OCCURRENCE OF AFLATOXIN CONTAMINATION IN *CYPERUS ESCULENTUS* L. (TIGER NUT) SOLD AND CONSUMED RAW IN KADUNA NIGERIA

BY

AMINU HAMZA AMINU
(SPS/12/MMB/00008)

Being a dissertation submitted to the Department of Microbiology, Faculty of Science Bayero University, Kano, Nigeria in partial fulfillment of the requirements for the award of the degree of Master of Science (M. Sc.) Microbiology (Industrial)

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DECLARATION

I AMINU HAMZA AMINU (SPS/12/MMB/00008) hereby declare that this work **OCCURRENCE OF AFLATOXIN IN *CYPERUS ESCULENTUS* (TIGER NUT) SOLD AND CONSUMED RAW IN KADUNA** is the product of my own research efforts undertaken under the supervision of Dr. Shamsuddeen Umar in Department of Microbiology, Faculty of Science, Bayero University, Kano, Nigeria and has not been presented elsewhere for the award of degree or certificate.

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AMINU HAMZA AMINU Date
SPS/12/MMB/00008

CERTFICATION

This is to certify that this work titled **'OCCURRENCE OF AFLATOXIN IN *CYPERUS ESCULENTUS* (TIGER NUT) SOLD AND CONSUMED RAW IN KADUNA'** was carried out by Aminu Hamza Aminu (SPS/12/MMB/00008) and is adequate in both scope and quality for the partial fulfillment of the requirements for the award of a degree of Master of Science (M Sc.) in Industrial Microbiology Faculty of Science, Bayero University, Kano, Nigeria.

Dr. shamsuddeen Umar
(Supervisor)

Date

(External examiner)

Date

(Internal examiner)

Date

Prof. A.H. Kawo
(P.G. Coordinator)

Date

Dr. A.M. Magashi
(H.O.D/chief Examiner)

Date

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DEDICATION

This work is dedicated to the memories of my late father Mallam Hamza Aminu may Allah grant him jannatil Firdausi amen.

ABSTRACT

Twenty (20) samples of tiger nut collected from different food stuff markets in Kaduna were subjected to proximate analysis, mycological analysis to determine the fungal load and to isolate the aflatoxin producing fungi as well as to carry out aflatoxin analysis using, thin layer chromatography (TLC), enzyme linked immunosorbent assay (ELISA) and fourier transform infrared spectroscopy (FTIR). The results of the mycological analysis showed that the samples had an average fungal load of 1.80 X 10^3 $cfug^{-1}$. The aflatoxin producing fungi isolated were *Aspergillus flavus*, *A. parasiticus* and *A. nomius.* The TLC results showed that 10% of the samples analysed fluorescence blue while the ELISA result showed that 35% of the samples contain aflatoxin with concentration varying from 0.2µg/kg to 23.0µg/kg. This FTIR spectral confirms the presence of conjugated aromatic double bond, C=O, C-O, and C-H, found in the structure of aflatoxin using FTIR. This shows that even though some tigernut samples were found to be contaminated with the aflatoxin, the presence of some highly contaminated samples therefore, suggested that caution should be exercised when consuming the tigernut for any mouldy sign.

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CHAPTER ONE

1.0 INTRODUCTION

Tiger nut or tigernut (*Cyperus esculentus* L.) is an edible perennial grass-like plant native to the Old World, and is a lesser-known vegetable that produces sweet nut-like tubers known as "earth almonds" (Coşkuner *et al.*, 2002). Tiger nut is also known by various other names as chufa (in Spanish), earth nut, yellow nut sedge, groundnut, rush nut, and edible galingale (Oderinde and Tairu 1988). Tiger nut (*Cyperus esculentus* L.) is an underutilized crop which belongs to the division – Magnoliophyta, class – liliopsida, order – cyperales and family – cyperaceae and was found to be a cosmopolitan perennial crop of the same genus as the papyrus plant. Tiger nut has been cultivated since early times (chiefly in south Erope and West Africa) for its small tuberous rhizomes which are eaten raw or roasted, used as hog feed or pressed for its juice to make beverage. Non-drying oil is also obtained from the rhizome. The egyptians used it as a source for food, medicine as well as perfumes. Tiger nut (*Cyperus esculentus* L.) grows freely and is consumed widely in Nigeria and in various parts of West and East Africa as well as Europe (Ejoh *et al.,* 2006). Tiger nut is not a real nut; despite its name. However, its chemical composition shares characteristics with tubers and with nuts. It has long been xiiiecognized as one of the best nutritional crops used to augment diets, since a substantial intake decreased reported cases of various health related conditions such as cardiovascular disease, diabetes, cancer, and obesity, and also ideal for children, older persons and sportsmen (Martinez 2003), as well as an excellent source of iron and calcium for body growth and development (Oladele and Aina 2007). It was found to be rich in myristic acid, oleic acid, linoleic acid (Eteshola and Oraedu, 1996). It is also reported that a quantity (33.33%) of tiger nuts was included in the diet of cockerel

starters (Bamgbose *et al.,* 2003). Again, tiger nuts with its inherent nutritional and therapeutic advantage, could serve as alternative to cassava in baking industry (Ade-Omowaye *et al.,* 2008). How ever since tiger nut is actually a tuber which is widely consumed raw and uncooked, it is believed to harbor many soil micro orgarnisms and their products such as toxins which when consumed by human or other animals pose a potential health concern.

A toxin can be defined as a substance that is synthesized by plants, animal or micro-organisms which is harmful to another organism. The naturall occurring toxins are grouped into five depending on their source.

Bacterial toxins: toxins produced by bacteria. Many bacterial toxins are proteins, which are not heat-stable. Some well-known bacterial toxins are botulin, *Staphylococcus aureus* enterotoxin and *Bacillus cereus* enterotoxin.

Phycotoxins: toxins produced by algae that, through food chains, end up in fishery products, such as shellfish. The significant phycotoxins for human health are diarrhoeic shellfish poisoning toxins and the amnesic shellfish poisoning toxins. Plant toxins (also named phytotoxins): toxins that are produced by edible plant species. Some play a role in the defence mechanism of plants against attacks of insects and fungi. Examples are the potato glycoalkaloids and toxins occurring in herbs, such as pyrrolizidine alkaloids, and anisatin in certain varieties of staranise.

