies.

- To communicate or publicize the research finding and create awareness to concerned bodies.

2. Literature Review

2.1 Overview of Tella Making

In Africa, fermented alcoholic beverages are consumed in different social occasions such as wedding, naming and rain making ceremonies, at festivals and social gatherings, at burial ceremonies and settling disputes (Mooha et al., 2015). The case is so peculiar in Ethiopia, that a deep rooted and age old practice of the tradition exists.

In Ethiopia, villagers prepare a wide variety of traditional fermented foods and beverages from different raw materials such as cereals, ensete (false banana), honey, milk etc. Some of the known Ethiopian traditional fermented foods and beverages are Injera, Dabo (Bread), Ambasha, Kocho, Bulla, Ergo, Siljo, Tella, Tej, Areki (katikala), Borde, Cheka, Shamita, Korefe, Bukire, kineto, Merissa and, Keribo. Traditional fermented beverages, are those that are indigenous to a particular area and have been developed by the local people using age old traditional techniques and locally available raw materials (kebede et al, 2002; Mooha et al., 2015; Rashid, 2013).

Among these traditional foods and beverages, the over view of this study paper focuses on Tella and Areki production. The volume produced in the country as well as in the study areas where this research encompasses is not known and difficult to estimate.

The way of preparing tella differs by the ethnic group and depends on tradition and the economic situation. Although the basic processing steps are similar, every tella-maker seems to have her own recipe. The clay container (insera) is washed with water and fresh leaves of grawa (*Vernonia amygdalina*) several times. The well-cleaned container is then inverted over smoking splinters of weyra (*Olea europaea*) for about 10 minutes (Mogessie, 2006). This will eliminate microorganisms sensitive to antimicrobial components of wood smoke. It also contributes to the desirable flavor of the fermented product. To make “bikil” (malt), grains of barley or wheat are moistened while in a container and left to germinate for about three days. And this is finally sun-dried. Bikil is the source of amylase for the fermenting cereals used in tella preparation. The gesho plant (*Rhamnus prinoide*s), which is different from hop (*Humulus lupulus*) similar herb ingredient in beer, is widely cultivated in Ethiopia and is available dried in the local market.

Although gesho may have antibacterial effect against some groups of bacteria, its main purpose in the process is to impart the typical bitter taste and flavor to tella (Berhanu, 2013) The fermentable grains for tella preparation are usually prepared in two forms. Flours of finger millet, barely, maize or teff (dark variety) are toasted, milled, mixed in water and baked on a wide metal pan into kita (unleavened bread). The kita is broken into small pieces. Barley flour is separately toasted on a metal pan sprinkling water on it during toasting until it turns dark brown. This is called enkuro. The color of tella, which may vary from light yellow to dark brown, is determined by the extent of baking the kita or toasting the enkuro (Berihu et al. 2015).

The fermentation is divided into four phases. During the first phase, powdered leaves of gesho are mixed with water in a small earthen pot and allowed to ferment for four days. The fermenting material is commonly called tijet (Berhanu, 2013). This is transferred to a large earthen pot and the second stage begins by mixing it with barley/wheat malt, pounded stems of gesho, pieces of kita and water which is called tensis (Berhanu, 2013; Berihu et al. 2015). This is left to ferment for two more days. During the third stage, chopped pounded stems of gesho, bikil, enkuro and water are added to the container and the contents are mixed into a thick slurry called difdif. This is also allowed to ferment for two more days. At the final stage, the container is filled with water to the brim and the contents are again mixed thoroughly.

The container is then sealed to create anaerobic conditions and left to ferment for two more days. At the end of the fermentation, most suspended materials settle to the bottom of the container. The clear liquid is tella. In general, about 1 kg of gesho (leaves and pounded stems), 0.5 kg of bikil, 15 kg of grains, in the form of kita (5 kg) and enkuro (10 kg) are mixed with 30 liters of water to prepare tella. Good quality tella has a final ethanol content of 2-8% (v/v) and the pH is 4-5 (Belay & Awraris, 2014). When the clear tella is completely decanted from the sediment, fresh water is added to the sediment and mixed well. This is left to ferment. The resulting beverage is known as kirari and is weaker than the regular tella. It is most often used for family consumption, and sometimes is given to children. The better quality is often kept for guests.

Sometimes, at the end of the third stage, a smaller volume of water is mixed with the difdif and a more concentrated tella is obtained by filtering the difdif through a cotton cloth and keeping it in a closed container. Such tella is known as filtered tella (Berihu et.al 2015). Additionally, it should

be noted that other cereal varieties like maize (*Zea mays*), sorghum (*Sorghum bicolor*) are also widely used in tella making (Mooha et al. 2015).

When we see the microorganisms involved in Tella fermentation, mainly Saccharomyces spp., (mostly *S. cerevisieae*) and *Lactobacillus* spp. (mostly *Lactobacillus pastorianumi*) and aerobic mesophilic bacteria are some of the microbiota in Tella. The yeasts dominate the fermenting flora after the end of the first stage till the completion of fermentation (Mogessie, 2006; Mooha et al. 2015). In another study also, the microbial flora such as molds and bacteria were almost disappeared at the end of tella fermentation phase, especially in tella made with gesho. The possible reason for this phenomenon may be due to the synergic effect of both Gesho (*Rhamnus prinoides*) antibacterial substance, high alcohol concentration, reduction of pH as fermentation time increases and reduction of nutrient content of tella. However, Acetic acid bacteria which is able to convert ethanol to acetic can ruin tella to sour taste under aerobic conditions. Local societies, try to reduce the acidification of Tella by avoiding free air circulation (cover the brewing container with tight cloth) and they also increased the quantity of gesho with reduction of water and malt used for brewing of tella to elongate the shelf life (Berhanu, 2013).

2.2 Areki Processing

Areki is a distilled local liquor. It is colorless, traditional alcoholic beverage which is distilled from fermentation products prepared in almost the same way as tella except that fermentation mass in this case is more concentrated. Traditionally, Areki is divided in to two, Terra-areki and Dagim-areki. The term " dagim" in Amharic refers to 'second time' and, indicates that it is distilled second time or the redistilled Terra -areki, whereas the term terra in Amharic refers to "ordinary". The Alcohol content of Terra-Areki reaches to 34.0 % (v/v) and commonly varies between 22.0- 28.0 % (V/V). The mean Alcohol content in dagim Areki was reported up to 46.6 % (v/v) ethanol content (Getachew, 2013; Tadele et al., 2015).

In conclusion Tella and Areki, are prepared from different cereals like teff (rarely), wheat, sorghum, millet, maize, barley, and traditional malt (bikil - usually from wheat and barley) and herb called Gesho used in the fermentation for its aroma, flavor and bactericidal activity. A number of microorganisms' also involve to ferment the mix. While, Areki is a distilled traditional beverage

of the more concentrated tella like preparation. Tella and Areki making are summarized in the following flow chart.

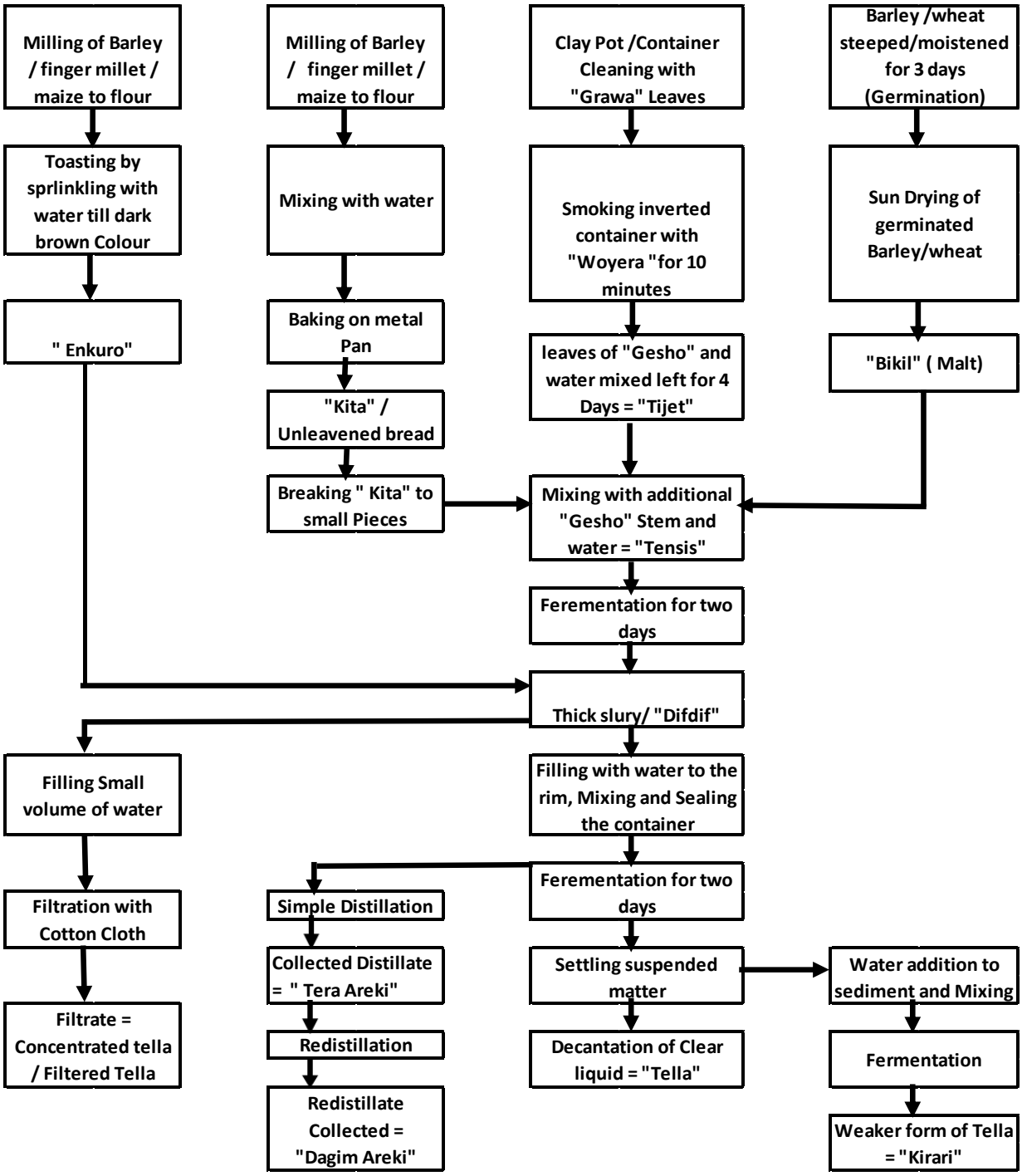


Fig 1. Flow chart of Tella and Areki Processing

Fig 2. Areki distillation in Awi –zone (Enjibarra town) picture taken during sample collection.

2.3 Mycotoxins

The presence of mycotoxins in grains and other staple foods and feedstuffs has serious implications for human and animal health. Exposure to mycotoxins can produce both acute and chronic toxicities ranging from death to deleterious effects on the central nervous, cardiovascular, pulmonary and digestive systems. Mycotoxins may also be carcinogenic, mutagenic, teratogenic and immunosuppressive. It also has a synergistic effect with the hepatitis B virus in the etiology of liver cancer and could interact with HIV/AIDS (WHO for Africa, 2006).

Mycotoxins are issues of food safety, health and agriculture. Sub-Saharan Africa where Ethiopia is in the domain is the most frequently affected by hunger. In 1978 mycotoxins responsible for gangrenous ergotism broke out in Ethiopia. In Kenya aflatoxicosis broke out in 1981, 2001 and also in 2004. Chronic exposure to mycotoxins can lead to cancers, growth retardation and immunosuppression (Mycotoxin control in food grains, 2004). According to the research in USA, on Global Burden of Aflatoxin-Induced Hepatocellular Carcinoma (HCC). Liver cancer or HCC, is the third leading cause of cancer deaths worldwide, with prevalence 16–32 times higher in developing countries than in developed countries (Yan, Felicia, 2010).

Mycotoxins are metabolic products of fungi which are capable of producing acute or chronic toxic effects (e. g carcinogenic, mutagenic, and teratogenic) on animals and probably on men at the levels of exposure. Toxic syndromes resulting from the intake of mycotoxins by man and Animals are known as mycotoxicosis. Although mycotoxicosis caused by mould *Claviceps purpurea* have been known for a long time mycotoxins remained neglected till 1960 when the Aflatoxins were discovered. Mould growth on foods is very common especially in warm and humid climates. It can occur in fields or in storage after harvest. Mould infection of foods such as grains, seeds and nuts is often localized in pockets especially in bulk storage and warehouses Currently a few hundred mycotoxins are known, often produced by genera, *Aspergillus, Penicillium and Fusarium* (William & Carl, 2000).

Aflatoxins, Ochratoxin A, Patulin, Deoxinivalenol, Citrinin, Fumonisins, T-2 Toxin, and Zearalenone are some of the notable mycotoxins.

Ochratoxins are a group of related compounds that are produced by *Aspergillus ochraceus* and related species, as well as *Penicillium verrucosum*, and certain other Penicillium species. These toxins have been found in corn, wheat, barley, flour, rice, oats, rye, beans, peas, green coffee beans, pancake mix, and mixed feeds. Ochratoxin A has been associated with porcine nephropathy, and also has been reported to be teratogenic to mice, rats, and chicken embryos. Despite the lack of conclusive evidence, Ochratoxin A has been suggested as a possible causative factor in a human disease known as Balkan Endemic Nephropathy.

Patulin is a highly reactive unsaturated lactone (4-hydroxy-4 H-furo [3, 2-c] pyran-2 (6H)-one) produced by certain species of *Penicillium*, *Aspergillus*, and *Byssochlamys*. It is of public health concern because of its potential carcinogenic properties. Patulin contaminates numerous agricultural products that are commonly consumed by both humans and animals. Strains of Patulin-producing mold have been isolated from grain, chick starter, malt feed, flour, moldy bread, bakery goods, sausage, cheese, and fruit, but the most common sources have been apples and apple products.

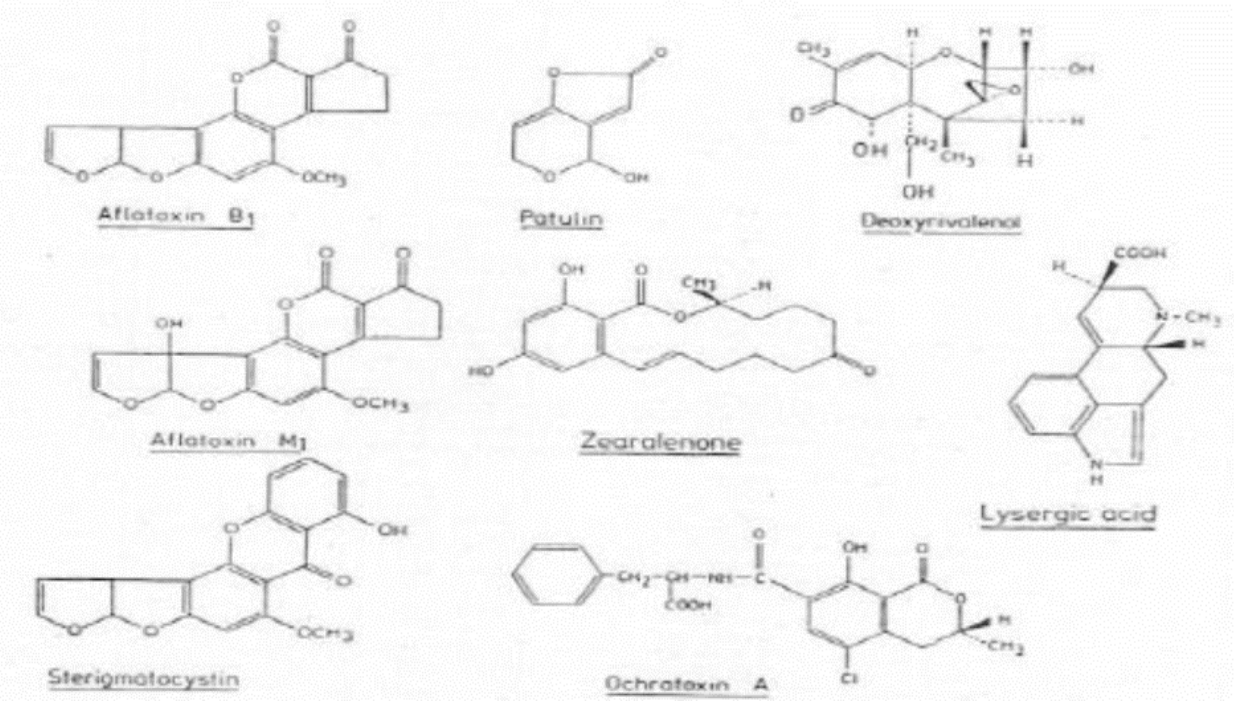


Fig 3. Chemical Structure of some important mycotoxins.

Deoxinivalenol (DON) is the most common of over 50 identified trichothecenes toxins. Trichothecene mycotoxins are mold metabolites produced by Fusaria sp. These compounds are of concern because of their frequent presence in agricultural commodities such as wheat, corn, barley, and oats. Some of the adverse health effects for contaminated crop consumption include reduced weight gain and feed consumption, feed refusal, diarrhea, emesis, immune suppression, gastrointestinal irritation, oral lesions, and death.

Citrinin is a secondary metabolite produced by *Penicillium citrinum* and *P. viridicatum* that usually accompanies ochratoxin A; it is also a metabolite of some *Aspergillus* species. Citrinin is an unstable mycotoxin in grains and apple juice, so it degrades at a fast rate. The most commonly affected commodities are mixed barley, oats, corn, and yellow peanut kernels. Citrinin has been related to kidney damage in laboratory animals and may be involved in cases of swine nephropathy. Some studies have addressed its potential for immunotoxicity.

Fumonisins are the most recently characterized toxins produced by Fusarium moniliforme. Although other Fusaria sp. produce fumonisins, F. moniliforme section Liseola is the most toxigenic. In humans, fumonisins have been epidemiologically linked to human esophageal cancer. Recently, the International Agency for Research on Cancer classified F. moniliforme toxins as potential carcinogens (class 2B carcinogens) to humans.

T-2 toxin, a Trichothecene mycotoxin produced by *Fusaria* sp., is primarily associated with moldy millet, but also with wheat, rye, oats, and buckwheat, Due to its lipophilic nature, T-2 toxin appears to be transmitted to milk when dairy cattle are fed contaminated grain. This toxin has been shown to be an inhibitor of protein and DNA synthesis in mammalian cells, a potent dermal irritant, and an impairing immune function agent. It is cytotoxic and has a radiomimetric effect on rapidly dividing cells. T-2 toxin has been implicated in alimentary toxic aleukia disease (ATA).

Zearalenone (ZEN) is produced by *Fusarium* graminearum and *F. sprotrichoides* in the field and during storage of commodities such as corn, barley, pig feeds, silage, sorghum, and hay. Hyperestrogenism is the most common biological effects associated with zearalenone (William & Carl, 2000).

2.4 Aflatoxins

Aflatoxin is probably the most common and widely known mycotoxin contaminant. It is produced by the moulds *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. In fact, the name is a composite word derived from 'A. flavus toxin'. There are six aflatoxins of analytical interest. Four occur in foods and two as metabolites in the milk of animals who have been fed contaminated feed. Aflatoxin B1, B2, G1, and G2 refers to toxins which fluoresce blue (B) or green (G) under ultraviolet light and are separable by thin layer chromatography. The only structural difference between B and G toxins is the inclusion of an oxygen in the cyclopentanone ring.

Aflatoxin M 1 and M 2 represent the toxin B 1 and B 2 which have been metabolized within the body of a lactating animal. Their finding in milk led to their M designation. The obvious structural difference between B and M is the addition of the hydroxyl group.

A. flavus is a common constituent of the microflora in air and soil throughout the world. It is prevalent in stored wheat, corn, cottonseed, rice, barley, bran, flour, peanuts, soybeans, sorghum, chili peppers, copra, millet, tree nuts, and green coffee beans, among other commodities. Growth can occur even when products are stored under relatively low moisture, which eliminates the growth of competing species such as *Penicillium* and *Fusarium*. However, storage in hot or humid conditions can aggravate toxin formation. Aflatoxin contamination also may be severe when developing crops are exposed to drought conditions.

The carcinogenicity, mutagenicity, teratogenicity, and acute toxicity of aflatoxins have been well documented. AFB1 is the most important in terms of occurrence and toxicity, and the most potent of the naturally occurring carcinogens.

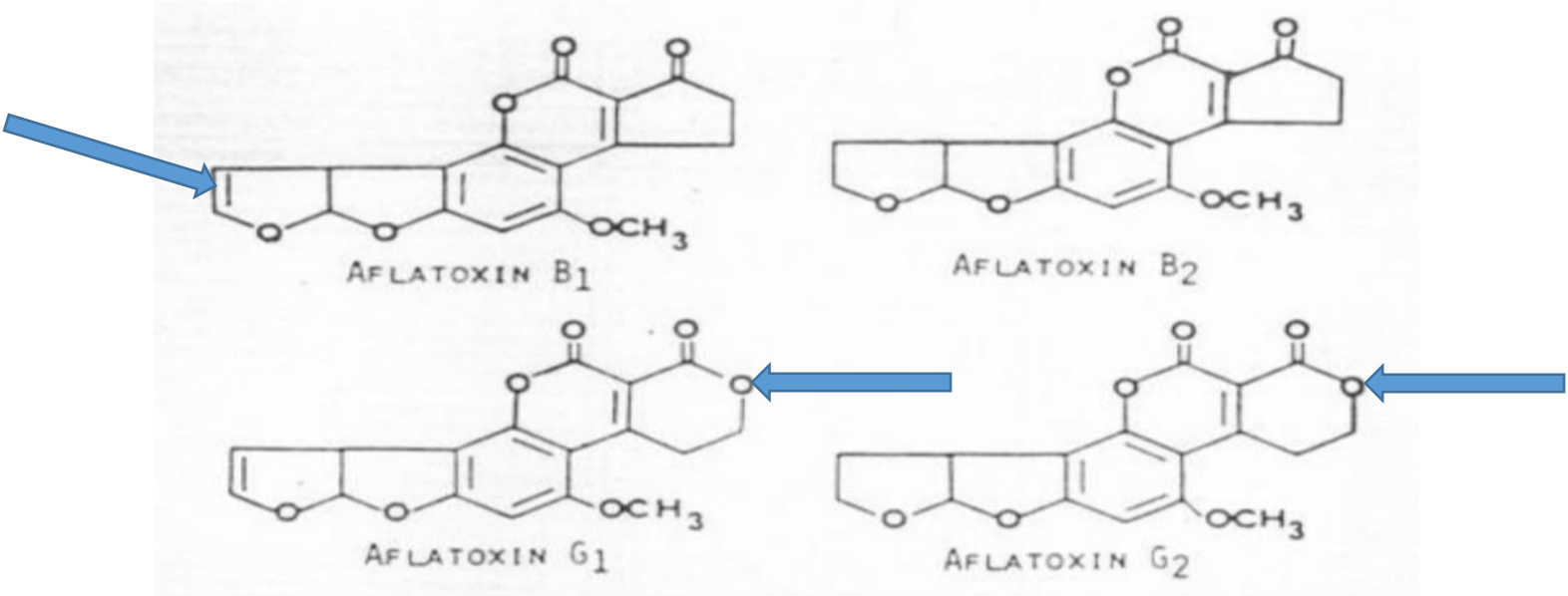


Fig. 4 The chemical structure of different types of Aflatoxins (Arrows are to show some differences in the chemical structure)

2.5 Toxicology of Aflatoxin

Aflatoxins are a group of closely related compounds with small differences in chemical composition. Aflatoxin B1 (AFB1) is the most prevalent form and also the most potent of these toxins. Aflatoxicosis is the poisoning that results from ingesting aflatoxins. Two forms of aflatoxicosis have been identified: the first is acute severe intoxication, which results in direct liver damage and subsequent illness or death, and the second is chronic sub symptomatic exposure.

The chronic incidence of aflatoxin in diets is evident from the presence of Aflatoxin M1 in human breast milk in Ghana, Nigeria, Sierra Leone and Sudan as well as in umbilical cord blood samples in Ghana, Kenya, Nigeria and Sierra Leone. Epidemiological studies have shown a strong correlation between exposure to aflatoxins and primary liver cancer. Aflatoxin exposure in children is also associated with child stunting and child neurological impairment. A cross-sectional study in Benin and Togo investigated aflatoxin exposure in children and showed that exposure to this toxic contaminant increases markedly following weaning and was associated with reduced growth (Phelix, 2006).

Acute Aflatoxicosis

The symptoms of severe aflatoxicosis include hemorrhagic necrosis of the liver, bile duct proliferation, edema, and lethargy.

Chronic Aflatoxicosis

Cancers: For humans, aflatoxin is predominantly perceived as an agent promoting liver cancers, although lung cancer is also a risk among workers handling contaminated grain. The increased risk of hepatomas is caused by deletion mutations in the P53 tumor–suppressing gene and by activation of dominant oncogenes. The risk of cancers due to exposure to the various forms of aflatoxin is well established and is based on the cumulative lifetime dose.

In many developing countries, epidemics of hepatitis B virus (HBV) and hepatitis C virus (HCV) affect ≤ 20% of the population. A strong synergy is observed between aflatoxin and these biological agents for liver cancer. In hepatitis surface antigen–positive subjects, aflatoxin is ~30 times more potent than in persons without the virus, and the relative risk of cancer for HBV patients increases from ~5 with only HBV infection to ~ 60 when HBV infection and aflatoxin exposure are combined. In some areas where aflatoxin contamination and HBV occur together, hepatomas are the predominant cancer (64% of cancers), and they may be a predominant cause of death: ~10% of males in Gambia die of liver cancer. Thus, to minimize the risk of liver cancer, it is critically important that exposure of HBV- and HCV-infected persons to aflatoxin is minimized.

A factor in this greater potency of aflatoxin in HBV-positive people is the finding that HBV positivity reduces the person's ability to detoxify aflatoxin. Whereas this synergy is recognized as an important factor for cancer, it is also of great potential importance for immunologic and nutritional toxicities, because it increases the level of biological exposure.

Moreover, as to the different studies done on farm animals and animal models conducted in poultry, pigs, and rats showed that exposure to aflatoxin in contaminated food results in suppression of the cell-mediated immune responses. Chronic aflatoxin exposure has major effects on nutritional status in animals, but, as with the immunotoxicities, thresholds for these effects are not defined for any species (Jonathan et al., 2004).

Thus, it is well established in animals that dietary aflatoxin reduces the rate of growth and other measures of productivity. Recent human research also confirms that this effect applies to humans: a dose-response relation was seen between aflatoxin exposure and the degree of stunting and underweight in children ≤ 5 years old in Benin and Togo, where all members of the study population had aflatoxin exposure (aflatoxin-albumin adducts between 5 and 1064 pg/mg albumin in 99%of the children). The toxin has also been shown, as a logical outcome of the effect of aflatoxin on protein synthesis, to be a factor modulating the rate of recovery from protein malnutrition although it has not been shown to be responsible for the development of the condition (Jonathan et al., 2004).

2.6 Aflatoxin Decontamination Approaches

Pre-harvest prevention of aflatoxin formation is difficult; therefore, aflatoxins in foods and feeds are considered a continuous risk. There is a need to manage the risks associated with aflatoxin contamination before using these products as animal feed or human food.

Several methods for decontamination and postharvest control have been reported. The use of ammonia-heat treatments has shown effective reduction of aflatoxin. Other chemicals such as monomethyl amine, sodium hydroxide, sodium hypochlorite, and hydrogen peroxide also have resulted in acceptable detoxification in several commodities. During fermentative production of ethanol, little degradation of the toxin was achieved. Other decontamination approaches include food and feed processing such as thermal inactivation, irradiation, solvent extraction, mechanical separation, density segregation, and reduction in bioavailable aflatoxin by selective chemisorption. Biocontrol methods and microbial inactivation have been also suggested (William & Carl, 2000).

2.7 Degradation of Aflatoxin during Fermentation

A number of studies have been conducted if fermentation has an effect on the concentration of aflatoxins, a study in South Africa demonstrated, decreased concentration of aflatoxin B1 in natural lactic acid fermented maize porridge (amahewu) samples. They spiked maize meal with 60 µg g-1 AFB1, and fermented, with or without starter culture, for 4 days at 25^{o}C. Unbound AFB1 in solution and the pH of the media were monitored daily. A significant decrease ($P < 0.05$) in the level of unbound AFB1 was observed (75% in the fourth day). The researchers of this study

concluded that lactic acid fermentation can significantly reduce the concentration of AFB1 in maize to trace levels. However, the safety of fermented products has not been well studied, as the mechanism of AFB1 removal is not well understood (Mokonea et al., 2006).

In a similar study in the republic of Korea, the study which was performed to investigate the inhibitory effects of four bacteria (*Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Bacillus subtilis*) which are found in fermented foods on the growth and aflatoxin production of *A. parasiticus*. Reduction of mycelial growth of *A. parasiticus* as a result of co-inoculation of the four bacteria was observed to range between 20.9 to 86.2% while reduction of aflatoxin production ranged from 21.6 to 70.4%. The great reduction was found when the mold was co-inoculated with *B. subtilis*, then with *Leu.mesenteroides*, then with *L. casei*, and the least reduction with *L. plantarum* (Jong, 2007).

Moreover, in a study in Morocco of a fermented sourdough bread, the ability of some selected strains of lactic acid bacteria isolated from traditional sourdough ferments to remove aflatoxin B1 (AFB1) was studied. Results showed that, Lactobacillus strains could remove more AFB1 than *Pediococcus* and *Leuconostoc* strains and the reduction of the initial amount of AFB1 ranged from 1.80 to 44.89% AFB1 in of all the strains studied (Abdellah, 2005). Also, a study in samples of Kutukutu, a fermented maize-based dough largely consumed in the Northern part of Cameroon, The incubation of Kutukutu contaminated with aflatoxin showed after 120 hours , the AFB1 was degraded by the Lactic Acid bacteria (LAB) in the following order *L. buchneri* M11 (64.2%) > *L. brevis* G25 (63%) > *L. fermentum* N33 (57.2) > *L. cellobiosus* M41 (52.3%) > L. fermentum N25 (45.3%) > *L. brevis* G11 (43.9%).The findings highlight the possibility of exploiting the LAB potential in the control of aflatoxinogenic strains of *A. flavusin* in Kutukutu (Tchikoua, et al., 2015).

The degradation of Aflatoxins by the fermentation process is not implicated with bacteria species only. Nevertheless, a study revealed strain of *Aspergillus niger* to have activity in the degradation of Aflatoxins. Here, 26.3 % of AFB1 degraded in 24hr fermentation in a nutrient broth and by optimization of fermentation conditions for AFB1 by *Aspergillus niger* impacted 58.2 % of AFB1 to be reduced (Wei, et al., 2014).

However, in another study of degradation of Aflatoxin B1 during the fermentation of alcoholic beverages of beer and wine using laboratory-scale bottom and top beer fermentation, and wine fermentation. During fermentation, cool wort beer samples and wine must samples were artificially spiked with AFB1 and the levels of AFB1 remaining after fermentation were analyzed. AFB1 levels were unchanged during both types of fermentation used for beer but were reduced to 30% of their initial concentration in wine. Differential analysis of the spiked and un-spiked wine samples showed that the degradation compound was AFB2a, a hydrated derivative of AFB1. Therefore, the results showed that risk of AFB1 carryover was still present for both types of beer fermentation but was reduced in the case of wine fermentation because of hydration (Tomonori et al., 2013).

In a review paper it is summarized also as, although, several papers dealing with the inhibition of mycotoxin biosynthesis by LAB have focused on aflatoxins. During cell lysis, it is possible that LAB releases molecules that potentially inhibit mould growth and therefore lead to a lower accumulation of their mycotoxins using a dialysis assay, a study demonstrated the occurrence of a metabolite that inhibits aflatoxin accumulation in *Lactobacillus* cell-free extracts. It was suggested also that this inhibition of aflatoxin biosynthesis was not the result of a hydrogen peroxide production or a pH decrease. A significant reduction of aflatoxin biosynthesis by *Lactobacillus* cell free supernatants, and suggested that this inhibition was related to a heat stable, low-molecular-weight inhibitory compound. Although Lactobacillus spp. were found to delay aflatoxin biosynthesis, other lactic strains such as *L. lactis* were found to stimulate aflatoxin accumulation (Grazina et al., 2012).

2.8 Masked Mycotoxins

Historically, the topic of conjugated or masked mycotoxins first caught attention in the mid-1980s because in some cases of mycotoxicosis, clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed. It has been hypothesized that the unexpected high toxicity could for instance be attributed to the occurrence of undetected, conjugated forms of mycotoxins that are hydrolyzed to the precursor toxins in the digestive tracts of animals (Andrea, unpublished).

It has been recently shown that, analogously to animals, also plants are able to counteract fungal invasion modifying the chemical structure of mycotoxins, producing different kind of metabolites. Among them, particular attention has been raised by so called masked mycotoxins, secondary metabolites produced by plants via conjugation mechanisms. Masked mycotoxins are so called because usually they escape routine analysis on account of their different chemical behavior in comparison to their parent compounds (native forms) (Andrea, unpublished). In 2011, "The, International Life science Institute (ILSI)" has adopted the following definition "Mycotoxin derivatives that are undetectable by the conventional analytical techniques because their structure has been changed in the plant are designated masked mycotoxins (Michael et al. 2014).

However, for harmonizing future scientific works and subsequent legislations it was suggested also that the term "modified mycotoxins" to be used and the term "masked mycotoxins" to be kept for the fraction of biologically modified mycotoxins that were conjugated by plants (Michael et al. 2014). Despite, this the majority of reviewed documents use the term masked toxins widely. For instance, in a paper it described mycotoxins may occur as conjugated form, either soluble (masked mycotoxins), or incorporated into/ associated with/attached to macromolecules (bound mycotoxins). These conjugated mycotoxins can emerge after metabolization by the host plant, fungi and mammals or after food processing (Laura et al., Unpublished). Additionally, a review paper which was focused to resolve the ambiguous definition of masked mycotoxins over the comprehensive definition of modified mycotoxins, as a new proposal reiterated that, modified mycotoxins were relevant rather than the term masked, which has draw backs (Michael et al. 2014).

Plants use detoxification mechanisms via enzymatic transformations as a resistance to counter act pathogen invasion. Therefore, this detoxification process by plants includes the conjugation of mycotoxins to polar substances such as sugars, amino acids or sulfate and subsequent storage of this metabolites in vacuoles of cells or conjugated to biopolymers such as cell wall components. This structural modification of mycotoxins may be also similar to transformations which may occur during food processing and fermentation (Andrea, unpublished).

Nowadays, toxicological data on masked mycotoxins are scarce, although several studies highlight the potential threat of these compounds for consumer safety. From mycotoxin determination point

of view, it remains still a challenge for the scientific community to apply the conventional analytical methods for this emerging mycotoxins (Natalia et al., 2014)

The recognition of the toxicological relevance of masked mycotoxins in food commodities provides a new impetus for the establishment of overall toxicity estimates to be used by regulatory bodies, food manufacturers and monitoring authorities to protect consumers' health (Franz et al., 2012).

As to the reviewer of this paper, the efforts made to find studies focused on masked Aflatoxins was not appealing besides to the overall scarce data and studies available on masked mycotoxins. Masked Aflatoxins were not as such specifically implicated in a number of studies reviewed. However, masked mycotoxins other than Aflatoxin were given attention in many of the papers. For example masked mycotoxins of, Fusarium mycotoxins (deoxynivalenol, zearalenone, fumonisins, nivalenol, fusarenon- X, T-2 toxin, HT-2 toxin, fusaric acid), (ochratoxin A, patulin, destruxins) were well described [6]. Also, in another study DON-3-glucoside or ZEN-4-glucoside, were described (Elisabeth et al., 2013).

Therefore, to be reliable on the data generated despite the challenges in the determination of masked mycotoxins, it was mentioned LC/MS-MS (Liquid chromatography- tandem mass spectrometry) was a better choice in selectivity and specificity (Elizabeth et al., 2013; Franz et al. 2012; Rudolf et al., 2008) Moreover, to analyze modified mycotoxins hydrolysis in gastrointestinal tract of animals UHPLC–MS/MS was also used (De Boevre et al., 2015).

In regard to Aflatoxin degradation / modified products during food processing, for instance in a study of degradation of Aflatoxin B1 during the Fermentation of Alcoholic Beverages, where the presence of the hydrated form of AFB1 product called AFB2a, was identified by LC-MS/MS (Tomonori et al. 2013).

On top of this, in a study conducted to explore the detoxification potential of Corymbia citriodora plant extracts against aflatoxin B1 and B2 (AFB1; 100 μg L−1 and AFB2; 50 μg L−1) in In vitro and In vivo assays. Though the qualitative and quantitative detoxifying potential of the plant extract was determined by TLC and HPLC respectively, the structural elucidation of degraded toxin products was done by LC - MS/MS analysis. HPLC chromatograms confirmed that after

C. citriodora leaf extract treatment, trace amount of aflatoxin was present along with other peaks whose footprints were not found in chromatogram of parent compounds which may be attributed to toxins degradation products (Wajiha et al., 2015).

To date, no antibodies have been specifically targeted and selected against masked mycotoxins in connection with Immunoaffinity columns. However, antibodies developed against the parent mycotoxin can potentially cross-react with masked forms if the epitope is not sterically hindered by the metabolisation (Franz et al. 2012).

2.9 *A. flavus* and *A. parasiticus* and their toxicological significance on Aflatoxins types.

The identification between *A. flavus*, *A. Parasiticus* and *A. nomius* is not straight forward due to the close similarities. However, a detailed morphological study revealed for the typical characteristics. Contemporary diagnosis of the two species of *A. flavus* and *A. parasiticus* the primary separation being the presence of metulae and phialides (biseriate conidial head) for *A. flavus* and phialides only (uniseriate conidial head) for *A. parasiticus*. Conidial wall ornamentation is now regarded as the primary diagnostic character for separation of these two species. Conidia of *A. flavus* have relatively thin walls which are finely to moderately roughen. Their shape can vary from spherical to elliptical. Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose.

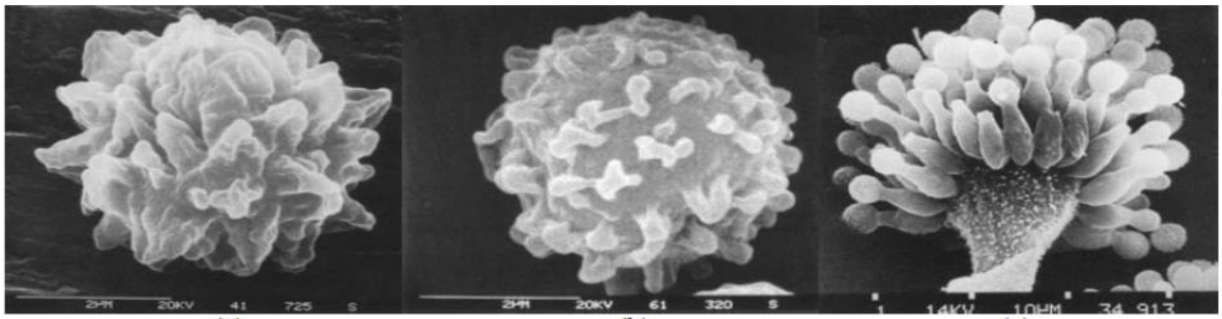


Fig 5. Scanning Electron Microscopy pictures of (a) *A. parasiticus* and (b) *A. flavus*, spores, where spore ornamentation differences are clearly seen; and (c) of *A. parasiticus* conidial head.

The other closely related species *A. nomius* is confusing in identification because of its morphological resemblance with *A. flavus*. However, it is different in its small bullet-shaped sclerotia; those in *A. flavus* being more globose. Moreover, *A. nomius* is different in aflatoxin types production and lack to produce a distinctive secondary metabolite called cyclopiazonic acid.

Molecular studies also rendered a specific difference to exist among the species (Rodrigues et al., 2007). A molecular study entitled as differentiation between *Aspergillus flavus* and *Aspergillus parasiticus* from Pure Culture and Aflatoxin-Contaminated Grapes Using PCR-RFLP Analysis of aflR-aflJ Intergenic Spacer, revealed the difference in the aflatoxin genic profile (Andr´e et al., 2011)

In light of the *Aspergillus species* specificity on the Aflatoxin family, which is covered in a number of studies. *A. flavus* typically produces AFB1 and AFB2, whereas *A. parasiticus* produces AFG1 and AFG2 as well as AFB1 and AFB2 (Ayhan & Ufuk, 2013; David & Durham, 1984; Kgomo et al., 2015).

In another paper it reiterates the specificity of *Aspergillus* species to the Aflatoxin types and their distinctive metabolite in such a way that, Aflatoxins are produced primarily by the common fungus *Aspergillus flavus* and the closely related species *A. parasiticus*. These are well defined species: *A. flavus* produces only B aflatoxins and sometimes the mycotoxin cyclopiazonic acid (CPA), while *A. parasiticus* produces both B and G aflatoxins but not cyclopiazonic acid (IARC Monograph, 2002).