Zootoxins: toxins produced by animals, e.g. snakes, scorpions and certain frogs. Zootoxins are generally of lesser significance to human health by oral exposure, although exceptions occur, such as bufotoxin, a toxin excreted by *Bufo marinus*. Licking the head

of this toad (a dangerous practice of drug addicts in the Netherlands) leads to hallucinations.

Mycotoxins: The toxins produced by fungi. Most have relatively small molecular weights, and mostly heat-stable compounds. The significant mycotoxins in terms of their toxicity and occurrence are aflatoxins, ochratoxins, trichothecenes, patulin, fumonisins and zearalenone. The term mycotoxin is a combination of a Greek word “mykes” means fungus and a Latin word “toxicum” meaning by poison. Mycotoxins are toxic secondary metabolites naturally produced by molds (fungi) that may contaminate agricultural commodities and cause health hazards to animals and human beings when environmental conditions are favorable (Stroka *et al.* 1999). The adverse economic effects attributed to mycotoxin contamination and losses are widely felt in all sectors of food production and particularly in agricultural commodities. Because molds are present in soil and plant debris, and are spread by Wind currents, insects, and rain, they are frequently found in/on foods especially tubers that are exposed to soil and wind together with their associated mycotoxins (Zheng *et al.,*2006). A fungus proliferates after getting favorable conditions and secretes mycotoxin but actual reason of mycotoxin production is yet unknown (Stroka *et al.,* 1999). Severity of toxins greatly varies and depends on the organism infected and its susceptibility, metabolism, and defense mechanisms (*Krska et al.*2005). Soil is a natural factor that exerts a powerful influence on the incidence of fungi. Crops grown in different soil types may have significantly different levels of mycotoxin contamination. For example, peanuts grown in light sandy soils support rapid growth of the fungi, particularly under dry conditions, while heavier soils result in less contamination of peanuts due to their high water holding capacity which helps the plant

to prevent drought stress. The hazardous health effects found in animals and humans includes identifiable diseases and some health problems, weakened immune systems without specificity to a toxin and as allergens. Since up to date we do not know how to detoxify chemically or physically crops and foods that are contaminated by mycotoxins in ways that retain their edibility. Our safety, therefore, relies on our ability to detect, quantify and avoid them. The public health concerns resulting from the finding of mycotoxins and the observation of both acute and chronic effects in animals has prompted the research effort focusing on analytical methods development. Analysis for mycotoxins is essential to minimize the consumption of contaminated food and feeds. However, method development and evaluation for mycotoxins is not a simple task. Determining the concentrations of toxins in grains at the μg/kg or parts-per billion levels required for the most important mycotoxins is difficult. The approach generally followed consists of obtaining a relatively large primary sample representing a lot, reducing it in bulk and particle size to a manageable quantity, and finally performing the analysis on a small representative portion (Pohland *et al.,* 200l). Given the vast number of methods that have been developed for the determination of mycotoxins in a variety of foods and feeds, a great deal of judgment is required for the selection of the optimum protocol of analysis. Besides performance criteria such as precision and trueness (contributing jointly to the accuracy), analytical procedures are characterized by three very practical criteria: (a) the speed with which the analysis can be performed, (b) the level of technical skills required to perform the assay, and (c) whether the assay provides a qualitative or quantitative result (CAST 2003). Clearly, the most desirable methods incorporate all three: they are rapid, easy to use, and quantitative. In reality, most methods are a

compromise and it is left to the users to determine the relative importance of each criterion for their application. This decision is the basis for selection of an analytical method that, in turn, will determine the technical expertise required to run the assays and the overall cost. Regardless of the method chosen, mycotoxin analysis usually involves extraction, cleanup, and detection.

Aflatoxins are highly toxic, mutagenic, teratogenic, and carcinogenic compounds that are produced as secondary metabolites by fungi belonging to several *Aspergillus* species, (O'Riordan and Wilkinson, 2008) as well as *A. nominus*. *A. flavus*, however, is the most common producer (Bradburn *et al*., 1993). The term secondary metabolites refers to the compounds produced by organisms which are dispensable while primary metabolites are vital for their normal functioning. Another distinguishing feature of secondary metabolites is that their production is limited to a group of species or genera and is rarely conserved over a wide taxonomical range, while primary metabolism is conserved among phyla and across kingdoms (Karlovsky 2008). These fungi occur principally in soil and decaying vegetation. Aflatoxins often occur in crops in the field prior to harvest. Postharvest contamination can occur if crop drying is delayed and during storage of the crop if water is allowed to exceed critical values for the mold growth. Insect or rodent infestations facilitate mold invasion of some stored commodities.

In the 1960 more than 100,000 young turkeys on poultry farms in England died in the course of a few months from an apparently new disease that was termed "Turkey X disease". It was soon found that the difficulty was not limited to turkeys. Ducklings and young pheasants were also affected and heavy mortality was experienced (Romognoli *et*

al., 2007). Speculations made during 1960 regarding the nature of the toxin suggested that it might be of fungal origin. In fact, the toxin-producing fungus was identified as Aspergillus flavus and the toxin was given the name Aflatoxin by virtue of its origin (A.flavus→ Afla).

The four major aflatoxins are B1, B2, G1 and G2. Aflatoxins M1 and M2 are hydroxylated metabolites of aflatoxins B1 and B2, respectively in animals. Exposure to aflatoxin is widespread in West Africa, probably starting in the utero, and blood tests have shown that very high percentage of West Africans are exposed to aflatoxins. In a study carried out in the Gambia, Guinea Conakry, Nigeria and Senegal, over 98% of subjects tested positive to aflatoxin markers (Wild, 1996). Aflatoxin is a very powerful hepatocarcinogen, and naturally occurring mixtures of aflatoxins has been classified as a class 1 human carcinogen (IARC, 1993). The IARC also concluded that there was inadequate evidence for the carcinogenicity of aflatoxin M1. Aflatoxin contaminated diet has been linked with the high incidence of liver cancer in Africa (Oettle, 1964). In a recent study in China, Li *et al.* (2001) found that the levels of aflatoxins B1, B2, and G1were significantly higher in corn from the high incidence area for human hepatocellular carcinoma, and the average daily intake of aflatoxin B1 from the high risk area was 184.1µg. Aflatoxin synergies other agents such as hepatitis B in the causation of liver cancer (Turner *et al*., 2000). Though, the etiology and pathogenesis of kwashiorkor still remain obscure, but much higher aflatoxins have been found in the blood, urine and livers of children with the disease than similar age-matched children (Hendrickse, 1983) and the presence of the toxin was established in the autopsy brain tissue of some Nigerian children (Oyelami *et al*., 1996). Nutritional deficiencies are quite prevalent in