Aspergillus species environmental interactions and Aflatoxin production are also worth reviewing. A review article on environmental influences on maize *Aspergillus flavus* interactions and Aflatoxin production signifies environmental factors can have a significant effect on maize resistance to *A. flavus* and aflatoxin production, particularly abiotic stresses such as drought and heat stress. Maize genotypes possessing drought tolerance, such as Lo964 and Tex6, tend to be less susceptible to aflatoxin contamination. Additionally, the oxidative responses of plants to pathogen infection and insect herbivory under varying environmental conditions (e.g., drought and the elevated CO2) are critical for the management of pest outbreaks and the reduction of mycotoxin contamination in agricultural crops (Jake et al., 2014).

Furthermore, in another review paper on the effect of climate change on *A. flavus* and Aflatoxin B1 production both in in vitro and maize grain, the potential impacts of key environmental factors and their interactions on the molecular ecology, growth and Aflatoxin production studied. The impact of water activity (a_w) × temperature on aflatoxin biosynthesis and phenotypic aflatoxin production revealed, there was a direct relationship on the relative expression of key regulatory and structural genes under different environmental conditions which correlate directly with aflatoxin B1production. Therefore, by modelling, the impact of a_w × temperature × elevated CO2 (2× and 3× existing levels) in the Aflatoxin biosynthetic path way, while such interacting environmental conditions have little effect on growth they do have a significant impact on aflatoxin biosynthetic gene expression (structural aflD and regulatory aflR genes) and can significantly stimulate the production of AFB1. While the individual factors alone have an impact, it is the combined effect of these three abiotic factors which have an impact on mycotoxin production (Angel et al., 2014).

Finally, the effect of agricultural practices on the Aflatoxin production is also reviewed very relevantly in this paper, cropping pattern influences community composition and, thereby, the epidemiology of aflatoxin contamination. *Aspergillus parasiticus* was isolated from two fields previously cropped to sugarcane but not from 23 fields without recent history of sugarcane cultivation in Texas. *A. parasiticus* was not detected in counties that do not produce sugarcane. *Aspergillus section Flav*i soil communities within sugarcane-producing counties differed significantly dependent on sugarcane cropping history (Garber & Cotty, 2013).

2.10 Management of Mycotoxins

Factors influencing the presence of mycotoxins in foods or feeds include environmental conditions related to storage that can be controlled. Other extrinsic factors such as climate or intrinsic factors such as fungal strain specificity, strain variation, and instability of toxigenic properties are more difficult to control (Atanda et al., 2012). In general, mycotoxigenic fungi are often divided into two broad categories: “Pre-harvest” (field fungi) –Fusarium “Post-harvest” (storage fungi) – Aspergillus, Penicillium aflatoxins are generally more common in tropical or semi-tropical regions while fusariatoxins are more common (David, 2009).

Economic impacts of Mycotoxins

Producer costs

Crops, Yield loss, restricted markets, non-marketable products, price discounts. Increased product cost, increased post-harvest cost, difficulty obtaining loans on stored grain. Disposal of useless grains, monitoring and sampling cost

Livestock and dairy, higher mortality rates, reproductive failures (abortions), lower egg and meat production , reduced feed efficiency, higher feed cost, reduced disease immunity, vaccine failures, increased medicine cost. Lower milk production, unmarketable milk, monitoring and testing

Handler/distributor cost, extra drying cost, excess storage capacity, losses in transit • Loss of markets, monitoring and testing.

Processor cost, restricted markets, loss of markets, reduced demand, product loss. Insurance premium, litigation costs, monitoring and testing.

Consumer and social costs, Less nutritious food, higher product price, possible health problems. Regulatory costs, research and education, lower foreign exchange earnings, increased cost of imports

Grain molds on poultry feed quality, the severity of mycotoxin contamination is less in sorghum as compared to that of maize owing to its hard seed coat. Occurrence of fumonisins is higher in sorghum than on maize, but the tolerance levels of fumonisins in chicken are at higher levels than aflatoxins (Waliyar, 2007).

Therefore, integrated mycotoxin management systems are very important. However, Mycotoxins cannot be considered a group of toxicants on the basis of their mechanism of action because they are very chemically diverse. For the same reason, it would be impossible to develop one single control method that would ensure the reduction of every mycotoxin present in every agricultural commodity. In addition, mycotoxin contamination is heterogeneous in nature, so sampling and analysis are complicated by the presence of "hot spots". Considering all these factors, it can be

concluded that the development of food safety programs for mycotoxin control is not a simple issue (Lopez- Garcia, 2009).

However, the integrated management system is relevant for reduction in the level of mycotoxins. Therefore, the concept behind an integrated management system is similar to a "hurdle" effect, where at each phase of production, i.e. pre-harvest, harvest and post-harvest processing, the risks are minimized. Preharvest, harvest, and post-harvest control strategies are worth managing.

Preharvest Control Strategies are, Management of insect infestation, management of crop residues and crop rotation, irrigation and soil condition, irrigation and soil condition. Soil fertility and drought stress have been found to be contributing factors in Preharvest aflatoxin contamination of maize. Development of resistant plat verities.

Harvest Control, during harvesting, it is important to control factors such as timeliness, clean-up and drying of the agricultural product. Such control is essential for preventing mycotoxin formation during storage. Studies have shown that the timing of harvesting greatly influences mycotoxin production. In some geographical regions, the planting date should be selected to take advantage of periods of higher rainfall. Harvesting should take place as soon as the crop is fully grown and the crop cycle is completed. Studies have reported that crops left on the field for longer periods of time may present higher levels of toxic contamination. Adequate drying is also essential to prevent fungal proliferation during storage.

Post-Harvest control and decontamination, this involves physical, biological and chemical decontamination are among the strategies in mycotoxin management (Lopez- Garcia, 2009).

2.11 Determination of Aflatoxins (B1, B2, G1, G2)

Since the discovery of aflatoxins in the 1960s, much research has focused on detecting the toxins in contaminated food and feedstuffs in the interest of public safety. Most traditional detection methods involved lengthy culturing and/or separation techniques or analytical instrumentation and complex, multistep procedures that required destruction of samples for accurate toxin determination.

According to a review published in 2015, on the developments of determination of Aflatoxin. Most of the current methods for quantitative aflatoxins determination include chromatographic methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and more recently liquid chromatography tandem mass spectrometry (LC-MS/MS), suitable for use in regulatory laboratories. Several immunology-based semi-quantitative and qualitative methods including enzyme linked immunosorbent assays (ELISAs) and immunoaffinity column assays, were also developed for use at grain stations and silos.

Among the novel potential aflatoxins-detection systems are screening and detection methods for rapid in-field and laboratory applications including dip-stick kits, optical-based sensing methods (e.g. hyperspectral imaging and electronic noses) and other emerging experimental methods including biosensors.

The SPE (Solid Phase Extraction) column contains a bonding phase such as porous silica, modified to allow selective absorption of impurities or the substance of interest (analyte). Typically, the silica traps the analyte, the impurities are washed off and a test-specific rinse solution releases the analyte from the column. In a multifunctional SPE column, the impurities are retained in the column and the analyte flows through. A more recent addition to the SPE clean-up methods is the widely adopted IAC (ImmunoAffinity Column) which employs a specific monoclonal or polyclonal antibody binding to the analyte, in this case, mycotoxins or Aflatoxins (Yao et al. 2015).

HPLC using fluorescence detection has already become the most accepted method for the determination of aflatoxins due to its several advantages over other analytical methods. Both normal- and reversed-phase HPLC can be used. However, the reversed-phase HPLC methods are more popular. Therefore, Chromatographic analysis of aflatoxins is preceded by a sequence of broad and complex general operations that include sampling, sample preparation, extraction, purification and concentration of the extract obtained before the separation, quantitation, and confirmation steps (Jamiza, et al., 2000: Anna, unpublished).

For comparison purpose the different methods of Aflatoxin determination are presented as summarized in the table 1 below.

Table 1. Advantages and disadvantages of Aflatoxin detection technology, World Mycotoxin Journal.

Method	Pros	Cons	References
Thin Layer Chromatography	- Reliable quantification method when combined with densitometry. - Accuracy and Precission comparable to HPLC methods (HPTLC, OPLC). -Official reference methdology for aflatoxins	- Outdated equipment - destructive sample preparation - largely replaced with HPLC for quantitative analysis of aflatoxins	- Rahmani et al, 2009 - Shepered, 2009
High Performance Liquid Chromatography	- Reliable, Sensitive, and repeatable quantification methdology - May be automated - Official refernce method for aflatoxins	- expensive equipment requiring dedicated operator and specialist to interpret the results. - destructive sample preparation - may require derivatization	Cho et al. 2006 Shepard,2009,Turner et al, 2009
Liquid Chromatography/mass spectrometry	- Simultaneous analysis of mycotoxins - low limit of detection (LC-MS/MS) - confirmatory method - no derivatization required	- Very expensive equipment requiring dedicated operator and specialist to interpret the results - Sensitivity relies on ionization - matrix assisted calibartion for quantitative analysis. - lacks internal standards	krska et al, 2008, Li etal ,2013, Pascale , 2009, Shepered 2009
Enzyme- linked Immunosorbent Assay	- Specific, rapid and relatively easy to use - inexpensive equipment - low limit of detection - Simultaneous analysis of multiple samples - Semi quantitative (screening) or quantitative analysis possible - limited use of organic solvents	- possible cross reactivity with related mycotoxins. matrix interfrence - possible false positives/negatives - narrow detection range - Confirmatory LC analysis may be required	Pascale, 2009, Pillet, 2006, Turner et al,2009
Immunoaffinity Assay	- IAC in combination with liquid flurometry is comparable to LC for determination of Aflatoxins - Official methods	- sample destrucion - limied to analysis of total aflatoxins	Pillet,2005
Fluoresence polarization immunoassay	- rapid, no cleanup required - mycotoxin - specific tracer for analysis - Very sensitive - Portable	- limited validation with ELISA or HPLC - Possible cross reactivity with related mycotoxins - matrix interference - limited to detecting one mycotoxin at a time	(Lattanzio et al , 2011, Lippolis and Maragos, 2014, Pascale,2009
Capillary electrophoresis	- useful for separating closely related mycotoxins - highly sensitive - capable of multi constituent analysis when combined with immunoassays	- limited to lab use due to cumbersome instrumentation	Maragos,2004
Biosensors	- rapid, no cleanup - high selectivity and low limit of detection - ease of use , low cost and portability - self contained, simple design	-extraction sample prep for solid samples - extract cleanup needed to improve sensitivity - Cross reactivity with related mycotoxins - Variation in reproduciability and repeatability. (I improved with use of novel materials)	Mathora etal ,2014 : Meneely and Eliott 2014, Pascade 2009, Rubert etal 2012a,Tolhil 2011
Near infrared spectroscopy	- rapid non destructive - no extraction or cleanup - user friendly operation	- calibration model must be validated - Knowledge of statistical methods - poor sensitivity (high limit of detection) - Costly equipment	- Bernardo etal 2005 : Dowel ert al , 2002 , FAO,2004 ; Gordon etal , 1999 , Hussien and Goto , 2014, pearson and Wicklow , 2006 . Pearson etal 2001. Taliada et al 2011.
Hyperspectral imaaging	-rapid non destructive - no extraction or clanup - user friendly operation - high spectral and spatial resolution - potential for in-line detecting applications	- calibration model must be validated - knowledge of statistical methods - poor sensitivity (high limit of detection) - low segnal level (for flouresence) - costly equipment	- Del Fiore etal 2010 , Hruska etal 2013, Yao etal 2008, 2010
Electronic nose	- rapid means for controling the microbiology of food	- need to improve selectivity and reduce interfrences (eg to humidity) - compensate for drift efects -limited feaibility studies and poor validation	De Lucca etal 2012 Gardner and Barlett , 1994

2.12 Determination of Aflatoxin with HPLC and Immuno- Affinity Column

2.12.1 High Performance Liquid Chromatography

Chromatography is one of the most popular methods to analyze mycotoxins such as aflatoxins. The most common techniques of chromatography are Gas chromatography (GC), liquid chromatography (LC), High performance liquid chromatography (HPLC) and Thin layer chromatography (TLC). From these methods, LC and HPLC are the most used. In many cases, they are followed by fluorescence detections stage. LC, TLC and HPLC are the most used quantitative methods in research and routine analysis of aflatoxins these techniques offer excellent sensitivities but they frequently require skilled operators, extensive sample pretreatment and expensive equipment (Aljandro, et al., unpublished).

Analytical laboratories moved away from TLC to HPLC determination with advances in HPLC methods in 1980s. High performance liquid chromatography is a very precise and highly automated quantification technique for aflatoxins analysis with high selectivity and sensitivity. Now-a-days, HPLC methods are widely used because of their superior performance and reliability as compared with TLC. HPLC methods have been developed for all major mycotoxins in cereals and other agricultural commodities. In the field of analysis of aflatoxins, HPLC is mainly used for final separation and detection of the analyte of the interest and extraction and clean-up techniques have to be applied prior to detection with HPLC (Irineo, 2011). HPLC, has been used jointly with techniques such as UV absorption, fluorescence, and mass spectrometry and aerometric detectors. Also derivatization with a fluorophore enhances the natural fluorescence of aflatoxins and improves detectability. The pre-column approach uses the formation of the corresponding hemiacetals using trifluoroacetic acid (TFA), while the post-column one utilizes either bromination by an electrochemical cellular in addition of bromide, or pyridinium hydrobromide perbromide, for the mobile phase and the formation of an iodine derivative (Aljandro, et al., unpublished).

Two types of HPLC methods are commonly used i.e., normal phase chromatography and reversed phase chromatography. In normal phase chromatography, a polar stationary phase e.g. silica gel and a non-polar solvent e.g. hexane are used. Whereas reversed-phase chromatography (RP-

HPLC) employs non-polar stationary phase e.g., C-8 or C-18 hydrocarbons and polar mobile phase e.g. water, methanol or acetonitrile. In HPLC, detection is mainly accomplished by using ultra violet (UV) detector, diode array detector (DAD) or a fluorescence detector (FLD). Fluorescence detection utilizes the emission of light (435 nm) from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation (365 nm) for aflatoxins. Fluorescence detection has superior Aflatoxins – Detection, Measurement and Control (Irineo, 2011).

Even though, High performance liquid chromatography (HPLC) combined with fluorescence detection is proved to be very accurate and has been extensively studied in different materials. However, in order to improve detection limits of Aflatoxins, a tedious pre- or post-column derivatization must be done in conventional HPLC methods. These problems have been successfully solved by introducing HPLC-MS method.

HPLC-MS system is equipped with an auto sampler, the HPLC system, the ionization source (which interfaces the LC to the MS) and the mass spectrometer. There are several types of mass spectrometers available for interfacing with HPLC. Single quadrupole mass is a common system used for the HPLC-MS, this system can provide a mass spectrum for each chromatographic peak that elutes from the LC column and is analyzed by the MS system. Time-of-flight (TOF) mass spectrometer, which has the added capability of providing a higher mass resolution spectrum from each component that is assayed. The triple quadrupole MS-MS system and ion-trap mass spectrometer are important tools in quantitative analysis and qualitative analysis. HPLC-ESI-MS/MS has become the most emerging analytical tool for the determination of aflatoxins and their metabolites. Single quadrupole mass spectrometer and ion-trap mass spectrometer were also used in the determination aflatoxins. In general, LC-MS provides decisive advantages in performing identification as well as determination of analytes at trace levels (Peiwu, unpublished)

2.12.2 Immuno- Affinity columns

Affinity chromatography is a high resolution, high capacity technique. And one of the most powerful and diverse methods for separating proteins and other biological molecules of interest on the basis of a highly specific, reversible biological interaction between two molecules an affinity ligand attached to a solid matrix to create a stationary phase , and a target molecule in a mobile

phase. Specifically, immunoaffinity chromatography (IAC) relies on a solid stationary phase consisting of an antibody coupled to a chromatographic matrix or to magnetic beads, and harness the selective and strong binding of antibodies to their targets (Daad & Luc, Unpublished).

In light of Aflatoxin analysis using Immuno affinity columns (IAC), it occupies a special place among the immune analytical approaches, being used many years as a method of sample purification and concentration in the aflatoxin analysis.

The principle of the IAC is that, an antibody (polyclonal or monoclonal) recognized the analyte is immobilized onto a solid support such as agarose or silica in phosphate buffer, all of which is contained in a small column. The clean-up procedures are completed in four steps: The column is initially conditioned with phosphate buffered saline (PBS) and reaches room temperature. The crude sample extract is applied to the IAC containing specific antibodies to aflatoxin at slow steady flow rate of 2-3 mL/min. Gravity or vacuum system can be used to control flow rate. The aflatoxin binds to the antibody and is retained in the IAC. The crude sample extract must be in aqueous solution because organic solvents can damage the antibody and can interfere with the antibody-aflatoxin interaction.

The binding strength of the antibody-aflatoxin will influence recovery of the IAC. The specificity of antibody is important to remove the structurally closely compounds which can cause interferences in the quantitation of aflatoxin. The capacity of the IAC (the total number of antibody sites available for binding aflatoxin) is also important as overloading the column will lead to poor recovery. The column is washed with washing solution (water or phosphate buffered saline) to remove impurities. After washing completely, the IAC is blown to dryness by N_2 stream. Elution is done by passing a solvent such as acetonitrile/methanol through the IAC, breaking the antibody aflatoxin bond, the captured aflatoxin is removed from the antibody and thus eluted from the column. The eluate containing aflatoxin is then further developed by addition of fluorescence enhancer or directly measured by HPLC method (Peiwu, unpublished).

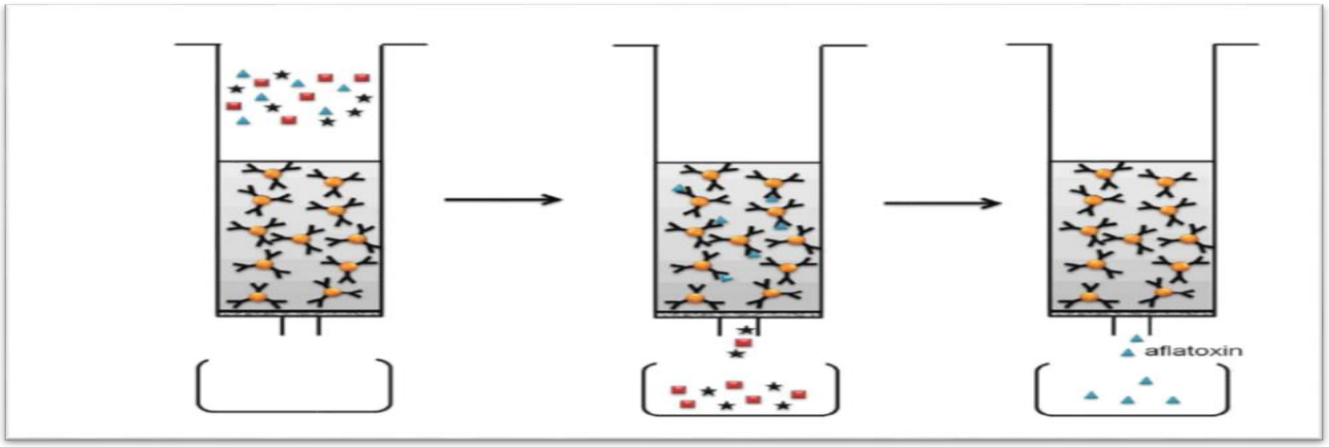


Fig 6. Scheme of aflatoxin immunoaffinity column for sample pretreatment (clean-up and enrichment).

In this study Aflaclean select from LC-TECH, Germany was used. This new LCTech immunoaffinity column AflaCLEAN TM was developed for the sample preparation of foods for the aflatoxin analysis using HPLC. With a maximum loading capacity of 200 ng aflatoxin B1 and a selectivity against aflatoxins B1, B2, G1 and G2 nothing is left to be desired for. AflaCLEAN TM columns are available in the practical 3 mL polypropylene format. In many accredited laboratories, these columns are successfully employed worldwide for the most diverse matrices such as cereals, nuts and spices (Daad & Luc, Unpublished).

Fig 7. 3 mL Aflaclean™ Immunoaffinity columns. (Source: LC Tech Application Note)

2.13 Method Validation in HPLC

In research, to get comparable data, the research methodology is subjected to validation procedure that the method produces reliable results and meets the set performance criteria.

12.13.1 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation, and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. Precision may be performed at three different levels: repeatability, intermediate precision, and reproducibility. Repeatability (intra-day assay precision) is the results of the method operating over a short time interval under the same conditions. (Intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (Ghulam, 2004; Bioanalytical Method Validation, 2013).

12.13.1.1 Repeatability

Repeatability (intra-day assay precision) is the results of the method operating over a short time interval under the same conditions (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels three repetitions each), or from a minimum of six determinations at 100% of the test or target concentration. A precision criterion for an assay method is that the instrument precision (RSD) will be ≤1%, and for the impurity assay, at the limit of quantitation, the instrument precision (repeatability) will be ≤ 5%. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and confidence interval (Ghulam 2004; Bioanalytical Method Validation, 2013)

12.13.1.2 Intermediate Precision

Intermediate precision (inter-day variation) is the results from within lab variations, due to random events, such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed, so that the effects (if any) of the individual variables can be monitored. Precision criteria for an assay method is that the intra assay precision will be ≤ 2%,

and for impurity assay, at the limit of quantitation, the instrument precision will be ≤ 5%, and the intra-assay precision will be ≤10% (Ghulam,2004).

12.13.1.3 Reproducibility

Reproducibility, is determined by testing homogeneous samples in multiple laboratories, often as part of inter-laboratory crossover studies. An example of reproducibility criteria for an assay method could be that the assay results obtained in multiple laboratories will be statistically equivalent, or the mean results will be within 2% of the value obtained by the primary testing lab. For an impurity method, results obtained in multiple laboratories will be statistically equivalent, or the mean results will be within 10% (relative) of the value obtained by the primary testing lab for impurities. Reproducibility is not normally expected if intermediate precision is performed (Ghulam 2004; Bioanalytical Method Validation, 2013).

In this research under the same experimental condition and in the same day repeatability test performed by choosing an interim standard in the working range that was 30 ppb of mixed aflatoxin standard by injecting ten times.

12.13.2 Linearity and Range.

The linearity of the method should be tested in order to demonstrate a proportional relationship of response versus analyte concentration over the working range. The linearity range for evaluation depends on the purpose of the analytical test method. Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. The regression coefficient (r2) is > 0.998 is generally considered as evidence of acceptable fit of the data to the regression line (Ghulam, 2004).

12.13.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy, is usually determined in one of four ways. First, accuracy can be assessed by analyzing a sample of known concentration (reference materials), and comparing the measured value to the true value. The second approach is to compare test results from the new method with

results from an existing alternate well-characterized procedure that is known to be accurate. The third approach is based on the recovery of known amounts of analyte. This is performed by spiking analyte in blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over a range of 50-150% of the target concentration. The percent recovery should then be calculated. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte. Accuracy criteria for an assay method (FDA) is that the mean recovery will be 100 ± 2% at each concentration over the range of 80-120% of the target concentration (Ghulam 2004; Bioanalytical Method Validation, 2013).

2.13.4 Limit of Detection (LOD), Limit of Quantification (LOQ)

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) tests for the procedure were performed on samples containing very low concentrations of analyte. LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level LOQ is defined as the lowest amount of analyte which can be reproducibly quantitated above the baseline noise, that gives S/N = 10 (Ghulam, 2004; Bioanalytical Method Validation, 2013).

2.14 Laboratory Safety Requirement in determination of Aflatoxins

With reference to the Indian Mycotoxin analysis manual and ORA laboratory manual taken from the official method of analysis (AOAC 2000) regarding to laboratory safety requirements, as aflatoxins are subject to light degradation. Protect analytical materials adequately from daylight and keep aflatoxin standard solutions protected from light by using amber vials or aluminum foil.

Care must be to given while handling samples suspected of mycotoxin contamination. While handling pure aflatoxin reference material, extreme precautions are to be taken as they are electrostatic. Work preferably in a hood. Swab any accidental spill of toxin with 1% Sodium Hypochlorite bleach (NaOCl), leave 10 minutes and then add 5 % aqueous acetone. Rinse all glassware exposed to aflatoxin with methanol, add 1% Sodium hypochlorite solution and after 2 hours add acetone to 5 % of total volume. Let it react for 30 minutes and then wash thoroughly. Use a laboratory coat or apron soaked in 5% Sodium Hypochlorite solution overnight and washed in water (Lab Manual India 2015 ; ORA Lab. Manual 213).

2.15 Legislations

The International Cancer Research Institute identifies aflatoxin as a Class 1 carcinogen, resulting in the regulation of this toxin to very low concentrations in traded commodities [20 ppb in grains and foods and 0.5 ppb in milk] in the United States FDA Aflatoxin Action Level (Jonathan et al.,2004; FDA Mycotoxin Regulatory Guidance,2011).

According to the European Commission food safety Authority technical report, Maximum levels of aflatoxins (aflatoxins B1, B2, G1, G2 and M1) are laid down in Commission Regulation (EC) No 1881/2006 (EFSA,2013). Cereals and processed foods for aflatoxin B1 maximum level is 2 µg/kg and for total 4 µg/kg (EC Regulation, 2012) And also as to the codex alimantarius aflatoxin standard on ready to eat foods should be maximum level of 10 µg/kg total aflatoxin (CODEX standard 193 -1995).

In the case of reviewed previous traditional beverage studies the level of aflatoxin was compared both with the Codex alimantarius and the European total aflatoxin levels in the cases of Malawi and Kenya studies. (Limbikani et al., 2014; Mary A., et al 2013).

3. Materials and Methods

3.1 Study Sites

Tella and Areki samples were collected notably from areas of Debremarkos, Finoteselam and Enjibarra towns in Gojam. In these towns the traditions of brewing Tella, Areki distillation and their consumption were observably common. Besides, trans-regional trading of Areki is the business of the many.

Debremarkos, is located in north western Ethiopia, in Amhara National Regional State, its distance is 300 km from Addis Ababa. Its astronomical location is 10º 21" North Latitude and 37º 43' East Longitude, it is the East Gojam Zonal- administrative town. And, Finoteselam, is located in north western Ethiopia, in Amhara National Regional State, west Gojam zone, Jabatehnane woreda, at a distance of 387 km from Addis Ababa, Its astronomical location is 10º 41' North Latitude and 37º 16' East Longitude. It is the west Gojam Zonal- administrative town.

Enjibarra is located in northern Ethiopia, in Amhara National Regional State, Awi zone, Babja Woreda, at a distance of 420 Km from Addis Ababa. Its astronomical location is 10º 53' North Latitude and 36º 56' East Longitude. It is also the Awi- zonal- administrative town.

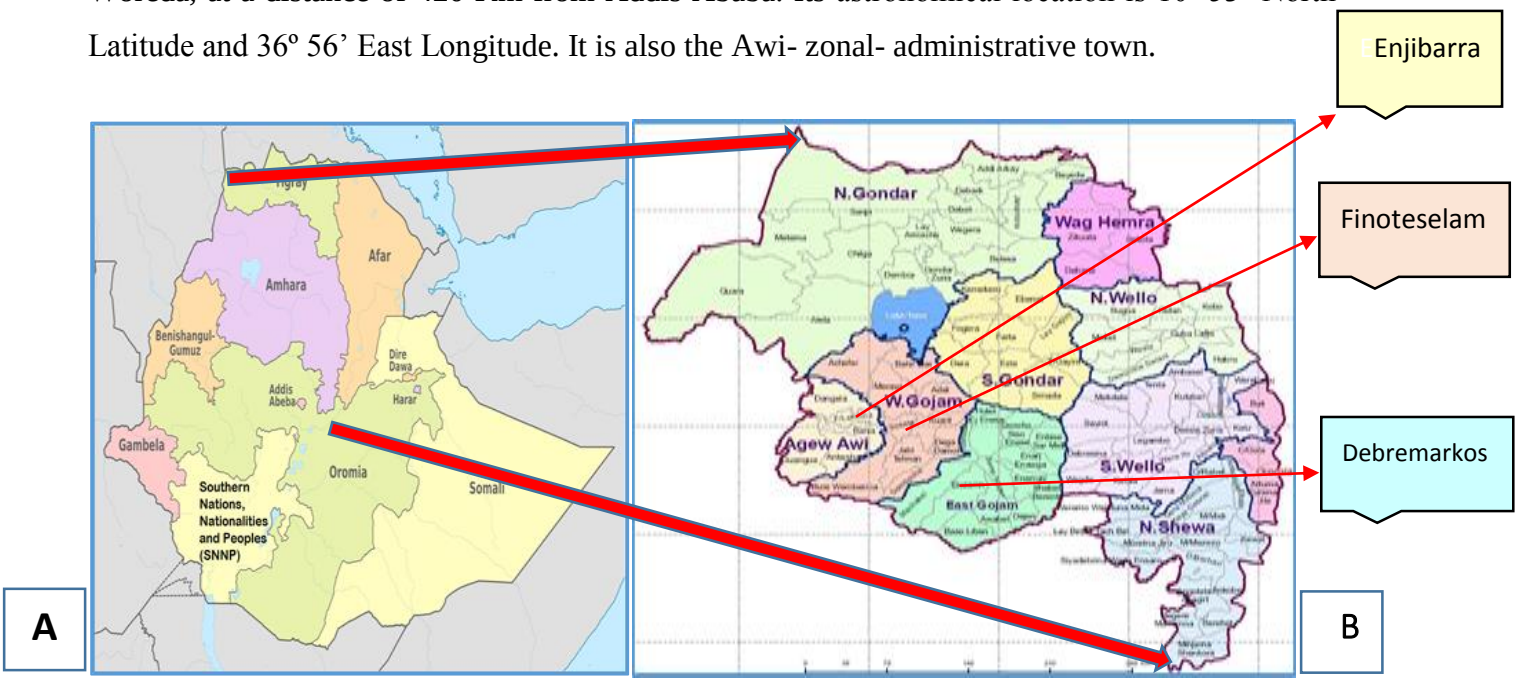


Fig. 8 (A) Map of the Amhara regional State in Ethiopia. (B) Zones, and Zonal-Administrative towns of the region

3.2 Study Design for KAP

The study design employed for the KAP survey was, a purposive sampling technique to assess the KAP by randomly selecting consumers, brewers and distillers (who are vendors also). Semi-structured questionnaires (Annex -1), were used to extract as much information as possible. The assessment carried out in parallel with the sample collection.

A total of 30 interviews were done for each consumers, brewers and distillers found in each study sites. The questionnaire used was translated in the local language. Interviews were done after briefing about the research objective and insisting on the confidentiality. It was emphasized also the genuine information they deliver have significant impact for the study finding.

3.3 Materials

Different laboratory apparatuses and instruments were used in the research. Here to list the major ones were : Laboratory Centrifuge, centrifuge tubes, vacuum pump, Immunoaffinity column, Whatman glass microfiber filter paper, lab stand with clump, 0.45 µm nylon filter ,Volumetric and Graduated pipettes (1ml, 5ml, 10ml, 25ml and 50ml), micropipettes of (100, 500, & 1000) µl, volumetric flasks (10ml, 25ml, 50ml, 100ml, 500ml and 1000ml), Measuring cylinders (50ml and 100ml), Beakers (50ml, 100ml and 500ml), conical flasks (250, 500 and 1000ml), Ultrasonic bath (Sonicator), Wash bottle, sample collecting bottles, ice-box, Electronic balance, syringes (5ml and 10ml), Vials with screw cap. SHIMADZU HPLC system setup containing auto sampler, injector, oven, column, Link, Degasser, fluorescence detector and desktop computer with chromatography software were used.

3.4 Chemicals and Reagents

Chemical and Reagents used in the sample preparation and analysis includes: Sigma Aldrich Aflatoxin Standards, HPLC grade Acetonitrile, Methanol, ultra-pure water, phosphate buffered saline solution (PBS: NaCl 8g/l, KCl 0.2 g /l, Na_2HPO_4 1.15 g /l, KH_2PO_4 0.2 g/l) and adjusted to pH to 7.4.

3.5 Sample collection and Preparation

Samples were collected from the aforementioned study sites, from randomly selected Tella and Areki vendors or who were brewers and distillers too. In this case, the researcher used the Addis Ababa to Bahirdar main road as a reference, as the road divides symmetrically the study sites to East and west. Therefore, samples collection was done at random in five households East of the road and also the same to the West aligned part of each towns. This made the total collected samples of both Tella and Areki 10 in each town, and on aggregate 30 samples of each Tella and Areki Samples collected for the study.

Samples were collected in unused and clean half liter plastic bottles by rinsing the bottles with the sample itself. Immediate labelling of the samples done with pre-formatted labels. All samples kept in Ice-Box to avoid the ongoing effect of fermentation on the sample characteristics in the case of Tella. Samples kept refrigerated around 4^0C after transport and in the course of analysis.

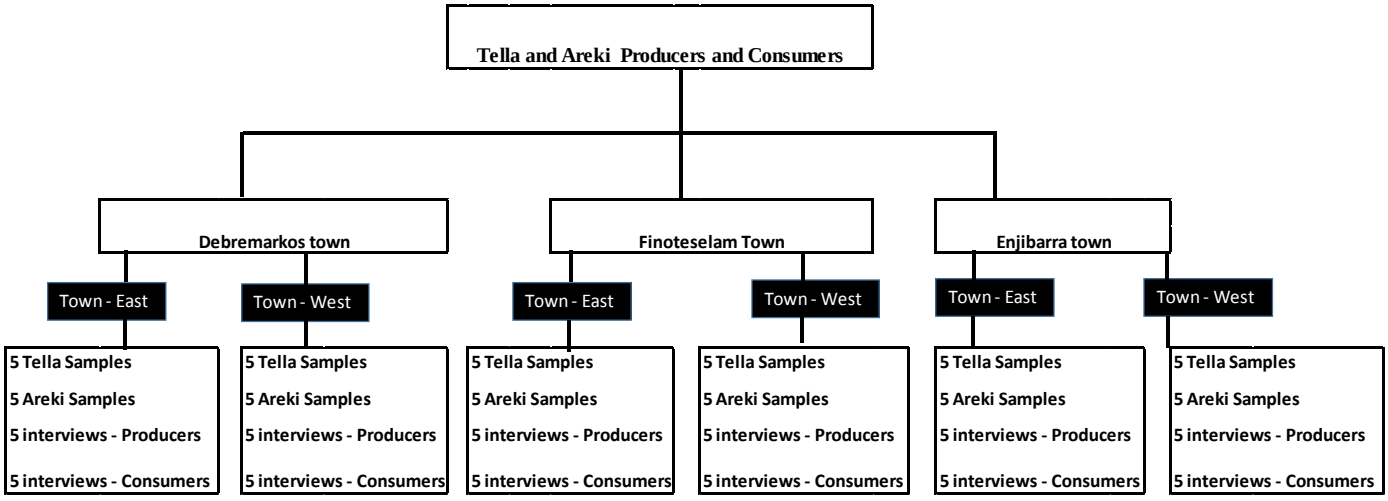


Fig.9 Sample collection and Interviews flow Chart

Fig.10 Sample collection and Parallel interview activity, picture taken in the study Sites.

3.6 Method Adaptation

3.6.1 Selection of HPLC

High performance Liquid chromatography coupled with fluorescence detection of used. Potential Aflatoxin was extracted by the Immunoaffinity column and injected in the HPLC. To evaluate the analytical performance of the instrument tests of, repeatability, LOD, LOQ, recoveries, asserting the working range and the detection of linearity of the Aflatoxins (AFG2, AFG1, AFB1, and AFB2) standards and were done before running samples.

3.6.2 Preparation of Standard Solutions

SIGMA – ALDRICH mixed aflatoxin standards were prepared in volumetric flasks by dissolving in HPLC grade Methanol at different desired concentrations of (2 ppb, 5 ppb, 10 ppb, 20 ppb, 30 ppb, 50 ppb and 100 ppb). And the spiking, LOQ and LOD, testing standards depending on the type of analysis, which required careful micro-pipetting of the solution prepared. The standards prepared transferred to vials and stored, cooled at 4^{o}C and protected from light to avoid deterioration of the Aflatoxins in solution.

Table 2. Total and Relative individual Aflatoxin concentration of standards

Total Mixed Standard concentration (ppb)	Relative concentration of individual Aflatoxins (ppb)			
	G2	G1	B2	B1
2	0.2	0.8	0.2	0.8
5	0.5	2	0.5	2
10	1	4	1	4
20	2	8	2	8
30	3	12	3	12
50	5	20	5	20
100	10	40	10	40

3.6.3 Identification of Aflatoxins Retention Time (RT)

Retention time was measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound. In this research identification of the RT for each specific Aflatoxin species was done to know the elution order of the specific Aflatoxin species peak to be revealed thereby to determine which of the Aflatoxin peak is detected first and sequentially to the desired last. Hence, from the prepared standards of 100 ppb (AFB2, AFB1, AFG2, and AFG1) of each Aflatoxin individually injected, after the mobile phase itself injected in vial as a blank or control. Then mixture of Aflatoxins standards were injected.

3.6.4 Precision

In this research under the same experimental condition and in the same day repeatability test performed by choosing an interim standard in the working range that was 30 ppb of mixed aflatoxin standard by injecting ten times.

3.6.5 Linearity and Range

In this study, linearity was studied by preparing mixed aflatoxin standard solutions of (2 ppb, 5 ppb, 10 ppb, 20 ppb, 30 ppb, 50 ppb and 100 ppb) and injecting in the HPLC. In these solutions the relative concentration of individual Aflatoxins were (0.2 ppb, 0.5 ppb, 1 ppb, 2 ppb, 3 ppb, 5 ppb, and 10 ppb for AFG2 and AFB2). And (0.8 ppb, 2 ppb, 4 ppb, 8 ppb, 12 ppb, 20 ppb and 40 ppb for AFG1, AFB1). As presented in table 2.

3.6.6 Limit of Detection (LOD), Limit of Quantification (LOQ)

In this study, 20 µl injection of the mixed aflatoxin at 0.05 ppb and 2 ppb concentration used. In reference to the S/N above 3 for Limit of Detection and S/N above 10 for limit of Quantification criteria applied to identify the LOD and LOQ of the analyte in the method developed.

3.6.7 Recovery

Recovery test, was done to check the accuracy of the method. In this study un-spiked sample of Tella used as a blank and different concentration of individually prepared Aflatoxin standards

spiked for recovery test. Concentrations of (0.25, 0.25, 0.9, 2.0 ppbs) used for AFG2, AFG1, AFB2, and AFB1, respectively for low level spiking. Also for high level spiking (2.5, 15, 10 and 12.5 ppbs.) concentrations of AFG2, AFG1, AFB2, and AFB1 respectively used.

Percent Recovery calculated according to the relation,

$$\% R = \frac{\text{(Spiked sample result - unspiked sample result)}}{\text{Known Spike added Concentration}} * 100\%$$

3.7 Determination of Aflatoxins in Tella and Areki

3.7.1 Sample Extraction and Clean Up

Analysis of Tella sample for Aflatoxins method adapted from a study paper on Mycotoxin occurrence in beer produced in several European countries and different beer brands sold in Canada (Mably etal. 2005; Terenzio et al., 2011). Slight modification of the method was validated in Addis Ababa University Center of Food science and Nutrition Food toxicology laboratory.

3.7.2 Sample Extraction and Cleanup in Tella

Tella samples centrifuged at (30,000 rpm for 5 minutes), and filtered through a Whatman glass microfiber filter paper by a vacuum pump this might underestimate the aflatoxin level but the analysis procedure allows only a clear sample. The filtrate collected for each sample were in duplicates by clean 100 ml sample tubes before clean up through Immunity Affinity columns. Filtered sample was ready for clean-up after sonicating/degassing the filtrate in Ultrasonic bath at room temperature for 30 minutes. Centrifugation and filtration procedures were to avoid particulate matters as Tella is an opaque traditional beverage. However, as Areki is distilled liquor no special sample preparation done except filtering through 0.45 µm nylon filter before injection in to HPLC to avoid trace of impurities.

In the ImmunoAffinity Column Assay, 20 ml of filtered Tella sample mixed with 10 ml Phosphate Buffer Solution (PBS) at pH 7.4. The mixed 30 ml solution was made to be cleaned up with Aflaclean select from LCTECH GMBH (Germany), with a maximum loading capacity of 200 ng aflatoxin for B1 and which shows selectivity for (B1, B2, G1, and G2).

This sample solution cleaned up for Aflatoxins through the ImmunoAffinity column (IAC) by gravity, this was done after sucking the sonicated sample solution by a syringe and filtering in 0.45 µm nylon Millipore filter and gently filling and passing it in the Aflaclean select.

After cleanup for Aflatoxins from the 30 ml sample and PBS solution in IAC, 3 ml PBS gently added through the 0.45 µm nylon filter to wash the trace of remaining sample impurities inside the IAC. Then, Aflatoxins eluted with 2ml methanol and the eluted volume collected in 2 ml vials before injection to HPLC. The same was done for the duplicate of the individual sample.