populations consuming high quantities of cereals. Aflatoxin positive kwashiorkor children showed significantly greater severity of edema, increased number of infections, lower haemoglobin levels and longer duration of hospital stay than aflatoxinnegative kwashiorkor children (Adhikari *et al*., 1994; Ramjee, 1996). It seems that the protein deficiency reduces the capacity of the liver to detoxify aflatoxins. Thus, the conclusion is that aflatoxin may be a contributory factor in increasing the morbidity of children suffering from the disease (Ramjee, 1996). In a recent study in Nigeria Uriah et al. (2001) found that blood and semen aflatoxin levels ranged from 700 to 1393 ng/ml and 60 to 148 ng/ml, respectively in infertile men and were significantly higher than that in fertile men. Gong *et al*. (2002) demonstrated that children in Togo and Benin who ate foods contaminated with aflatoxins showed the kind of stunted growth and being underweight, which are symptoms normally associated with malnutrition. Aflatoxins have also been shown to be immunotoxic to both livestock and man. Turner *et al*. (2000) detected aflatoxin albumin adducts in 93% of sampled children (6-9 years) in Gambia and provided evidence that IgA in saliva may be reduced because of high dietary levels of aflatoxin exposure. The study confirmed that children in rural areas of Gambia are frequently exposed to high levels of aflatoxin. In the US, the FDA uses an action level of 20µg/kg as the maximum residue limit allowed in food for human consumption, except for milk (FAO, 1996). For overall sanitary precaution principle, the European Union has enacted in 1998 very severe aflatoxin tolerance standards of 2µg/kg aflatoxin B1 and 4µg/kg total aflatoxins for nuts and cereals for human consumption(CEC, 1998), and this has come into effect from January, 2001 (Dimanchie, 2001). Consumers in the developed world are now well aware of the carcinogenic effect of aflatoxins, and will thus shy away

from a product from any supplier that has aflatoxin beyond the acceptance level. Exports of agricultural products particularly groundnuts from developing countries have dropped considerably in recent years resulting in major economic losses to producing countries (Bhat and Vashanti, 1999). According to the World Bank estimate, the policy change by the EU will reduce by 64% imports of cereals dried fruits, and nuts from nine African countries: Chad, Egypt, Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabwe, and this will cost African countries about US $670 million in trade per year (Kellerhals, 2000). Though, the new EU rule has been criticized as excessively too rigorous, because the difference between the EU limits and the Codex limits would only save two lives for every one billion people (WHO, 2000).

1.3 Justification for the Research

It is a known fact that tiger nuts are important source of nourishment and a vital ingredient in healthy and balanced diets (Oladele and Aina, 2007; Eteshola and Oraedu, 1996) but harbor varied loads of microbial flora and can be a potential source of acute or chronic food borne illness when contaminated with pathogenic microorganisms or microbial product such as toxins (Negedu *et al.,* 2011). Since the tuber grows in the soil it may contain several toxigenic moulds and their mycotoxins for which reason this study will be carried out to ascertain the level of contamination.

1.4 Aim of the Research

The aim of this research work was to determine the fungal load and the level of aflatoxin contamination in tiger nut sold and consumed raw in Kaduna.

1.5 Objectives of the Research

i. To determine the proximate composition of tigernut

ii. To determine the fungal load on the tiger nut samples.

iii. To isolate aflatoxigenic *Aspergillus* species in the tigernut.

iv. To detect the presence of the aflatoxin using TLC.

v. To determine the level of aflatoxin contamination in tiger nut using ELISA.

vi. To confirm the presence of aflatoxin using FTIR.

CHAPTER TWO

2.0 LITERATURE REVIEW

It is a common knowledge that the human food supply basically consist of plant and animals which are naturally in constant interaction with microorganisms and or their product. This is why it is safe to assume that our food supply contain various forms of microorganism. In most cases microorganisms use our food supply as a source of nutrients for their growth which of course can result in deterioration of food as well as food borne disease and intoxification. When microorganisms involved in food are pathogenic, their association with our food supply is of critical from the public health point of view (Adams and Whitaker, 2004).

The kind of food and nutrient in food are all important in determining what microorganism is most likely to grow consideration must be given to (1) food for energy, (2) foods for growth, and (3) accessory food substances, or vitamin, which may be necessary for energy and growth.

2.1.1 Tiger nut

Tiger nut or tigernut (*Cyperus esculentus* L.) with it inherent nutritional values can effectively serve as source for food with all those benefit even though Tiger nuts are under-utilized due to lack of information on their nutritional potential. Tiger nuts which

are incorrectly called “nuts” or “nutlets,” thus the origin of their common name, are actually small tubers about the size of a peanut growing at the rhizome of the plant which shares the characteristics of both nuts and tubers (Tigernuts Traders 2009). Tiger nut is one of the earliest domesticated crops first discovered some 4000 years ago. It is an edible perennial grass-like plant native to the Old World, and is a lesser-known vegetable that produces sweet nut-like tubers known as “earth almonds” (Coşkuner *et. al.*, 2002). Tiger nut is also known by various other names as chufa (in Spanish), earth nut, yellow nut sedge, groundnut, rush nut, and edible galingale (Oderinde and Tairu 1988). Tiger nut (*Cyperus esculentus* L.) Is an underutilized crop which belongs to the division – Magnoliophyta, class – liliopsida, order – cyperales and family – cyperaceae and was found to be a cosmopolitan perennial crop of the same genus as the papyrus plant. In Nigeria, tiger nut is available in fresh, semi-dried and dried form in the markets where it is sold locally and consumed even uncooked. It is known as Aya in Hausa, Ofio in Yoruba, and akiausa in Igbo. Tiger nut has been cultivated since early times (chiefly in south Erope and West Africa) for its small tuberous rhizomes which are eaten raw or roasted, used as hog feed or pressed for its juice to make beverage. Non-drying oil is also obtained from the rhizome. The egyptians used it as a source for food, medicine as well as perfumes and in fact, was found in vases and was used to embalm bodies of the Egyptian Pharaohs (Watt and Breyer-Brandwijk 1962). Tiger nut (*Cyperus esculentus* L.) grows freely and is consumed widely in Nigeria and in various parts of West and East Africa as well as Europe (Ejoh *et al*., 2006). Tiger nut is not a real nut; despite its name. However, its chemical composition shares characteristics with tubers and with nuts. It has long been recognised as one of the best nutritional crops used to