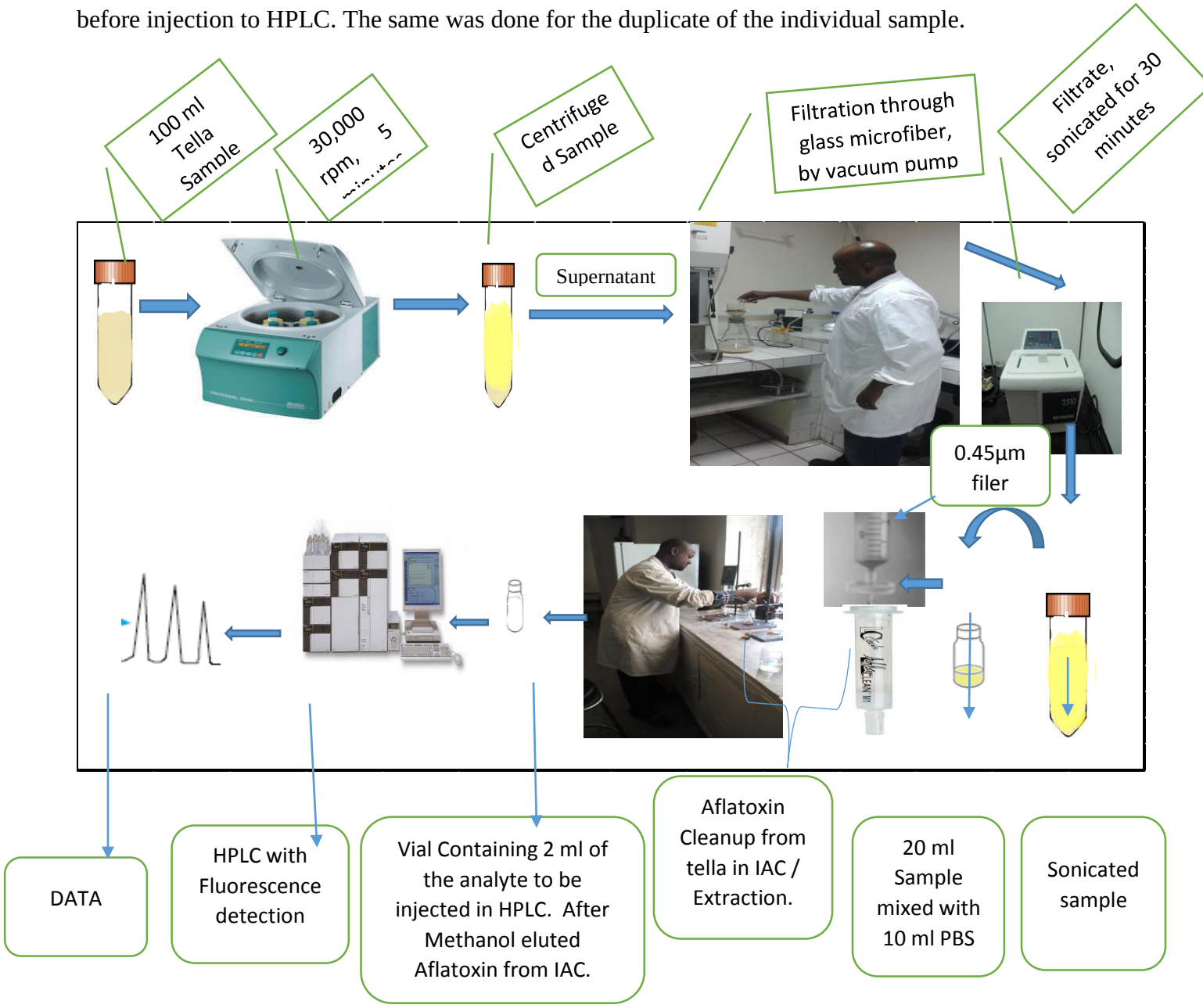


Fig. 11 Work flow in Tella Sample preparation and Analysis of Aflatoxins.

3.7.3 Sample Extraction in Areki

For Areki samples, no need to cleanup with the IAC except filtering through in 0.45 µm nylon Millipore filter the 2 ml sample, and collecting in the vials before injection in the HPLC the method was in reference to previous study (Ian et al., 2012).

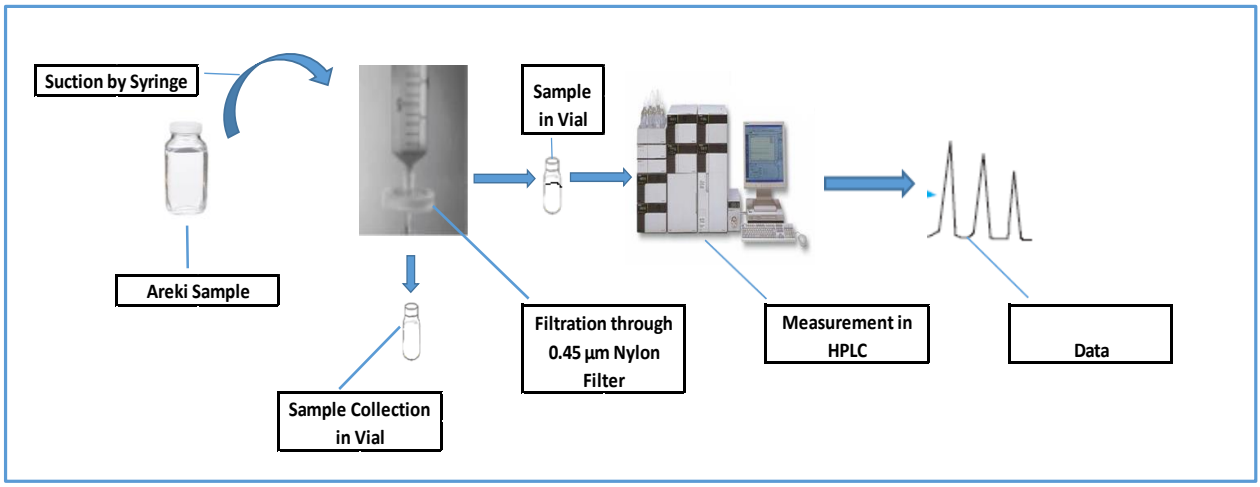


Fig. 12 Work Flow in Areki Sample Preparation and Analysis of Aflatoxins

3.8 Analysis of Aflatoxins

Mobile phase used were a mixture of ultra-pure water, Methanol, and Acetonitrile, (60, 25, 15) % Acetonitrile. Mobile phase was sonicated to bubble out any gas molecules so as not disturb the Aflatoxin determination process in the HPLC.

Analysis performed by SHIMADZU HPLC, first by conditioning the HPLC. Meaning purging the line, and cleaning the HPLC column with in the pre-set time before sample run. The Oven temperature was set also at 25^{o} C. The excitation and emission wave lengths were at 365 nm and 440 nm respectively in the fluorimeter column. The injection time per sample was15 minutes.

Mobile phase was in the Isocratic method, and this mobile phase solution made in 2 ml vial and run as a blank during the Aflatoxin Analysis before sample injection. Samples and Aflatoxin standards were injected together in the HPLC by standardized 1.3 ml / min flow rate. Therefore,

keeping the aforementioned parameters 20 µl of the sample volume and standard solutions used for injection.

Aflatoxin level in the sample calculated after obtaining the necessary data from the developed chromatograms, and building the calibration curves and the regression equation, of the standards gained during the analysis.

3.9 Aflatoxin Level Calculation

Calculation

Aflatoxin concentration calculated based on the formula

$$W = W_a \times (V_f / V_i) \times (1/ Vs)$$

W = amount of aflatoxin in the test sample in µg/L
W_a = amount of aflatoxin corresponding to area of aflatoxin peak of the test extract (ng)
V_f = the final volume of elute (µL)
V_i = volume of injected elute (µL)
V_s = volume of test portion (Tella) passing through the column (mL).

3.10 Data Analysis

For Data analysis, Excel Microsoft office 2013 and IBM SPSS Statistics version 20 software used. In the SPSS method, one-way analysis of variance (ANOVA), was performed to evaluate the levels of total aflatoxin means comparison between the study sites. A P-value of less than 0.05 ($P < 0.05$) was considered to show statistical significance. Assumptions of ANOVA were checked. As there was one dependent variable one-way ANOVA used. Dependent variable, level of Aflatoxin in the study sites. As the independent variable the study sites were used to test if locality affected for the possible mean aflatoxin level difference.

4. Results and Discussion

4.1 KAP Survey Result on Aflatoxin among Producers and Consumers.

4.1.1 Socio-demographic Characteristics Among Producers and Consumers.

As we can infer below from the summary in table 3., the majority of Tella and Areki producers included in the study were above the age of 30 years, mostly married or widowed, and 76.7 % of them were illiterate. Their economic class can be categorized in the poor economic class where the majority 66.7 % have a family income of ≤ 1500 birr per month on the average.

The livelihood of these women was based only in the production of Tella and Areki making, which is a laborious and time taking job which occupies their full time for the preparation and hosting the consumers, in effect it hampered them from engaging in other economic activities.

The age structure in the socio-demographic characteristics of the consumers approached in the study, were almost similar to the producers in that the majority of the consumers were above the age of 30 years old. Even though, it was not pre-structured in the questioner as they were not target groups, it is the observational inference of the researcher that there was a considerable tradition of children's and young ones to have the habit of "kirari "consumption. "Kirari" is a sediment of Tella dilution with water after the main Tella was decanted, and it is weaker than the normal tella.

The majority of consumers were married, and illiterate. Although, a better mix of educational status was distributed than the producers grouped in this limited survey. The economic status can be regarded as low class for the majority and some of them can be taken as the working class of the society.

However, the occupational profiles of the consumers were skewed to some extent. 60 % were farmers. Probably this is due to the fact that the period the study conducted was in the weekend times and this was the time popular market days existed in the towns. It is the common ritual habits of the farmers especially after selling and buying crops and commodities to gather in Tella Vending houses to feast.

Table 3. Socio Demographic Characteristics of .producers and consumers in the study sites.

Socio Demographic Characterstics of Producers			
Statement	**Response**		
		Frequency (n)	**Percenage**
Age	20 -30	**5**	**16.7**
	30- 50	**13**	**43.3**
	> 50	**12**	**40.0**
Sex	Female	**30**	**100.0**
Marital Status	Single	**4**	**13.3**
	Married	**18**	**60.0**
	Divorced	**2**	**6.7**
	Widowed	**6**	**20.0**
Educational Status	Illiterate	**23**	**76.7**
	Able to write and read	**5**	**16.7**
	Elementary School	**2**	**6.7**
Family Income	≤ 1500	**20**	**66.7**
	≤ 2000	**10**	**33.3**
Occupation	Private workers	**30**	**100.0**
Socio Demographic Characterstics of Consumers			
Statement	**Response**		
		Frequency (n)	**Percenage**
Age	20 -30	**7**	**23.3**
	30- 50	**17**	**56.7**
	> 50	**6**	**20.0**
Sex	Male	**30**	**100.0**
Marital Status	Single	**3**	**10.0**
	Married	**24**	**48.0**
	Widowed	**3**	**10.0**
Educational Status	Illiterate	**13**	**43.3**
	Able to write and read	**7**	**23.3**
	Elementary School	**4**	**13.3**
	High School	**5**	**16.7**
	Higher Education	**1**	**3.3**
Family Income	≤ 1500	**12**	**40.0**
	≤ 2000	**14**	**46.7**
	≥ 3000	**4**	**13.3**
Occupation	Private workers	**5**	**16.7**
	Government Employee	**6**	**20.0**
	Pensioned	**1**	**3.3**
	Farmer	**18**	**60.0**

4.1.2 Results on the Survey of Aflatoxin KAP among Producers

The questionnaire developed tried to get a snapshot insight on the KAP about Aflatoxin both among producers and consumers. Different probing questions were administered so as to get an informative picture of the KAP with this limited survey.

As summarized in table 4 below, results revealed the producers were genuinely responsive on the cereals used which made the researchers pre-assumption and doubt of deliberately hiding the recipe to be on the contrary. This was due to the fact, the researcher's prior knowledge and experience in another setting, to include maize in the brew recipe is highly gossiped for its unhealthiness and hangover effect. However, none of the respondents deny the fact that maize is their main ingredient for Tella and Areki, except some use millet for special Areki only in a known and "labeled ", way as "yedagussa Areki". Therefore, Maize was the main recipe cereal ingredient unanimously in all of the study sites. They, even reaffirmed that due to the cost of barley and its unavailability by the computing and growing interest of breweries, the tradition of using barley for commercial Tella and Areki producers was abandoned long ago.

The producers buy the cereals from the market, not from their farm land and most don't use exclusion criteria to avoid mouldy cereals if encountered. However, it seems they have a good knowledge on how cereals can get mouldy. Inconsistently, they use either hand picking or more roasting of the cereals as heat treatment or both of these practices in their preparation this is the effort to tackle the effect of mouldy cereals in relation to inferior quality of the products. This exemplified, their knowledge of mouldy cereals was in relation to the quality of the product. There doesn't seem to exist the tradition of storing in ground pits in these localities, a good number of the respondents also know that farmers before marketing the cereal, did store in muddy silos.

To assess their attitude on mouldy cereals, the question administered was in another way to know their opinion, if it is better to use "mouldy cereals for Tella and Areki making than to use for Injera or Bread making". 76.7 % of the respondents prefer to use for Tella and Areki making.

Moreover, their attitudes towards the traditional beverages production and consumption over the industrially produced ones, were related to their belief that it is the culture and tradition, as well as the assumption of its healthiness as they are freshly crafted in households without any chemical

additive. However, a considerable number of the respondents also were price sensitive in that, traditional beverages are less in price and preferred because affordable by the majority of the dwellers in the towns.

With regard to the practice of Tella and Areki making, in the light of the vulnerability to Aflatoxin contamination, even though they store for a limited time and freshly buy from the market, they store the purchased cereals in polypropylene bags.

To magnify the knowledge, attitude and their practice in relation of using mouldy cereals, they were interviewed if they use left over and mouldy Injera when available to mix with the ingredients, 70 % of the respondents had the habit of this practice. While, some of them seem to be reserved that it may not be good to the product if the Injera is contaminated with stew/" wot".

Most of the producers are a good customer of their product, they consume their produce per day mostly not less than two cups of Tella/Areki. Nevertheless, with the probing question on their health status, none of them had ever diagnosed for liver related sickness. This may be masked by the low tendency and health care seeking behavior of our rural communities.

A notable finding of the survey also may be on the Tella/Areki making practice, it looked the tradition of Enjibarra and Fintoselam brewers and distillers to use only Barley malt for their recipes while those in Debremarkos, the locality towards the south of the study towns were Wheat malt users in their recipe. This may be related to the agro ecological distribution of the cereals, availability, and community tradition at large.

Though, limited number of strict distillers of Areki reached in the survey, to relate the fact Areki could be potentially contaminated with Aflatoxins during the traditional distillation technique. Questions were raised with regard to the effect of possible gushing of the fermentation concentrate to the "columns", some of them responded to face so while others not. Those who admits to face gushing, tries to avoid gushing by gently heating of the fermentation concentrate and redistilling if encountered, to keep the required clarity and flavor of Areki.

Table 4. Summary of Aflatoxin KAP among Producers

Statement	Response		
Knowledge		**Frequency (n)**	**Precent (%)**
Cereals Used for Tella and /Areki	Maize *	30	100
	Millet **	5	16.7
Purchase cereals for Tella and/Areki from Market	Yes	30	100
Knowledge on cereals storage by farmers	Muddy Silos	23	76.7
	Don't Know	7	23.3
Use Mouldy cereals exclusion criteria during Purchase ?	Yes	12	40.0
Knowlede on how cereals to be mouldy ?	No	18	60.0
	In the field	15	50
	Storage condition	25	83.3
Special treatments done when mouldy cereals encountered	Hand Picking	13	33.3
	Roasting	22	73.3
	No special treatment	6	20.0
Attitude			
What makes Traditional Beverages a better choice than Industrially produced ones ?	Culture /Tradition	19	63.3
	Health	8	26.7
	No Additive/Fresh	3	10.0
	Price	16	53.3
Do you prefer Mouldy cereals for Tella and Areki Making than to use for Bread and Inejera ?	Yes	23	76.7
	No	7	30.4
Practice			
Storage Conditions of purchased cereals	Polypropylene Bags	30	100
Do you store or Purchase on demand cereals ?	Purchase on demand	30	100
Mouldy" Injera" left over use for Tella / Areki	Yes	21	70
	NO	9	30
Do you purchase if mouldy cereals encountered in the market ?	Yes	18	60
	No	12	40
Volume of Tella /Areki Produced Per Month	Tella - 320 liter	16	53.3
	Tella - 400 liter	13	43.3
	Tella- 480 liter	1	3.3
	Areki- 80 litr	4	13.3
	Areki- 360 liter	1	3.3
Number of Tella /Areki consumed per day	≤ 2	24	80
	≤ 4	6	20
Have you ever Diagnosed of Liver related illness	No	30	100.00
Type of Malt used for Tella and /Areki	Barley Malt ***	20	66.7
	Wheat Malt ****	10	33.3
Do you face gushing during Areki Distillation	Yes	3	10
	No	2	6.7
Measures taken during gushing ?	Gentle Heating	3	13.3
	Redistilling	1	3.3

Notes: * = Result for Tella, **= Result for Areki, ***= Barley malt existed in Enjibarra and Fintoselam only, and ****= Wheat Malt tradition only in Debremarkos.

4.1.3 Results on the Survey of Aflatoxin KAP, among Consumers

The Assessment of the KAP about Aflatoxin was done among consumers and results revealed that, they didn't seem to have knowledge of Aflatoxins. They didn't look also to be conscious enough on the inclusion of mouldy cereal usage of the commercially produced traditional beverages as Survey results summarized below in Table 5.

The attitude assessment results also showed, the majority were not far knowledgeable from the producers whether mouldy cereal presence is concerning in the brews.

Most believe that consumption of traditional beverages is healthy, even if a good number of the respondents prefer to drink the industrially produced ones over the traditional ones had the cost not been high enough.

For the many, it is their usual practice to consume Tella and Areki one after the other, and even a considerable number are highly addicted to consume ≥ 5 cups of Tella and/Areki.

Most of the respondents about 70 % have no body reactions like vomiting and abdominal discomfort after consumption even though, some had such experiences. On top of this, the significant number of the interviewees had not ever diagnosed with liver related disease, only one person admit to comply with the question of interest. However, these findings may be inconclusive as they may not confirm whether the health defects arise from health issues in relation to gastritis, hepatitis or exposure to aflatoxins.

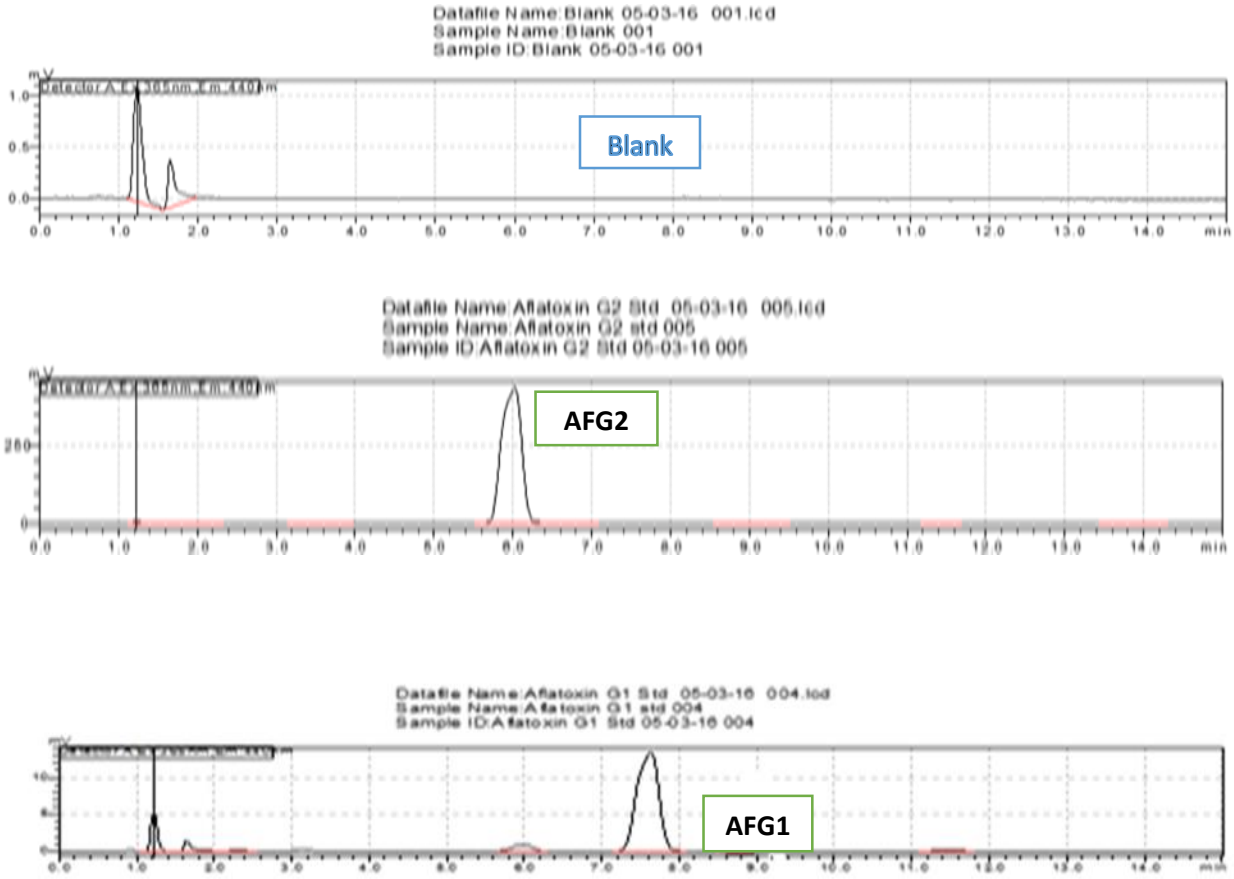
Table 5. Summary of Aflatoxin KAP Among Consumers.

Knowledge		Frequency (n)	Precent (%)
Tella/Areki produced from mouldy cereals is safe to drink	Yes	17	56.7
	No	6	20.0
	Don't Know	7	23.3
Cereals can get mouldy in the field	Yes	30	100.0
Cereals can get mouldy during storage	Yes	30	100.0
Cereals can get mouldy during tansport conditions	Yes	27	90.0
	Don't Know	3	10.0
Does using mouldy cereals for traditional beverages negative impact on health ?	No	17	56.7
	Don't Know	13	43.3
Special treatments done when mouldy cereals encountered	Hand Picking	13	33.3
Have you ever heard of Aflatoxins ?	No	30	100.0
Attitude			
Do you think Traditional beverage producers use mouldy cereals ?	Yes	9	30.0
	No	5	16.7
	Don't Know	16	53.3
Prefernce of drinking taditional beverages than industrially produced ones	Economic Reasons	20	66.7
	Health Advanatge	24	80.0
	Cultural/ Traditional	18	60.0
Practice			
Do You consume traditional beverages often	Yes	30	100
Habit of consumption of tarditional bevearges from sellers	Yes	30	100
Consumption of Tella or Areki on average Per day ?	Tella ≤ 4	17	70
	Tella ≥ 5	13	30
	Areki ≤ 4	21	70
	Areki ≥ 5	9	30
Do you consume Tella and Areki together per day	Yes	16	53.3
	No	14	46.7
Do you face some reactions after consumption of Tella or Areki	Yes	9	30.0
	No	21	70.0
Reactions faced after consumption among who report to have	Abdominal Discomfort	5	16.7
	Vomiting	4	13.3
Have you ever Diagnosed of Liver related illness	Yes	1	3.3
	No	29	96.7

4.2 Results of Method Validation

4.2.1 Identification

After running of the individual standards for identification of retention time or elution order of the specific Aflatoxin peaks, retention time recorded / gained for the individual Aflatoxin types. This enabled for identification of which Aflatoxin type is in what order of the peak revealing sequence and RT. The chromatograms below further elicit the work of identification better.



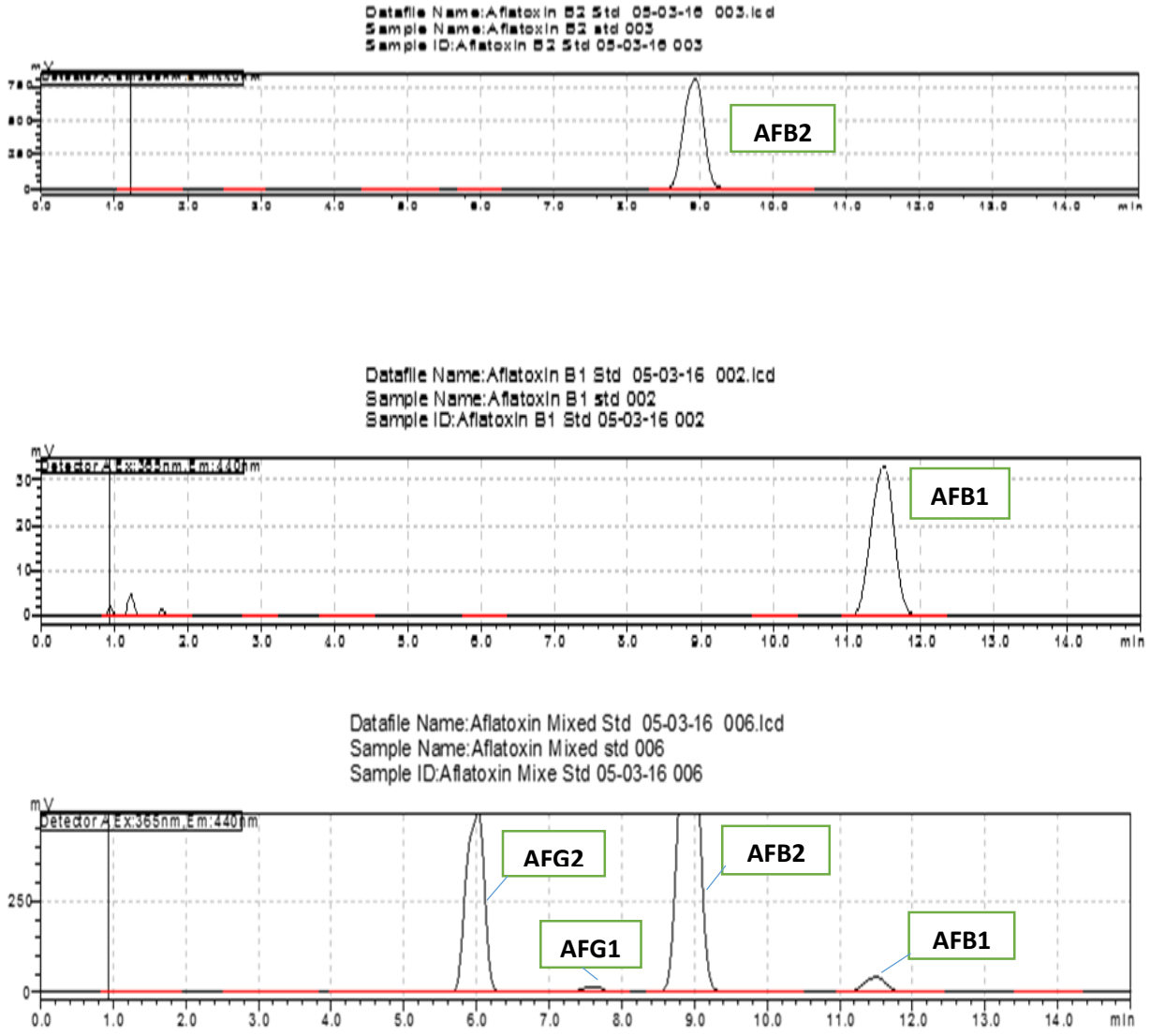


Fig.13 Chromatograms of Individual and Mixed Aflatoxin standard solution for Identification Test

According to the retention times gained of each individual standards, the detection sequence was in the order of AFG2 - AFG1- AFB2- AFB1 with their specific RT. Finally, mixed Aflatoxin standard chromatogram superimposition had also coherent Retention time as to the individual Aflatoxin types retention time.

Table 6. Summary of Retention time (RT) for individual and mixed Aflatoxin standards in Identification of Aflatoxins

Aflatoxins Retention Time (RT) in Identification, Statistics					
Types of Aflatoxins standards	Retention Time (Min.),after 100ppb Injection		Mean	STD	% RSD
	Individual Aflatoxin	Mixed Run			
AFG2	5.824	5.806	5.82	0.01	0.22
AFG1	7.312	7.273	7.29	0.03	0.38
AFB2	8.487	8.421	8.45	0.05	0.55
AFB1	10.828	10.706	10.77	0.09	0.80

As in Table % of RSD was in the range of (0.22 – 0.8) %, which was < 2 % RSD of the acceptable standard for identification.

4.2.2 Precision

Results of repeatability test obtained as measure of precision are presented below,

Table 7. Demonstration of the Repeatability of the HPLC assay for Aflatoxins as shown by the results of 10 replicate injections of 20 ppb of mixed aflatoxin standard.

	AFG2		AFG1		AFB2		AFB1	
Injection Number	Ret. Time (min)	Peak Area	Ret. Time	Peak Area	Ret. Time	Peak Area	Ret. Time	Peak Area
1	5.407	133174	6.785	17773	7.858	323899	10.001	63361
2	5.408	129140	6.789	17577	7.858	319784	10.003	60981
3	5.407	130822	6.788	17048	7.855	313918	10.001	60209
4	5.406	129679	6.783	17085	7.854	310444	9.999	59300
5	5.407	130659	6.784	16764	7.855	307200	9.997	59146
6	5.406	126666	6.781	16865	7.853	305415	9.995	59077
7	5.405	127697	6.787	16651	7.854	304136	9.998	58519
8	5.407	127464	6.784	17016	7.855	303315	9.997	58550
9	5.408	127106	6.783	16606	7.858	302208	9.999	58554
10	5.408	123807	6.785	16566	7.857	301622	10.001	58141
Mean	5.41	128621.40	6.78	16995.10	7.86	309194.10	10.00	59583.80
SD	0.00099	2637.35	0.00	405.42	0.0019	7723.03	0.00	1580.58
RSD %	**0.02**	**2.05**	**0.04**	**2.39**	**0.02**	**2.50**	**0.02**	**2.65**

The instrument repeatability was therefore with RSD % of < 5 % at the limits of detection. Which signified the instruments workability for the research.

4.2.3 Linearity and Working Range

After injecting the solution, data obtained of the peak area against the concentrations. Calibration curve drawn by linear regression of the least-square method using the peak area of standard as response versus concentration. To portray the curve in condensed and summarized form all four curves are shown below in one graph.

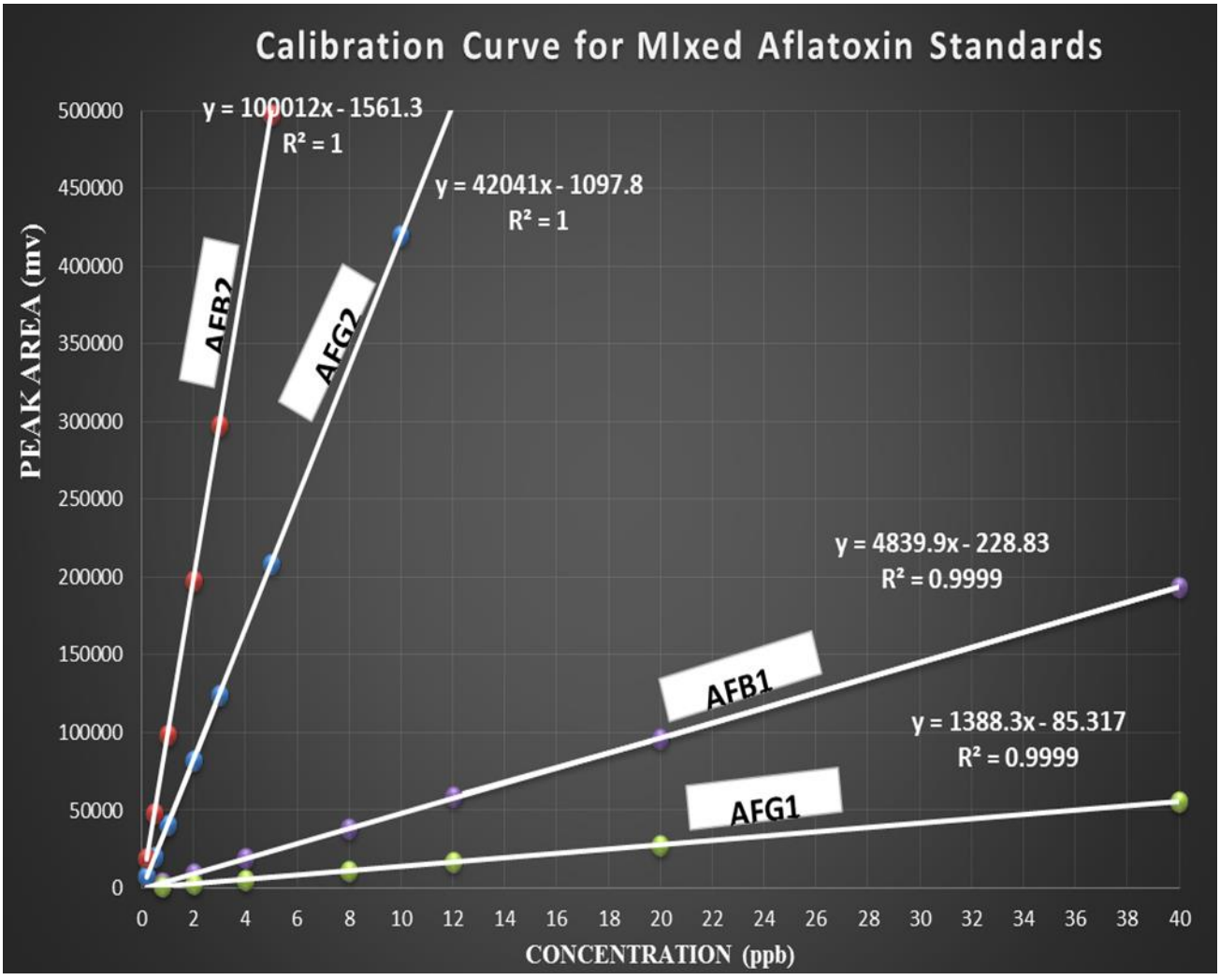


Fig. 14 Calibration Curve for Linearity test at different concentration of Aflatoxins.

As can be seen the correlation coeffiecnt (r^2) gained was 1.0000 for AFB2 and AFG2, 0.9999 for AFB1 and AFG1. This illustrated there existed linear relation of the response of the analyte concentration over the working range. And the regression line is fit and acceptable for the data as it was in line with the generally accepted (r^2) > 0.9998 criterion.

- **Working Range**

Moreover, the working ranges refereeing to table 2, the ranges obtained were, AFG2 (0.2 – 10) ppb, AFG1 (0.8 – 40) ppb, AFB2 (0.2 – 10) ppb and AFB1 was (0.8 – 40) ppb.

4.2.4 Limit of Detection (LOD), Limit of Quantification (LOQ).

The results done for the LOD and LOQ taking the criteria S/N > 3 for limit of detection and S/N > 10 for limit of quantification results revealed summarized in the table below.

Table 8. Level of Aflatoxins in (ppb) for LOD and LOQ.

	LOD		LOQ	
Aflatoxin Type	**Relative Individual concentration of Aflatoxin (ppb)**	**S/N > 3, Results**	**Relative Individual concentration of Aflatoxin (ppb)**	**S/N > 10, Results**
AFG2	**0.01**	**4.7**	**0.05**	11.92
AFG1	**0.20**	**3.3**	**0.80**	10.41
AFB2	**0.01**	**5.4**	**0.05**	12.86
AFB1	**0.20**	**4.1**	**0.80**	11.16

Therefore, according to the criteria LOD of AFG2 and AFB2 = 0.01 ppb and AFG1 and AFB1 = 0.2 ppb. And similarly the LOQ of AFG2 and AFB2 = 0.05 ppb and AFG1, AFB1 = 0.8 ppb. LOD of AFG2 and AFB2 = 0.01 ppb and AFG1 and AFB1 = 0.2 ppb. And similarly the LOQ of AFG2 and AFB2 = 0.05 ppb and AFG1, AFB1 = 0.8 ppb.

4.2.5 Recovery Test.

After spiking the sample and data obtained for the corresponding spiking and un-spiking level, percent recovery calculation done and results summarized as in the table below.

Table 9. Summary of the % mean recovery data in recovery test

Aflatoxin Type	Spiking Low Level (ppb)	Spiking High Level (ppb)	Mean % Recovery Low level	Mean % Recovery High level	Mean % Recovery (ppb)	STD	% RSD
AFG2	**0.3**	**2.5**	74.0	121.0	97.5	33.23	34.09
AFG1	**0.3**	**15.0**	118.0	102.0	110.0	11.31	10.29
AFB2	**0.9**	**10.0**	81.0	83.0	82.0	1.41	1.72
AFB1	**2.0**	**12.5**	94.0	87.0	90.5	4.95	5.47

The mean % recovery was in the range of (82.0 – 110.0) %, which was in line with (80 -120) % of FDA criteria as presented in the literature review.

4.3 Level of Aflatoxin in Samples of Tella and Areki.

4.3.1 Aflatoxin Level in Areki

As explained earlier, in the sample extraction and clean up subtitle, no IAC cleanup was done in the analysis. Therefore, after filtering the samples with 0.45 µm nylon filters level of Aflatoxin was analyzed by the HPLC. This was a methodology adopted as in the case in other study (Ian et al. 2012). The fact that Areki were not made to be cleaned up by IAC was because, the high ethanol content in the Areki (up to 48 % V/V) as reviewed before, may interfere in the cleanup process. In a paper which discusses about Immune Affinity Chromatography it was explained as after having retained the compound or macromolecule of interest on the immobilized antibody, elution can be accomplished by a variety of methods. If the antibody is covalently bound to the support, relatively strong eluotropic conditions can be used including organic solvents such as ethanol (Wilson and Stevenson). Therefore, cleaning in IAC can cleave the bond of the antibody or leaves the Aflatoxin un- bound interfering in affinity. Hence, Aflatoxin from Areki may be washed out Therefore, direct injection after filtration from impurities was demonstrated.

However, the samples of Areki revealed for Aflatoxin to be inconclusive. This research finding was not consistent with other studies conducted in another setting. A study conducted in Mississippi State University, USA on the fate of Aflatoxin in corn fermentation results indicated no Aflatoxin was detected in the distilled ethanol (Ian et al., 2012). Their experiment was, a lab scale fermentation of corn samples with high level of Aflatoxin (ranging from 7750 - 17,208 parts per billion), fractions were taken from the fermented mash, distilled ethanol, stillage, and dried corn solids (DCS) for analysis. While no Aflatoxin was detected in distilled ethanol, some aflatoxin (13%) was detected in the stillage, but most of the toxin was recovered in the DCSs ranging from 31% to 58%.

In another review paper from university of Kentucky, USA. Implicated, Aflatoxin didn't appear in distilled Alcohol even when corn has relatively of high Aflatoxin (Stuckey, Unpublished University of Kentucky). But also indicated that little toxin degradation occurs during the distillation process and the toxin remain to be concentrated in distiller's grain. Technically, distiller's grain is to mean cereal by product of the distillation process. Moreover, in a study which was targeted to assess the IAC relevance to Aflatoxin detection in distiller's grain it confirmed indirectly the relative presence of Aflatoxin in the distiller's grain (McDaniel et al., 2011)

Additionally, study conducted in China, which used 100 samples of domestic liquor products from the Taiwan Tobacco and Wine Monopoly Bureau and from Mainland China. No Aflatoxin detected in all alcohol liquors (Shiow & You 1997).

Nevertheless, these supporting researches seems to embolden the absence of Aflatoxin in distilled alcoholic products. Areki, is an alcoholic liquor distilled traditionally which may not comply with engineering design of standard distillation column setup. This brings the notion of possible gushing, and splash of fermentation concentrate bumping during distillation and possible transfer of potential Aflatoxin contamination through the condenser to the distillate. This is evident as indicated in the pre-thought KAP survey, that there was sometimes gushing and transfer of splash. They do gentle heating and re-distillation measures to keep the quality of Areki clarity and flavor.

In light of this fact, the chromatograms obtained in the Areki samples, have several peaks as presented below in Fig. 13, although the peaks were not aligned and within the comparative

retention times of the peaks of the concomitant Aflatoxin standards run for the analysis and were not able to lead to the conclusion of aflatoxin detection. These peaks may be the result of different fermentation metabolites or Aflatoxin degradation compound peaks masked in the determination as reviewed in (Elisabeth et al. 2013; Franz et al.; 2012, Tomonori et al., 2015). This may pave the way for confirmatory future researches to combine HPLC-MS technique and evaluate the peak mass in relation to Aflatoxins. As the methods importance discussed in the literature review of this paper (Peiwu, Unpublished).