augment diets, since a substantial intake decreased reported cases of various health related conditions such as cardiovascular disease, diabetes, cancer, and obesity, and also ideal for children, older persons and sportsmen (Martinez 2003). Tiger nuts are also regarded as a digestive tonic, having a heating and drying effect on the digestive system and alleviating flatulence and also promote urine production and menstruation (Wills, 1962). The tubers are said to be aphrodisiac, carminative, diuretic, stimulant and tonic, and in ayurvedic medicine are used in the treatment of flatulence, indigestion, colic, diarrhea, dysentery, debility and excessive thirst. (Pengium, 1984). The nuts are valued for their highly nutritious starch content, dietary fibre, digestible carbohydrate (mono, di and polysaccharides). The nut was reported to be rich in sucrose (17.4 to 20.0%) and fat (25.50%) which are resistant to peroxidation, and protein (7-8%) [9, 20, 21] as indicated in Table 2.1. The nut is also fairly rich in mineral content (iron, Sodium, Calcium, Potassium, phosphorous, Magnesium, Zinc and traces of Copper as well as vitamins A and C) (Oladele and Aina 2007). It was found to be rich in myristic acid, oleic acid, linoleic acid (Eteshola and Oraedu, 1996). A quantity (33.33%) of tiger nuts was included in the diet of cockerel starters (Bamgbose *et. al. 2003)*. Again, tiger nuts with its inherent nutritional and therapeutic advantage, could serve as alternative to cassava in baking industry (Ade-Omowaye *et. al.*).

2.1.2 The plant

Tiger nut is a tough erect fibrous-rooted perennial plant, 1 to 3 ft high, reproducing by seeds and by many deep, slender rhizomes, which form weak runners above the ground, and small tubers or nutlets at the tips of underground stems (Kelley 1990). This native perennial sedge is 0.5–2 inches tall and unbranched. The central stem is erect, 3-angled,

and mostly covered by the sheaths of the leaves. The leaves tend to congregate toward the base of the plant. The leaf blades are up to 1.5 inches long and 0.75 inches across; they are light green and glabrous, spreading outward from the stem. There is a conspicuous channel along the central vein of each leaf blade, especially the larger ones. The leaf sheaths are whitish green, closed, and hairless; sometimes they become pale red towards the base of the plant (James *et al*., 1991). The central stem terminates in an umbel or compound umbel of floral spikes; the size and shape of the umbel is rather variable (on larger plants, it is usually several inches across). Each umbel has 1-3 sessile spikes and 6-10 non-sessile spikes on straight branches of varying length. At the base of each umbel or compound umbel of spikelets, there are several leafy bracts of varying length; the largest bract is usually longer than the inflorescence. Each floral spike is about 2-3 inches long, consisting of 4 ranks of spikelets along its central stalk (or rachis). The central stalk is flattened and narrowly winged. The spikelets are perpendicular to this stalk and about 0.5 to 0.75 inches long. The spikelets are yellow to golden brown, narrowly linear, and flattened in shape; they consist of 10-30 florets and their scales. The overlapping scales are slightly spreading along the length of each spikelet; each scale is 2.0–3.0 mm. in length. Each floret has a white tripartite style and yellowish brown anthers; the tips of the styles are curly. The blooming period occurs from mid-summer to early fall.

Pollination of the tiger nut plant is by wind (*Stroka et al., 1999*). The florets are replaced by small achenes that are 1.0–1.5 mm. long, oblongoid or oblongoid-obovoid, and flattened. The shallow root system is fibrous, rhizomatous, and tuberous. The white rhizomes have a slightly segmented appearance from the brown margins of their outer

membranes; the rhizomes are connected to small globoid tubers up to 0.5 inch across. Young tubers are white, while older tubers are covered by a yellow outer membrane;

2.1.3 Economics and Nutritional benefits of tiger nut

According to Mason 2009 tiger nuts have long been recognized for their health benefits as they have a high content of soluble glucose and oleic acid, along with high energy content (starch, fats, sugars and proteins), they are rich in minerals such as phosphorous and potassium, calcium, magnesium and iron necessary for bones, tissue repair, muscles, the blood stream and for body growth and development and rich in vitamins E and C. Sugar-free tiger nut milk is suitable for diabetic people and also helps in weight control (Martinez 2003), due to its content of carbohydrates with a base of sucrose and starch (without glucose), and its high content of Arginine, which liberates the hormone that produces insulin (Tiger nut Traders, 2005, Chevallier, 1996). It is recommended for those who suffer from indigestion, flatulence and diarrhoea because it provides digestive enzymes like the catalase, lipase and amylase. The high content of oleic acid has positive effect on cholesterol, thereby preventing heart attacks, thrombosis and activates blood content of soluble glucose. Tiger nut reduces the risk of colon cancer. It prevents constipation. Tiger nut contains a good quantity of vitamin B1, which assists in balancing the central nervous system and helps to encourage the body to adapt to stress (Tiger nut Traders, 2005, David, 2005). The milk supplies the body with enough quantity of Vitamin E, essential for fertility in both men and women. Vitamin E also delays cell aging, improves elasticity of skin and helps to clear the appearance of wrinkles, acne and other skin alterations.

In China, tiger nut milk is used as a liver tonic, heart stimulant, drank to heal serious stomach pain, to promote normal menstruation, to heal mouth and gum ulcers, used in Florida, Georgia, and Alabama. Tubers of tiger nut have also been identified as valuable food for waterfowl and cranes. Ducks dive for them when wetland fields are flooded. It is also used in seed mixes for wetland restoration, mitigation, and erosion control. The caramel from malted tubers of *Cyperus esculentus* may be used to add body, flavor, or color to certain baked products, non-alcoholic malt beverages and dark beers, and in the production of condiments. The starches obtained from tiger nut and rice showed similar properties; the solutions of the starch exhibited a good paste stability, clarity, and adhesive strength. The starch can be used in many starch-based foods as well as in the cosmetic industry, and for laundry, glazing and stiffening. The waste residue after oil extraction could be further modified to produce syrups, flours, or livestock feeds (Umerie and Enebeli, 2005, The spectators 2005).