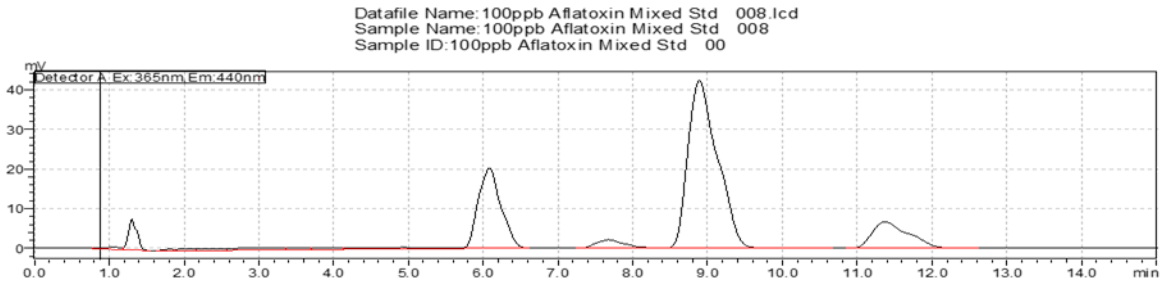


Fig. 15 Chromatogram of mixed Aflatoxin standard of 100 ppb for identification of RT and calibration curve.

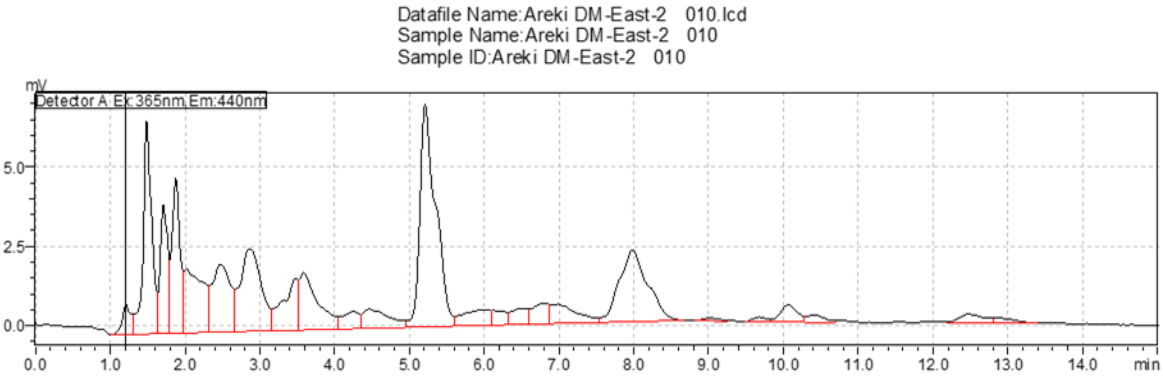


Fig. 16 Chromatogram of Areki samples with various "peaks" of non-aflatoxin detected.

4.3.2 Levels of Aflatoxin in Tella samples

As shown below in table 10 , the level of Aflatoxin analyzed in Debremarkos town samples of Tella, the mean Total Aflatoxin level was 12.8 ± 4.43 µg/kg ranging from (6.4 – 20.1) µg/kg. No Aflatoxin of the aflatoxin B family detected, while the G1 and G2 contributed to the total Aflatoxin level where G1 is the predominant type of toxin detected.

In Finoteselam Tella samples, the mean Aflatoxin level was 14.4 ± 8.86 µg/kg ranging from (3.2 - 28.3) µg/kg total aflatoxin level. This was the highest level of total Aflatoxin level compared from the three study site samples. Strikingly, also the aflatoxin B family detected in four samples where some are quantifiable and some in the sample are below the limit of quantification. Aflatoxin B2 was quantifiable.

Tella sample from Enjibarra, has the lowest mean total Aflatoxin level 11.4 ± 3.38 µg/kg, ranging from (4.12- 16.8) µg/kg compared in the three study samples. Here also Aflatoxin B2 detected in one of the samples even if it was below the limit of quantification.

There were vivid peaks in the chromatograms of Tella samples other than the standard Aflatoxin retention time, which might be the degradation of Aflatoxins or different microbial metabolites during the fermentation which probably appeared as intermediate peaks of the chromatograms.

As to the results, no Aflatoxin of the B family detected from samples of Debremarkos town while it was detected in Finoteselam and Enjibarra towns samples of tella. Referring to the KAP survey result in connection with the recipe cereals, there existed a variability in malt type usage. None of the Debremarkos brewers use barley malt unanimously, while on the contrary both Enjibarra and Fintoselam brewers used exclusively barley malt. This may signify and give insight to, the variability in the trace of B family Aflatoxins detection might have arose from the difference in malt type recipes. As all of them used maize as their main ingredient which is a shared cereal in the recipes of all the brewers. Probably, the low level of the B family Aflatoxin detected is due to the practice of low amount of malt proportion used for the fermentation.

In light of the Aflatoxin type's findings, the highest contribution of the Aflatoxin type for total Aflatoxin level was contributed from the G Aflatoxin families and mainly from Aflatoxin type G_1, which was detected and quantified in all of the samples from the three study locations.

Pursuing to the fact that, Aflatoxin types are dependent on the Aspergillus species infecting the cereal. And, the case in this research finding, only the G families of the toxin dominate, and only one main cereal is shared in all of the recipes. Moreover, the research finding that the B family aflatoxins detected might have arose from the variability in the malt type used. On top of these, the fact that *Aspergillus parasiticus* produces both the B and G Aflatoxin families and Aspergillus flavus produces the B Aflatoxin families only as referenced in the literature review (Andr´e et al., 2011; Kgomo et al., 2015; Rodrigues et al 2007).

Enlightened the researcher that, the maize used in the recipe might be contaminated by the specific *Aspergillus parasiticus* type of fungus. However, this may leave a gap for future research for the agriculturists, mycologists and food scientists etc. to consider primarily the predominant fungal species in the different agro ecological zones for Aflatoxin contamination studies.

Furthermore the agriculture practice of sugar cane plantation versus maize harvest, in relation to fungal species characteristically affects the Aspergillus species type as reviewed in the literature (Garber & Cotty, 2013). Therefore, the fact that in Ethiopia there is sugarcane cultivation in the backyards of small holder farmers since ancient times in general (Essayas et al.,2016), and the engagement of small holding farmers in sugar cane farming in East Gojam and West Gojam zones in particular. Where, the Ethiopian Central Statistical survey of 2010/2013 agricultural sample survey on peasant holdings of "Meher" season plotted the localities to had 561.22 Quintals in East Gojam and 2,607,567.05 Quintals of sugar cane production in West Gojam (CSA, 2010/2011). It might Strengthens the argument of the researcher for the *Aspergillus parasiticus* predominance in relevance to the result findings and might highlight the agricultural practice factor not to be neglected for future thorough studies.

Tella fermentation is composed of different dynamics of microorganisms, including the worth mentioning of lactic bacteria species presence. As well presented in the overview of Tella preparation literature reviews (Mooha etal, 2015; Mogessie, 2006). It might be evident that the

potential lactic acid bacteria species might probably had participated in the degradation of Aflatoxins especially in the B family aflatoxins as referenced in a number of studies and as presented in the sub title degradation of Aflatoxin during fermentation in this paper. Therefore, the research findings showed none in many of the samples and if any, at a very low level of B aflatoxins might elucidate the effect of lactic bacteria species. However, this finding and the concept on the effect of lactic acid bacteria in the degradation, opens further research to be conducted on the different phases of Tella fermentation for level of Aflatoxins follow- up in the course of fermentation thereby, get a strong evidence for this insight.

When it is sought to compare the level of Aflatoxins obtained in Tella samples with similar studies on traditional and industrial beverages, and the limited cereal based study conducted in Ethiopia. Corn samples were found to have greater than 20 µg/kg of total Aflatoxin level in Ethiopia (Habtamu & Kelbessa, 2001). And the research finding on this paper revealed, at least five of the thirty samples of maize derived tella to have > 20 µg/kg aflatoxin level.

Moreover, when it was compared with African traditional beverages, the grand mean Aflatoxin level of the thirty Tella samples which was 12.9 ± 1.6 µg/kg, was, higher than a similar traditional maize derived beverage (Busaa) of Kenya, where the mean Aflatoxin level was 5.2 ± 0.2 µg/kg (Mary et al. 2014). However, it was significantly lower than maize based traditional beverages studies of Malawi, where the Aflatoxin level in thobwa (opaque and sweet traditional beverage) was, 22.32 µg/l (Limbikani et al. 2011) and in the other traditional beverage from Malawi for the samples to had Aflatoxin detected 90 ± 95 µg/kg (Limbikani et al., 2014). Moreover, it was also lower than another non-maize based Nigerian traditional beverage from cocoa powder which had 16.01 µg/kg (Mary et al., 2013). Additionally, from another traditional beverages of Nigeria, the maize based beverage called, kunu-zaki analyzed had Aflatoxin level ranging from (< LOD – 123 µg/kg), which is high from Tella Aflatoxin level ranges. Nevertheless, Pito, sorghum based beverage of Nigeria had level ranging from (<LOQ – 5 µg/kg) which was lower than Tella aflatoxin level ranges (Chibundu et al., 2014).

In the studies of beer samples for Aflatoxin from different country studies, the mean level of total Aflatoxin in Tella compared. In South Africa, for sorghum beer analyzed from pilot scale brewing and industrially produced ranged from (0.3 – 10) µg/kg and < 0.1 µg/kg respectively, which was

lower than the level of aflatoxin in Tella (Trinder, 1988). Moreover, Aflatoxin study of beer samples in Czech Republic, ranged (1.5 – 4.7) ng/l (Karolina et al., 2012). In a study of 106 beer samples produced from several European countries, Aflatoxins were no detected in any of the samples (Terenzio et al., 2011). Besides, study of different beer samples sold in Canada imported from different countries and domestically produced ones, only 12 of 304 samples were positive for Aflatoxins with level above LOQ of , B1= 4.4 ng/l, B2= 3.4 ng/l , G1= 11.2 ng /l and aflatoxin G2= 6.2 ng/l) (Mably et al., 2005) . Therefore, Aflatoxin level in Tella was higher than all beer samples results reviewed. However, the detection of Aflatoxin B1 in beer samples was common although most were below the limit of quantitation in the specific study.

In light of international standards comparison, Tella aflatoxin levels compared to the European Legislation, (Commission of the European Communities, 2010), setting the maximum allowable limit for aflatoxin B1, 2.0 µg/kg and for total content of all aflatoxins (Sum B1, B2, G1, G2), 4.0 µg/kg as used in beer Aflatoxin studies (Karolina et al., 2012). Twenty-nine samples, out of thirty, that is 96.67 % of the samples had Aflatoxin level above 4.0 µg/kg, the set European level for total Aflatoxin in cereal based foods. On the contrary, none of the samples had Aflatoxin level above 2.0 µg/kg for specifically to the limit of AFB1which is the most carcinogenic of all Aflatoxin types. Although, it was detected in five samples or 16.67 % of them, while it was below the level of LOQ, or LOD.

ANOVA data analysis by SPSS done to know if there was a significant difference in the mean total Aflatoxin level among the study sites. ANOVA results revealed a significance Probability-value of (Sig) = 0.509. Taking alpha (α) level of significance 0.05, and P= 0.509 > 0.05. The decision was made to accept the null hypothesis. Hence, it was possible to conclude there was no significant difference in the total Aflatoxin mean level among the three study sites.

In table 10 the sample codes refer to the short hand writing form of the study sites and the hyphenated East or West in the codes refer to the sampling places East of the symmetric main road and West of the road places in the specific study towns. Additionally, the Numbers in the code refer to sequence of sampling done. For instance (DM-East-01), meant the first sampling household in Debremarkos town which is found East of the road.

Table 10. Aflatoxin Level of Tella Samples from the three study sites.

		Aflatoxin Level, (µg/Kg)				
Location	Sample code	G2 (µg/Kg)	G1 (µg/Kg),	B2 (µg/Kg)	B1 (µg/Kg)	Total AFs (µg/Kg)
Debremarkos	DM-EAST-01	0.42	9.82 ± 0.82	ND	ND	10.2
	DM-EAST-02	< LOD	7.32 ± 0.27	ND	ND	7.3
	DM-EAST-03	< LOD	13.8 ± 0.27	ND	ND	13.8
	DM-EAST-04	0.8 ± 0.25	5.62 ± 0.19	ND	ND	6.4
	DM-EAST-05	0.51	11.76	ND	ND	12.3
	DM-WEST-01	< LOD	16.95 ± 0.19	ND	ND	17.0
	DM-WEST-02	< LOD	15.91 ± 1.39	ND	ND	16.0
	DM-WEST-03	< LOD	14.97 ± 0.17	ND	ND	15.0
	DM-WEST-04	ND	20.09 ± 1.69	ND	ND	20.1
	DM-WEST-05	0.81	9.27 ± 0.69	ND	ND	10.1
					Average	12.8 ± 4.37
Fintoselam	FS -EAST-01	ND	16.49 ± 1.13	ND	< LOD	16.5
	FS -EAST-02	ND	5.22 ± 3.29	ND	ND	5.2
	FS -EAST-03	0.11 ± 0.08	5.08 ± 0.78	ND	ND	5.2
	FS -EAST-04	0.92 ± 1.04	13.88 ± 4.57	ND	ND	14.8
	FS -EAST-05	ND	11.46 ± 2.19	0.88 ± 0.02	< LOD	12.3
	FS-WEST-01	ND	2.21 ± 0.3	1.03 ± 0.06	< LOQ	3.2
	FS-WEST-02	ND	12.18 ± 0.3	ND	ND	12.18
	FS-WEST-03	ND	18.91 ± 2.08	ND	ND	18.9
	FS-WEST-04	ND	26.18 ± 1.78	1.2	< LOQ	27.4
	FS-WEST-05	ND	27.21 ± 1.24	1.06	ND	28.3
					Average	14.4 ± 8.76
Enjibarra	EB -EAST-01	0.68	8.44 ± 2.5	ND	ND	9.12
	EB-EAST-02	0.62	11.36 ± 1.43	ND	ND	11.98
	EB-EAST-03	ND	4.12	ND	ND	4.12
	EB-EAST-04	ND	11.89 ± 2.05	ND	ND	11.9
	EB-EAST-05	ND	13.25 ± 1.29	ND	ND	13.3
	EB-WEST-01	ND	11.13 ± 3.08	ND	ND	11.1
	EB-WEST-02	< LOQ	16.77 ± 4.89	ND	< LOQ	16.8
	EB-WEST-03	< LOQ	9.81 ± 0.19	ND	ND	9.8
	EB-WEST-04	ND	14.25 ± 0.44	ND	ND	14.3
	EB-WEST-05	0.44	10.59 ± 4.0	ND	ND	11.03
					Average	11.4 ± 3.38

Grand mean (mean of the means), from the three localities Aflatoxin level = 12.9 ± 1.6 µg/kg

Note - ND: is to mean the Aflatoxin is not detected

5. Conclusions and Recommendations

5.1 Conclusions

Aflatoxin detection in Areki remained to be difficult to conclude for the absence of the toxin. But in Tella samples the mean total Aflatoxin types from the three study sites was 12.9 ± 1.6 µg/kg. And this figure might be underestimated by the very impact of the sample centrifugation procedure in the analysis while on the contrary people drink the product with the turbid sediments and might expose them to higher level of the toxin. In the Tella samples Aflatoxin type G_1 predominated the distribution found entirely in all of the samples. Aflatoxin B_1 detected in some of the tella samples while it was not above the limit of quantifications. Therefore, as it was an inconclusive finding, drinking Areki from the point of aflatoxin exposure is uncertain. Hence, the result should be cautiously interpreted until the masked aflatoxins peaks findings issue is resolved to rule out the absence of the toxin as in similar researches in liquors by future researches.

To conclude the KAP survey, the consumption level of the beverage and liquor was high and there existed a deep rooted culture of preference for traditional drinks. In general, the knowledge among producers and consumers was extremely low towards Aflatoxin contamination. And attitudinally, most didn't seemed to worry about the presence of mouldy cereals in their brews as well as consumption, if would be, for the sake of taste and flavor to the products. In terms of their practice a considerable number of producers prefers or had the habit of using mouldy cereals encountered for Tella and or Areki than to other food items.

None of the Tella samples had aflatoxin level B_1 above the European limit of legislation, while globally the samples had total Aflatoxin level above the legislation limit. Hence, Tella samples lacked of the presence of the most carcinogenic type of Aflatoxin type above the limits.

5.2 Recommendations

Eventually, the researcher would like to recommend based on the findings that

- It would be better to not utilize mouldy cereals for Areki and Tella. But, also keeping the fact the distillers' grain and Tella residues supply to animal feed should be excluded as it

had an in turn effect on the dairy products aflatoxin level as highlighted by different researches and potentially more aflatoxin level might be present in these residues.

- Much to be done on not advocating on utilization of mouldy cereals on traditional alcoholic drinks. Awareness creating on the supply chain of cereals and their utilization for traditional alcoholic drinks preparation in relation with mould contamination. And the knowledge attitude and practices towards Aflatoxin exposure is worth seeking and should be shared by the stake holders.

- The researcher also further insists for future larger studies, to consider the potential factors of variation in aflatoxin types and level especially in relevance to traditional alcoholic beverages and liquors, by working on from cereal material, through the phases of fermentation and contrasting the type of malt used as source of variability, and the effect of lactic lactobacillus species for degradation in Aflatoxin types with larger number sample size.

- It might be also worth mentioning for further studies to consider metrological data, agricultural practice in relation to sugar cane and maize, and interdisciplinary researches by the mycologists for primarily to study the predominant species of Aspergillus in the specific cereal and agro-ecological zone of the country would be recommendable to have a full picture of Aflatoxin in traditional alcoholic drinks in general.

- The integration of Mass Spectrometer with the HPLC will help in certain identification of the peaks developed during Aflatoxin determination of Areki and Tella samples thereby, could yield for confirmatory identification and level of Aflatoxin in these traditional alcoholic beverage and liquor.

6. References

Abdellah, Z., Mohamed F., Mohamed B., (2005). In vitro reduction of Aflatoxin B1 by strains of Lactic Acid Bacteria Isolated from Moroccan Sourdough Bread. *International Journal of Agricultural and Biological Engineering, Vol. 7, No. 1*.

Aflatoxin-Analysis, (2010). HPLC analysis and photochemical post column derivatization. *LC Tech Application note.*

Aflatoxins IARC. (2002). International Agency for Research on Cancer (IARC) on the Evaluation of carcinogenic risks to humans. *Monograph 100 F*.

Alejandro, E.C. Luis M., Contreras M., Rafael F., Muñoz H., Jesús R., Millán-A., Ramón G. Irineo T. (unpublished). Methods for Detection and Quantification of Aflatoxins. Retrived from https//:www.intechopen.com.

Andr´e, E.K., Ali A., Toufic R., Roger L., Mireille K., Ahmed L. (2011). Differentiation between Aspergillus flavus and Aspergillus parasiticus from Pure Culture and Aflatoxin-Contaminated Grapes Using PCR-RFLP Analysis of aflR-aflJ Intergenic Spacer. *Journal of Food Science, vol. 76 (n°4). pp. M247-M253*. ISSN 0022-1147

Andrea Dall'Erta, (Unpublished Doctoral Dissertation). Masked mycotoxins: occurrence, metabolism and role in plants. Universita' Degli Studi di Parma Di Parma Dottorato di ricerca in Scienze e Tecnologie Alimentari Ciclo XXV

Angel, M., Alicia R., Naresh M. (2014). Effect of climate change on Aspergillus flavus and aflatoxin B1 production. *Frontiers in Microbiology, Volume 5 Article 348*

Anna C. M. (Unpublished). Aflatoxins: Their Measure and Analysis. Department of Food and Feed Science, University of Teramo Italy. Retrived from https: /www.intechopen.com.

Atanda, S.A., Aina J. A. , Agoda S. A., Usanga O. E. and Pessu P. O.(2012). Mycotoxin Management in Agriculture: a Review. *Journal of Animal science advances 2(Suppl. 3.1)*: 250-260.

Ayhan, F., Ufuk T. S. (2013). Occurrence of Aflatoxins in Food. *In Tech* doi.org: /10.5772/51031

Belay, B., & Awraris W. (2014). Fermenter technology modification changes microbiological and physicochemical - parameters, improves sensory characteristics in the fermentation of Tella: An Ethiopian Traditional Fermented Alcoholic Beverage. *Journal of Food Processing and Technology,* 5-4

Berhanu, A. (2013). Microbial profile of Tella and the role of gesho (Rhamnus prinoides) as bittering and antimicrobial agent in traditional Tella (Beer) production. *International Food Research Journal (21) 1,* 357-365.

Berihu, T., Getachew G., Tewodros A., Harikrishna R.S. (2015). Determination of Alcoholic Content and Other Parameters of Local Alcoholic Beverage (Tella) at Different Stages in Gondar, Ethiopia. *International Journal of IT, Engineering and Applied Sciences Research Volume 4, No. 6,* 2319-4413

Bioanalytical Method Validation (2013). Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). *Center for Veterinary Medicine (CVM). Revision 1*

Central Statistical Agency Agricultural Sample Survey (2010/2011). Crop and Livestock Product Utilization (Private Peasant Holdings, Meher Season .) *FDRA Central statistical Agency Agricultural Sample Survey Report Volume VII*.

Chibundu, N. E., Wilfred A. A., Isaac M.O., Michael S.F., Benedikt W., Rudolf K. (2014). Fate of mycotoxins in two popular traditional cereal-based beverages (kunu-zaki and pito) from rural Nigeria. *LWT - Food Science and Technology,* 1-5.

CODEX General Standard for Contaminants and Toxins in Food and Feed (2010). *Codex Standard 193-1995*

Daad, A. A, Luc R.B. (Unpublished). ImmunoAffinity Chromatography a Review. Retrived from https //: www.intechopen.com

David, F.K. (2009). The impact of crop, pest, and agricultural management practices on Mycotoxin contamination of field crops. Prague, Czech Republic. *OECD Conference Challenges for Agricultural Research 5-8*

David, M. W., Durham K.B. (1984). Aflatoxin Production by Aspergillus flavus and A. parasiticus on Visibly Sound Rehydrated Peanut, Corn and Soybean Seed. *Peanut Science, 11*, 43- 45

De Boevre, M., Graniczkowska K., De Saeger S. (2015). Metabolism of modified mycotoxins studied through in vitro and in vivo models: an overview. *Toxicology Letters 233*, 24-28

Dereje, A., Muez T. &, Helge .S (2012). Natural Occurrence of toxigenic fungi Species and Aflatoxin in freshly harvested groundnut Kernels in Tigray, Northern Ethiopia. *Journal of the Dry Lands (1)*, 377-384

EC Commission Regulation (2012). Premier Analytical Services.

Elisabeth, S., Christina S., Michael S., Karin N., Rudolf K.Gerd S.(2013). Multi-Mycotoxin Screening Reveals the Occurrence of 139 Different Secondary Metabolites in Feed and Feed Ingredients. *Toxins 5, 504-523*; doi:10.3390/toxins5030504

Ephrem, G. (2015). Aflatoxin contamination in groundnut (Arachis hypogaea L.) caused by Aspergillus Species in Ethiopia. *Journal of Applied & Environmental Microbiology Vol. 3, No. 1*, 11-19.

Esayas, T. Firew M., Amsalu A. (2016). Molecular Analysis of Genetic Diversity and Population Structure of Local Sugarcane Genotypes in Ethiopia. *Plant Cell Biotechnology and Molecular Biology 17(3-4),* 120-139

Ethiopia Demographic Health Survey. (2011). Central Statistical Agency Addis Ababa, Ethiopia ICF International Calverton, Maryland, USA. *EDHS*

European Food Safety Authority (EFSA), (2013); Aflatoxins (sum of B1, B2, G1, G2) in cereals and cereal-derived food products. Supporting Publications: EN-406. [11 pp.]. Retrived from https//: www.efsa.europa.eu/publications.

FDA, Mycotoxin Regulatory Guidance (2011). Guide for Grain Elevators, Feed Manufacturers, Grain Processors and Exporters by National Grain and Feed Association.

Franz, B., Colin C, Chiara D.A., Sarah D. S., Geert H. Peter k. et al. (2012). Masked mycotoxins. *Molecular Nutrition Food Research.57,* 165–186

Garber, N.P., Cotty P. J., (2013). Aspergillus parasiticus Communities Associated with Sugarcane in the Rio Grande Valley of Texas: Implications of Global Transport and Host Association within Aspergillus Section Flavi. *Ecology and Epideomology Vol. 104*, No. 5

Getachew, T. (2015). A review on Traditional Fermented Beverages of Ethiopian. *Journal of Natural Sciences Research* Vol.5,No 15

Ghulam, A.S. (2004). A Practical Approach to Validation of HPLC Methods under Current Good Manufacturing Practices. *Journal of Validation Technology*

Grazina, J., Loreta B., Elena B., Paulius M., (2012). Mycotoxin Decontamination Aspects in Food, Feed and Renewables Using Fermentation Processes. *In Tech* doi.org: /10.5772/46184

Habtamu, F., & Kelbessa U. (2001). Survey of Aflatoxin Contamination in Ethiopia. Ethiopian *Journal of Health Sciences Vol. 11, No.1*, 17-26.

Ian, J.C., Rebecca S., Cedric R., Darrell S., Ashli B, Brian B., Stephanie H. Paul W.W. (2012). The Fate of Aflatoxin in Corn Fermentation. *Natural Resources,Vol 3*, 126-136.

Irineo, T.P. (2011). Aflatoxins Detection, Measurement and Control . Retrived from *Intech* WEB. ORG ISBN 978-953-307-711-6

Jaimeza, J. Fentea C.A., Vazqueza B., Francoa, C.A., Cepedaa A., Mahuzierb G., Prognon P.(2000). Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *Journal of Chromatography A, 882*, 1–10

Jake, C. F., Brian T.S., Xinzhi N., Robert C. K., Robert D. L., Zhi-Yuan C. (2014). Environmental influences on maize-Aspergillus flavus interactions and aflatoxin production. *Frontiers in Microbiology Vol. 5*, 1-22

Jonathan, H.W. Timothy D. P., Pauline E.J., Jonathan K.S., Curtis M. J, Deepak A. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *American Society for Clinical Nutrition 80*, 1106 –22

Jonathan, H.W., Timothy D.P., Pauline E.J., Jonathan K.S., Curtis M.J., Deepak A., (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *American Journal of Clinical Nutrition 80*, 1106 –22

Jong, G. K. (2007). Anti-aflatoxigenic activity of some bacteria related with fermentation. Department of Public Health, Keimyung University. *Daegu 704-701*.

Karolína B., Sylvie B., Renata M., Zdenék S. (2012). Monitoring of selected aflatoxins in brewing materials and beer by liquid chromatography/mass spectrometry. *Food Control 25*, 626-630.

Kebede, A., Fekadu B., Thor L., Judith .A.N. (2002). Indigenous processing and raw materials of borde, an Ethiopian traditional fermented beverage. *The Journal of Food Technology in Africa Vol.7 No. 2*, 59-64

Kgomo, H., Bwembya G.C., Mamba S.S., Thwala1 J.M. (2015). Assessment of Aflatoxins B1, B2, G1 and G2 Status of home grown maize in Swaziland. *African Journal of Food Agriculture Nutrition and Development, Volume15 No 5.* 10552-10541

Laura, M.R. et al (Unpublished). Aflatoxins Biochemistry and Molecular Biology – Biotechnological Approaches for Control in Crops. Facultad de Ingeniería, CA Ingeniería de Biosistemas, Universidad Autónoma de Querétaro, Centro Universitario Cerro de las Campanas s/n, Querétaro, Qro, México. Retrived from Intech web site, www.intechopen.com/.../aflatoxins-biochemistry-and-molecular-biolo.

Limbikani, M., Christof V.P., Timothy B., Maurice M., Jonas .M. Sarah D.S. (2014). A limited survey of mycotoxins in traditional maize based opaque beers in Malawi. *Food Control 36*, 253-256

Limbikani, M., Maurice M., Elenimo B. K., Deliwe D. L (2011). Aflatoxins in sorghum, sorghum malt and traditional opaque beer in southern Malawi. Food Control 22, 266-268

Lopez-Garcia R., Park D.L., Phillips T.D. (2009), Integrated mycotoxin management systems. FAO Food and Nutrition Division FNA/ANA 23.

Mably, M Mankotia M, Cavlovic P., Tam J. Wong L., Pantazopoulos P., Calway P., & Scott P.M. (2005). Survey of aflatoxins in beer sold in Canada. *Food Additives and Contaminants, 22(12)*: 1252–1257

Manual of Methods of Analysis of Foods Mycotoxins (2015). New Delhi, Food Safety and Standards Authority of India ministry of health and family welfare government of India Lab Manual 7, fissai.

Mary, A. E., Cynthia A.C., Judith Z.P., Mulunda M., Michael F.D., Co-contamination of Nigerian Cocoa and Cocoa-Based Powder Beverages Destined for Human Consumption by Mycotoxins. (2013). *Ethno Medicine 7(3)*, 187-194

Mary, C. K., Amos E.A., Keith K.T, Gonoi T., Christine C.B., (2014). Total aflatoxin, fumonism, and deoxynivalenol contamination of Bussa in Bomet County Kenya. *African Journal of Biotechnology Vol. 13(26)*, 2675 -2678

McDaniel1, A. Holmes W.E., Williams P., Armbrust K.L., Sparks D.S., Brown A.E. (2011). Effect of Matrix Clean-Up for Aflatoxin Analysis in Corn and Dried Distillers Grains. *Natural Resources, 2*, 250-257

Michael, R., Hans U.H., Doris M., Sven D., Angella M., Franz B., Horst K., Nicole L. (2014). A comprehensive definition of modified and masked "masked" mycotoxins. *Mycotoxin Research 30*,197–205

Mogessie, A. (2006). A Review on the microbiology of indigenous fermented Foods and beverages of Ethiopia. *Ethiopian Journal of Science 5(2)*, 189-245

Mokonea, M.P., Chelule P.K., Gqaleni N. (2006). The toxicity and decreased concentration of aflatoxin B1 in natural lactic acid fermented maize meal. The Society for Applied Microbiology, *Journal of Applied Microbiology 100*, 773–777.

Mooha, L., Meron R., Semeneh S. (2015). Uniqueness of Ethiopian traditional alcoholic beverage of plant origin, tella. *Journal of Ethnic Foods 2*, 100-114

Mycotoxin Analysis (2013). ORA Laboratory Manual. Volume IV, Section 7-

Natalia, A.-M., José F. H.P., AnaM. G.-C., and Laura G.G. (2014). Review Article: Mycotoxin Analysis: New Proposals for Sample Treatment. *Hindawi Publishing Corporation Advances in Chemistry Article ID 547506*.

Okobia, M.N (2003). Cancer Care in sub-Saharan Africa – Urgent Need for Population–based Cancer Registries. *The Ethiopian Journal of Health Development 17(2)*, 89 – 98

Peiwu, Li., Zhang Q., et al., (Unpublished) Aflatoxin Measurement and Analysis. Retrived from https //: www.intechopen.com

Phelix, M. et al (2004). Mycotoxin control in food grains. Proceedings of a small group meeting Nairobi, Kenya: *African Agricultural Foundation*.

Rashid, A. A., (2013). Indigenous processing Methods and Raw Material of keribo: An Ethiopian Traditional Fermented Beverage. *Journal of Food Resource Science 2 (1)*, 13-20

Rodrigues, P., Soares C., Kozakiewic Z., Paterson R.R.M., Lima N., Venancio A. (2007). Identification and characterization of Aspergillus flavus and aflatoxins. Communicating Current Research and Educational Topics and Trends in Applied Microbiology Formatex.

Rudolf, k., Patricia S.U., Alexandra M., Michael S., Susan M., Colin C. (2008). Mycotoxin analysis: An update. *Food Additives and Contaminants, 25 (2)*, 152–163.

Shiow, S.L., You M.F. (1997). Determination of Aflatoxins in liquor products by Immuno-Affinity column with Fluorometry and HPLC. *Journal of Food and Drug analysis*, 5(2)

Stuckey, R.E., Lane GT. LoerryeCr J. Miller C.E., Bitzer J. Aflatoxin in Corn. University of Kentucky College of Agriculture Cooperative Extension service.

Tadele, Y., Fekadu M., Khalid S., (2013). Preparation and physicochemical analysis of some Ethiopian traditional alcoholic beverages. *African Journal of Food Science Vol. 7(11),* 399-403

Tchikoua, R., Tatsadjieu N.L., Mbofung Carl M.F., (2015). Effect of Selected Lactic Acid bacteria on Growth of Aspergillus flavus and Aflatoxin B1 Production in Kutukutu. *Journal of Microbiology Research 5(3),* 84-94

Terenzio B., Silvia R., Annalisa M., Gianluca D., Amedeo P. (2011). Mycotoxin occurrence in beer produced in several European countries. *Food Control 22*, 2059-2064

Tomonori, I., Yasushi N., Atsuo U., Naoki M. (2013). Degradation of Aflatoxin B1 during the Fermentation of Alcoholic Beverages. *Toxins 5*, 1219-1229

Trinder D.W. (1988). A Survey of Aflatoxins in industrially Brewed South African Sorghum Beer and Beer Strainings. *Journal of the Institute of Brewing Brew. Vol. 95*, 307-309.

Wajiha, I., Tehmina A., Mazhar I., Abdul G., Mateen A. (2015). Mass spectrometric identification and toxicity assessment of degraded products of aflatoxin B1 and B2 by Corymbia citriodora aqueous extracts. Retrived from nature website www.nature,com/scinticreports

Waliyar F, Ravinder R., Alur A., Reddy S., Reddy B., Reddy A., Rai K., Gowda C.(2007). Management of Grain Mold and Mycotoxins in Sorghum. Patancheru 502 324, Andhra Pradesh, India: *International Crops Research Institute for the Semi-Arid Tropics*. 32pp

Wei, Z, Beibei X., Mengmeng L., Yang. M., Zhihui C., Jianping L., Anshan S. (2014). Screening a Strain of *Aspergillus niger* and Optimization of Fermentation Conditions for Degradation of Aflatoxin B1. *Toxins, 6,* 2072-6651.

William, H., &, Carl, K.W. (2000). Food Toxicology. Boca Raton, London, New York Washington, D.C: CRC Press LLC

Wilson I.D., Stevenson (2000).Immunoaffinity Chromatography. AstraZeneca Pharmaceuticals, Mereside, Alderfly Park, Macclesfield, Cheshire, UK., University of Surrey, Guildford, UK.

World Health Organization Regional Office for Africa (2006). Mycotoxins in African Foods. *AFRO Food Safety Newsletter Issue No. 2,* 1-10

Yan, L., Felichia W. (2010). Global Burden of Aflatoxin-Induced Hepatocellular Carcinoma: *A Risk Assessment. Environmental Health Perspective Volume 118 No 6*

Yao, H., Hruska Z., Diana D.M.J., (2014). Developments in detection and determination of aflatoxins. *World Mycotoxin Journal, 2015; 8 (2),*181-191

ANNEXES

Consent and Questioner Form

My name is Mr. Workineh Birhanu, who is doing research as partial fulfillment for the requirement of Masters of Science in Food Science and Nutrition in Addis Ababa University Center of Food Science and Nutrition. Here, I am interviewing traditional beverages venders and consumers about the knowledge, Attitude and practice (KAP) on Mycotoxins and specifically on Aflatoxin in relation with the preparation and consumption of Ethiopian traditional beverages of Tella and Areki. I am going to ask you some important questions for a few minutes that are useful for the success of this research in particular and in mitigation efforts of Aflatoxin exposure initiatives in general.

Your name will not be asked and written in this form and all the information you give will be kept confidential. You have the full right not to participate in the study. Unless you are not willing to participate you are not obliged to participate. During the interview you have the right to escape some of the questions that you don't want to give answer also; you have the right to quit the interview at any time.

However, your willingness to participate and answer genuinely all the questions is highly appreciated.

Thank You

Name of the interviewer ----------

Signature ----------

Date of Interview----------

QUESTIONNAIRE

PART I **SOCIO-DEMOGRAPHIC CHARACTERSICS**

Question Number	Question	Response	Instruction
1	Age (Years)		
2	Sex	1-Male 2- Female	
3	Marital Status	1-Single 2-Married 3-Divorced 4-Widowed	
4	Educational Status	1- Unable to Read and Write 2- Able to Read and write without formal education. 3- Elementary Education (1-6) grades 4- Secondary Education (6-12) grades 5- Higher Institution, Specify……..	
5	Monthly Family Income	…….Birr	
6	Occupation	1- House Wife 2- Government Employee 3- Private Employee 4- Merchant 5- Daily Laborer 6- Other Specify……..	

PART II

KAP QUESTIONNAIRE ON CONSUMERS

Question Number	Question	Response	Instruction
Knowledge (K)			
1	Do you think to drink Tella and Areki produced from mouldy cereals is safe to drink ?	1- Yes 2- NO 3- Don't know	
2	Do you think cereals get mouldy in the field ?	1- Yes 2- No 3- Don't know	
3	Do you think cereals could get mouldy during storage conditions?	1-Yes 2-No 3-Don't know	
4	Do you think cereals get mouldy during farm to market transport?	1-Yes 2-No 3 – Don't know	
5	Do you think using mouldy cereals for traditional beverage inputs ha12s negative Impact on health?	1-Yes 2- NO 3-Do Not Know	
6	Have you heard of Aflatoxins?	1- Yes 2- No	If yes go to Q.6
7	Where do you get the information?	1- Media 2- Trainings 3- Medical persons	
8	Do you think Aflatoxins can cause Liver Disease	1-Yes 2-No 3-Do not know	
Attitude (A)			
1	Do you think traditional beverage producers use mouldy cereals?	1- Yes 2- NO 3- Don't Know	
2	Do you prefer traditional beverages consumption than industrially produced ones?	1- Yes 2- No	If, yes Q-3,4,5

3	**Do you prefer to consume traditional beverage because of economic reasons?**	**1- Yes** **2- No**	
4	**Do you prefer to consume traditional beverages because of health advantage?**	**1- Yes** **2- No**	
5	**Do you prefer to consume traditional beverages because of cultural reasons?**	**1-Yes** **2-No**	
Practice (P)			
1	**Do You drink traditional alcoholic beverages often?**	**1- Yes** **2- No**	
2	**Do you have the habit to consume traditional beverages from sellers?**	**1-Yes** **2-NO**	
3	**How many Tella cups on average per day you consume ?**	**1- ≤ 2** **2- ≤ 4** **3- ≥ 5**	
4	**How many cups of Areki on average per day you consume?**	**1- ≤ 2** **2- ≤ 4** **3- ≥ 5**	
5	**Do you consume tella and Areki together Per day?**	**1- Yes** **2- No**	
6	**Do you face some reactions after consumption of tella/ Areki?**	**1- Yes** **2- No**	**If Yes Q-7**
7	**What reactions usually you feel ?**	**1- Abdominal Discomfort** **2- Vomiting** **3- Other, Specify…….**	
8	**Have you ever diagnosed or feel to have sickness related to liver ?**	**1- Yes** **2- No**	

PART III

KAP QUESTIONER ON TELLA BREWERS AND AREKI DISTILLERS

Question Number	Question	Response	Instruction
Knowledge (K)			
1	From what cereals you produce commonly Tella and Areki?	Tella……. Areki……	
2	Do you harvest your own cereals used for traditional beverages?	1- Yes 2- No	From Market to Q3.
3	Where do you think farmers store cereals used for tella or Areki?	1- Ground Pits 2- Muddy Silos 3- Wooden Silos 4- In Bags 5- Don't' know 6- Others, Specify……..	
4	Do you exclude mouldy cereals for Tella /Areki to purchase from the market?	1- Yes 2- No	
5	What factors you think Cereals to be mouldy?	1- In the field 2- Storage Conditions 3- Transport 4- Do not know	
6	What special treatments you use when you found mouldy cereals?	…… ……	
Attitude (A)			
1	In your opinion what makes Tella and Areki better than industrial beverages?	…… ……	
2	Do you recommend using mouldy/ weevil infested cereals for beverages than to use for food consumption is relevant?	1 – Yes 2 - No	

Practice (P)			
1	**How do you store the cereals used for Tella and /Areki making?**	**…… ……**	
2	**Do you buy for every preparation freshly in the market or buy and store for long periods?**	**1- Purchase on demand 2- Purchase and Store**	.
3	**What methods you use to protect the cereals from moulds / Weevil Infestation in your storage?**	**……. …….**	
4	**Do you think it is good to mix mouldy Injera (Derkosh) / Bread good for the fermentation?**	**1- Yes 2- No**	
5	**If you get cheap price of mouldy cereals by chance do you buy for Areki and or Tella preparation because it is good for the taste?**	**1- Yes 2- No**	
6	**How much volume you produce Tella / Areki per month**	**Tella…….. Areki……..**	
7	**Which undesirable cereal materials you sort during preparation?**	**……. …….**	
8	**How much cups of tella / Areki you consume per day?**	**1- ≤ 2 2- ≤ 4 3- ≥ 5**	
9	**Have you ever diagnosed or feel to liver sickness?**	**1 – Yes 2 - No**	
10	**What kind of malt material you use for fermentation**	**1- Wheat 2- Barley**	
11	**Do you face sometimes gushing during Areki Distillation?**	**1- Yes 2- No**	
12	**What do you do if you face gushing during distillation**	**…….. ……..**	
13	**Do you clean often the condenser before every distillation?**	**1- Yes 2- No**	

DECLARATION

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

Name: Workineh Birhanu

Date: _____

Signature: _____

Advisors:

Name: Ashagre Zewdu (PHD)

Date: _____

Signature: _____

Name : Emana Getu (PHD)

Date: _____