How ever since tiger nut is actually a tuber which is widely consumed raw and uncooked, it is believed to harbor many soil micro organisms and their products such as toxins which when consumed by human or other animals pose a potential health risk. It is also reported that the tiger nut contain the element lead (Pb) which inhibits haemoglobin synthesis.

2.2 TOXINS

A toxin (Latin word for poison) can be defined as a substance that is synthesized by plants, animals or micro-organisms which is harmful to another organism. The naturall occurring toxins are grouped into five depending on their source.

Bacterial toxins: toxins produced by bacteria. Many bacterial toxins are proteins, which are not heat-stable. Some well-known bacterial toxins are botulin, *Staphylococcus aureus* enterotoxin and *Bacillus cereus* enterotoxin.

Phycotoxins: toxins produced by algae that, through feed chains, end up in fishery products, such as shellfish. The significant phycotoxins for human health are diarrhoeic shellfish poisoning toxins and the amnesic shellfish poisoning toxins.

Plant toxins (also named phytotoxins): toxins that are produced by edible plant species. Some play a role in the defence mechanism of plants against attacks of insects and fungi.

Table 2.1: Proximate composition of Tiger nut tuber

Parameter	**Dry matter (%)**
Moisture (%wet wt)	5.77
Crude protein	7.00
Ether extract	25.70
Total ash	1.86
Crude fibre	5.50
NFE	60.00

Total carbohydrate	65.50
Caloric value (kcal)	524.6

Source; tigernut traders 2013

Examples are the potato glycoalkaloids and toxins occurring in herbs, such as pyrrolizidine alkaloids, and anisatin in certain varieties of staranise.

Zootoxins: toxins produced by animals, e.g. snakes, scorpions and certain frogs. Zootoxins are generally of lesser significance to human health by oral exposure, although exceptions occur, such as bufotoxin, a toxin excreted by *Bufo marinus*. Licking the head of this toad (a dangerous practice of drug addicts in the Netherlands) leads to hallucinations.

2.3 MYCOTOXINS

These are toxins produced by fungi. They have relatively small molecular weight and mostly heat-stable compounds. The significant mycotoxins in terms of their toxicity and

occurrence are aflatoxins, ochratoxins, trichothecenes, patulin, fumonisins and zearalenone. The concentration and target of the metabolite are both important. Fungal products such as penicillin that are mainly toxic to bacteria are usually called antibiotics. commodities such as groundnuts, maize, sorghum, rice, yam, cassava, tiger nut, soyabeans, cotton seeds, fruits, vegetables spices can be contaminated with toxins of fungal origin such as aflatoxins, ochratoxins, fumonisins, patulin, sterigmatocystin, deoxynivalenol, zearalenone and other mycotoxins which pose serious economic and health risks (Negedu *et al* 2011). Mycotoxins even in low concentration are toxic to vertebrates and other animal groups. Fungal metabolites of low-molecular-weight such as ethanol that are toxic only in high concentrations are not considered mycotoxins (Bennett, 1987). Plant pathologists describe fungal products that are toxic to plants are called phytotoxins.

Mycotoxins are known as relatively small low molecular (MW ~700), natural toxic chemical products produced as secondary metabolites by a certain fungi that voluntarily colonize crops in the field as pre-harvest or post harvest (Zain, 2010). The definition of secondary metabolites is used to differentiate them from those compounds essential for all living organisms named primary metabolites, which are essential for the growth of the organism. Another distinguishing feature of secondary metabolites is that their production is limited to a group of species or genera and is rarely conserved over a wide taxonomical range, while primary metabolism is conserved among phyla and across kingdoms (Karlovsky, 2008). The term mycotoxin was coined for the first time in 1962 following an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died. This disease was found to be connected to a

peanut (groundnut) meal contaminated with secondary metabolites from the fungus *Aspergillus flavus*. Mycotoxin is a combination of two words *mykes* (Greek word for fungus) and Latin word *toxicum* meaning poison. Therefore, Toxic compounds produced by fungi and belongs to fungal origin are known as mycotoxins. Currently 400 different mycotoxins have been recognized (Zinedine and Mañes, 2009) that are formed by more than 200 different fungal species. Three genera are responsible for the majority of the mycotoxins with which food microbiology is concerned. These are the *Aspergillus sp*., *Penicillium sp*., and *Fusarium sp*. Alternaria sp., were also reported. Of the numerous mycotoxins elaborated by these fungi, regulatory agencies are actively concerned with the aflatoxins, fumonisins, trichothecenes, ochratoxins, patulin, and zearalenone. Because molds are present in soil and plant debris, and are spread by wind currents, insects, and rain, they are frequently found in/on foods together with their associated mycotoxins (Pittet, 2000). A fungus proliferates after getting favorable conditions and secretes mycotoxin but actual reason of mycotoxin production is yet unknown (Stroka *et al.,* 1999). Severity of toxins greatly varies and depends on the organism infected and its susceptibility, metabolism and defense mechanisms (Krska *et al.,* 2005). However, there are only 20 mycotoxins which are found at concentrations likely to pose health hazard for animals and peoples and are regulated in different food and food products which are also termed as "primary Exposure" (Anklam *et al.*, 2002). Exposure to mycotoxins in animals occurs through the consumption of mouldy feedstuff while humans, the exposure can be either direct, that is due to the consumption of mouldy plant products or indirect, through the consumption of contaminated animal products (meat, milk and eggs), containing considerable amounts of residual mycotoxins ingested by animals. Fungal contaminations

of crops and foods, however, are widespread in less-developed countries (Jelinek *et al*., 1989). The adverse economic effects attributed to mycotoxin contamination and losses are widely felt in all sectors of food production and particularly in agricultural commodities. Therefore, the concern for mycotoxin contamination in food commodities grown and produced in sub-Saharan Africa has grown considerably over the years with improved strategies for monitoring Soil is a natural factor that exerts a powerful influence on the incidence of fungi. Crops grown in different soil types may have significantly different levels of mycotoxin contamination. For example, peanuts grown in light sandy soils support rapid growth of the fungi, particularly under dry conditions, while heavier soils result in less contamination of peanuts due to their high water holding capacity which helps the plant to prevent drought stress. The potential for a product to contain a naturally incurred mycotoxin depends on whether the product contains and supports the growth of a mycotoxin producing mold species, and whether the optimum temperature and humidity are present. According to the Food and Agriculture Organization (FAO) an estimation of 25% of the world's agricultural commodities are contaminated with mycotoxins, a fact leading to significant economic losses (Aziz and Moussa, 2002).

The hazardous health effects found in animals and humans includes identifiable diseases and some health problems, weakened immune systems without specificity to a toxin and as allergens. Mycotoxins, when present in food and feed in sufficient levels, can affect both human and animal health. But depending on a number of factors like the intake levels, duration of exposure, toxin species, mechanisms of action, metabolism, and defence mechanisms, and the toxic effect will vary (Hussein and Brasel, 2001). The toxic

syndromes resulting from the intake of mycotoxins are known as mycotoxicoses. Mycotoxicoses have been responsible for the major epidemics in men and animals in recent historic times. The most important mycotoxicosis has been ergotism, alsoknown as St.Anthony's fire. It is caused by the ingestion of grains contaminated by sclerotia of *Claviceps purpurea* and it has been known since, at least, 1750. After periodic outbreaks, the disease became epidemic in central Europe during the middle ages.

Mycotoxins remain challenging to classify due to their diverse chemical structures, biosynthetic origins and their production by a wide number of fungal species (Groopman *et al*., 1994). A first approach can be to classify them according to their differences in their fungal origin, chemical structure and biological activity. Also the classification can be done according to how frequently they occur and in what amounts. This is a more complicated task because mycotoxin contamination of food and feed depends on environmental and climatic conditions, harvesting techniques, storage conditions and some others factors. Typically, the classification schemes reflect the scientific background of the person doing the categorising. For clinicians the classification is done depending on the organ they affect: hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, etc. For cell biologists the classification is done according to generic groups such as teratogens, mutagens, carcinogens and allergens. Organic chemists tend to classify mycotoxins according to their chemical structures, e.g., lactones, coumarines, etc.; biochemists according to their biosynthetic origins, e.g., polyketides, amino acid-derived, etc.; physicians by the illnesses they cause e.g. St. Anthony's fire, stachybotrytoxicosis, etc.; and mycologists by the fungi that produce them e.g. *Aspergillus* toxins, *Penicillium* toxins, etc (Scott, 1991).

Since up To date, we do not know how to detoxify chemically or physically crops and foods that are contaminated by mycotoxins effectively in a way that retain their edibility. Our safety, therefore, relies on our ability to detect, quantify and avoid them.

Because most of the people and animals under threat by mycotoxins live in third world countries, the development of fast, simple to perform and interpret, and inexpensive, yet sensitive methodologies for the detection of mycotoxins are of paramount importance to safety of the people. People and livestock in the developed, affluent countries enjoy aflatoxin-safe food and animal feed thanks to strictly enforced regulatory measures. The latter rely on a plethora of sensitive and accurate methods for the detection and quantization of aflatoxins and other mycotoxins. These assays require the use of sophisticated, expensive scientific equipment, and highly trained professional personnel to operate it.

2.3.1 Ergot and ergotism

Ergot is the alkaloid-containing product of a fungus, *Claviceps purpurea*, which grow on cereals, especially rye (Barug *et al.,* 2004). Ingestion of the sclerotia is poisonous and causes a disease called ergotism. Ergotism has two main manifestations: gangrene(referred to as chronic ergotism) and convulsions (acute ergotism). Also known as Holy Fire or St Anthony's Fire and is characterised by intense burning pain and gangrene of feet, hands, and whole limbs, due to the vasoconstrictive properties of ergot. In severe cases, affected tissues became dry and black, and mummified limbs dropped off without loss of blood. Spontaneous abortion frequently Occurred. Convulsive ergotism was often accompanied by manic episode and hallucinations.

2.3.2 Patulin

Patulin (PAT) is a toxic secondary metabolite produced by approximately 60 species of moulds belonging to 30 fungal genera like *Penicillium*, *Aspergillus* and *Byssochlamys*, which grow on fruit, including apples, pears, grapes. It has also been reported to grow in vegetables, cereal grains and silage. *P. expansum* seems to be the mould responsible for PAT in apple juice. The conditions in which *P. expansum* develops and produce patulin in apples have been the aim of several studies; as example (Morales *et al*., 2007) investigated this topic with a postharvest fungicide treatment, storage at low temperatures and controlled atmosphere storage. It was concluded that none of the factor tested avoided the fruit spoilage. Additionally, when ripe apples where stored at warmer temperature, a rapid increase (40%) of the lesion diameter was observed. Nevertheless, no patulin accumulation was reported after cold storage. This conclusion confirms the previously finding of Groopman *et al.,* (1994). Apparently the removal of decayed tissue or the washing before processing reduces levels of PAT in final products. However, some studies demonstrated that PAT is also found in healthy tissues. Therefore, it appears that the prevention of PAT contamination rather than trying to remove it is the best way to avoid further contamination. Alcoholic fermentation of fruit juices destroys PAT. Therefore, fermented products such as cider or vinegars will not contain PAT as long as apple juice was not used as an additive post-fermentation. PAT was originally isolated because of its wide-spectrum antibiotic properties and was tested in humans to evaluate its ability to treat common cold. But it was found to be carcinogenic, teratogenic and mutagenic. Therefore, it was recommended that the level of PAT in the food should be reduced to the lowest level technically achievable. By far the most

important source of PAT for humans are apples and apple juices and concern is expressed regarding the effect of PAT in the diet of young children.

2.3.3 Zearalenone

Zearalenone (ZON) is a mycotoxin described chemically as a phenolic resorcylic acid lactone in This secondary fungal metabolite is produced by several species of *Fusarium* fungi, mainly *F. graminearum* and *F. culmorum*, which are species known to invade maize, barley, oats, wheat, rice and sorghum. Co-occurrence with other *Fusarium* toxins like deoxynivalenol, nivalenol and fumonisin is often observed and depends on several factors like genotype, climatic condition, harvest season and storage condition. Nevertheless, available data indicate that maize has the highest risk of contamination while wheat, oats and soybean have been found to be contaminated occasionally (Cardwell 1995). ZON has important effects on the reproductive system because it binds to oestrogen receptors. Animal studies show that after oral exposure, it is metabolised mainly in liver to α- and ß-zearalenol, which are afterwards conjugated with glucuronic acid. Swine has been found to be the most sensitive domestic animal to ZON, showing symptoms of hyperestrogenism. Calves have been reported to show earlier sexual maturity, while cows are reported to suffer from vaginitis, prolonged oestrus and/or infertility when intoxicated with ZON.

2.3.4 Mycotoxin Regulations

Following the discovery of the aflatoxins in the 1960s, regulations have been established in many countries to protect consumers from the harmful effects of mycotoxins that may contaminate foodstuffs, as well as to ensure fair practices in food trade. This is because safety and security have generally remained basic human needs. Despite the difficulties, mycotoxin regulations have been established in many countries during the past decades,

and newer regulations are still being issued. National regulations have been established for a number of mycotoxins such as the naturally occurring aflatoxins and aflatoxin M1; the trichothecenes deoxynivalenol, diacetoxyscirpenol, T-2 toxin and HT-2 toxin, the fumonisins B1, B2 and B3; agaric acid; the ergot alkaloids; ochratoxin A; patulin, phomopsins; sterigmatocystin and zearalenone.

International inquiries on existing legislation on mycotoxins in foodstuffs and animal feedstuffs have been carried out several times, and details about tolerance levels, legal bases, responsible authorities, and official protocols for sampling and analysis have been published.

The Food and Agriculture Organization of the United Nations (FAO) has played a major role in providing information on worldwide regulations for mycotoxins in foods and feeds.

In 2002, an international inquiry on mycotoxins was initiated by the National Institute for Public Health and the Environment. As part of this inquiry, the Agricultural Services in Dutch Embassies around the world were requested to gather up-to-date information on the situation regarding mycotoxin regulations from local authorities (AOCS. 2003) in as many countries as possible.

2.3.5 Factors affecting the constitution of mycotoxin regulations in foods and feeds

Several factors, both of a scientific and socio-economic nature, may influence the establishment of mycotoxin limits and regulations. These include:

a. Availability of toxicological data;
b. Availability of data on the occurrence of mycotoxins in various commodities;

c. Knowledge of the distribution of mycotoxin concentrations within a lot;
d. Availability of analytical methods;
e. Legislation in countries with which trade contacts exist; and
f. Need for sufficient food supply.

The first two factors provide the necessary information for hazard assessment and exposure assessment respectively, the main ingredients for risk assessment. Risk assessment is the scientific evaluation of the probability of occurrence of known or potential adverse health effects resulting from human exposure to food-borne hazards; it is the primary scientific basis for the establishment of regulations (AOCS, 2003).

Regulations are primarily made on the basis of known toxic effects. For the mycotoxins currently considered most significant – aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and some trichothecenes including deoxynivalenol – the Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of FAO and WHO, has recently evaluated their hazards. JECFA provides a mechanism for assessing the toxicity of food additives, veterinary drug residues and contaminants. Safety evaluation of contaminants incorporates various steps in a formal health risk assessment approach.

The qualitative indication that a contaminant can cause adverse effects on health (hazard identification) is usually included in the information presented to JECFA for evaluation.

Similarly, qualitative and quantitative evaluation of the nature of the adverse effects (hazard characterization) is embodied in the data sets that are presented. The evaluation of toxicological data carried out by JECFA normally results in the estimation of a Provisional Tolerable Weekly Intake (PTWI) or a Provisional Tolerable Daily Intake

(PTDI). On a worldwide basis, at least 99 countries had mycotoxin regulations (Adams and Whitaker, 2004) for food and/or feed in 2003 an increase of approximately 30 percent compared to 1995. The total population in these countries represents approximately 87 percent of the world's inhabitants. This percentage had decreased to 13 percent in 2003, due to a slight increase in coverage in Latin America and Europe, and more significant increases in Africa and Asia/Oceania.

2.4 Detection and Determination of Mycotoxins in the Analytical Laboratory

Numerous analytical methods have been developed for the determination of mycotoxins which in clued thin-layer chromatography (TLC),high-performance liquid chromatography (HPLC), lateral flow immunochromatography, fluorescent polarization immunoassay, molecular imprinting, enzyme linked immuno sorbent assay (ELISA), arrays of biosensors, electronic noses.

2.4.1 Thin-layer chromatography

This technique is simple and fast, and although it might be used as a reference method it is often used as a mycotoxin screening assay. It is a powerful tool to determinate the presence of one or more mycotoxins in a sample involving simple equipment but on the other hand it requires some skills in its operation and interpretation. It has been widely used for the determination of mycotoxins but nowadays it has been replaced by other chromatographic methods because of their superior analytical performance.

The principle of TLC is based on a stationary phase attached to a glass or plastic plate and mixtures of solvents as mobile phase. The sample dissolved in a volatile solvent is deposited as a spot on the stationary phase. A standard will run simultaneously with the samples. The plate is immersed in a solvent reservoir and the solvent moves up the plate

by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. Different components in the sample move up at different rates due to their different chromatographic behaviour between the mobile liquid phase and the stationary phase. Typical stationary phases for normal phase TLC include silica gel, aluminium oxide and cellulose. The components of the mobile phase are usually chlorinated solvents such as chloroform or dichloromethane also other relatively non-polar solvents can be used in order to produce the selectivity necessary for the separation. These solvents are combined with polar solvents including acetone, alcohols, water and modifiers. The majority of TLC analyses of mycotoxins are performed on normal-phase silica gel plates, with the use of two different mobile phases and the development of plates in two different directions giving the greatest selectivity.

In case that the compounds of interest are not naturally fluorescent or don't absorb UV light, the application of a detection reagent by spraying or dipping is necessary in order to produce colour or fluorescence. A further development is the use of high performance thin-layer chromatography that involves a reduction of layer thickness down to 100µm and particle size down to 5µm. This leads to an improved separation within a shorter time but it has a very small sample capacity. Automation is possible with the use of commercially available spotters and plate scanner. TLC methods for a large number of mycotoxins (e.g. aflatoxins, PAT and ZON) are available via literature (Betina, 1985). Detection and identification procedures have been specifically developed for each single mycotoxin making use of molecular properties or reactions with spray reagents that are essential to be able to quantify the mycotoxin. On the other hand they normally use very

toxic reagents and therefore they are outdated. An important drawback of TLC methods is that the limits of detection they offer are sometimes out of the actual legislative limits.

2.4.2 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) of extracts of commodities, foods and feeds is the most prevalent and sensitive current method for the identification and quantization of mycotoxins. Uncanny sensitivity and precision in the detection of ppt (parts per trillion) concentrations of the fluorescent mycotoxins AFB2, AFB2, AFG2, AFG2 and sterigmatocystin, citrinin and ochratoxin A can be achieved by careful preparation and concentration of extracts of grain/fruit samples, followed by HPLC in an apparatus equipped with a fluorescence detector (Nguyen *et al.,* 2007). HPLC separates the mixture of compounds present in an extract of a sample by relative retention of the compounds to a stationary phase and mixtures of polar solvents as mobile phase. Subsequently, the compounds pass through a detector normally ultraviolet or fluorescence.

2.4.3 Chromatographic methods with mass spectrometric detection

Although LC/MS methods are fairly recent developments in mycotoxin determination they have become the state-of-art in the mycotoxin identification and quantification despite high costs and the need for experienced staff.

In addition, limitations of conventional HPLC methods, such as the need to derivatise samples before analyses have led to a more common use of LC/MS methods. A mass spectrometer is an instrument that separates charged gas phase species according to their molecular mass and charge. An appropriate inlet, ionisation mode and ion analyser must be selected. Examples of inlet methods are gas chromatography, capillary

electrophoresis, liquid chromatography, etc. Ionisation is provided by several techniques and the two most widespread for HPLC are atmospheric pressure chemical ionisation and electrospray ionisation. In the case of GC/MS, electron impact ionisation can be considered as 'hard ionisation' process and can produce many fragmented ions and possibly a poor abundance of the molecular ion. At the opposite, a 'soft ionisation' process as electrospray produces few fragmented ions with abundant molecular ion species. For atmospheric pressure chemical ionisation, a nebulizer sprays the eluate and the resulting droplets are pre-dried in a heated capillary before being ionised through a corona discharge needle. The corona needle inserts electrons into the haze causing an ionisation of the mobile phase. A secondary reaction follows with the ionisation of the analyte molecules. For the electrospray ionisation charged droplets are produced by forcing the analyte solution through a charged orifice. A potential is used to disperse the emerging solution into a very fine spray of charged droplets. Thereafter the solvent evaporates and the droplets shrink with an increase of the charge concentration at the droplet surface. Finally the droplet surface tension reaches a point that explodes and ends in smaller and lower charged droplets. This process is repeated until individually charged analyte ions are formed.

Several types of ion analysers are available such as ion trap, quadrupole or time-of-flight mass spectrometers.

Ion trap instruments are generally better suited for identification than triple quadrupole instruments because they allow successive series of trapping and fragmentation, but triple quadrupole instruments provide better selectivity in quatitative analysis. Hybrid

instrumentsalso exist and they provide the best of both setups with a linear ion trap in a triple quad instrument.

A significant advantage over conventional techniques is the development of multimycotoxin determination methods. Within a single run several mycotoxins belonging to different chemical families are detected.

2.4.4 HPLC-Mass Spectrometry (LC-MS) and HPLC-Tandem Mass Spectrometry (LC-MS-MS)

These are the ultimate methods for the identification/confirmation of the identity of mycotoxins, including those which do not fluoresce or do not absorb visible UV light. Such methods allow the identification and sometimes the quantization of many mycotoxins in a single sample (Lattanzio *et al.,* 2007). The techniques require much care and precision. Despite the fact that LC-MS and LC-MS-MS are the most sensitive methodologies for the detection of mycotoxins, it is difficult to achieve complete ionization in every measurement because the degree of ionization is finicky and is complicated by trivial details. Thus, whereas the method of choice for quantifying trichothecenes and fumonisins is LC-MS, mycotoxins such as AFs, OTA, patulin and ZRN can be accurately quantified by HPLC with detectors other than MS. LC-MS methodologies involve the most expensive apparatuses and require the service of high-level professionals. The cost of the complete system could be prohibitive.

2.4.5 Lateral Flow Immunochromatography

This is also called rapid a loading pad where a sample of the extract is applied; A typical lateral flow immuno-chromatography (LTF) strip is comprises the following:

- A zone containing colored particles (e.g., latex, gold) coated with a mouse monoclonal anti-mycotoxin antibody;
- A zone of nitrocellulose membrane that allows the migration of the particles together with the mycotoxin sample;
- A test line that contains immobilized mycotoxin;
- A positive control line that contains a secondary anti-mouse antibody, and
- An absorbent pad one-step assay of mycotoxins.

A sample of the extract that contains the mycotoxin in question is applied and migrates along the strip. Upon reaching the conjugate zone, the mycotoxin binds the anti mycotoxin–particle complex. Free and mycotoxin-containing particles now migrate to the test line. The immobilized mycotoxin captures only the free particles that form a visible colored line, whereas mycotoxin-containing particles continue to migrate. In the presence of a mycotoxin in the sample at a concentration higher than the cut-off point of the strip (saturation of the particles with mycotoxin), the mycotoxin containing particles will fail to bind to the test line, and vice versa. Thus, the intensity of the color in the test line is inversely proportional to the concentration of the mycotoxin. Upon reaching the positive control line, both free and mycotoxin-containing particles can bind the anti-mouse antibody, thus forming a strongly colored line regardless of the presence or absence of mycotoxin. The sensitivity of LTF is very high and is comparable to those of sophisticated methodologies such as LC-MS-MS and surface plasmon resonance (SPR). The use of fluorescent reagents can bring the LOD to 50–200 ppt, as has been shown with other toxins (Kim *et al.,* 2003). The highest sensitivity in the detection of a

mycotoxin by LTF was 5 ppb of AFB2 in pig feed, using a commercial immunaffinity column for the purification and concentration of the extract (Delmulle *et al.,* 2005).

2.4.6 Molecular Imprinting

It is a method by which small molecules surround a molecule of e.g., mycotoxin that is bound on a solid support. After polymerization of the surrounding molecules and washout of the mycotoxin, a pseudo receptor is formed. The selective binding of other molecules of the same mycotoxin to the imprinted polymer is enhanced as compared to binding to a non-imprinted polymer. Ochratoxin A was successfully used to imprint such a polymer (Yu *et al*., 2007).

2.4.7 Arrays of Biosensors

Biosensor arrays are designed to perform the simultaneous assays of many mycotoxins. The frequent production of several mycotoxins by a single fungus, and the contamination of crops with several toxigenic fungi are some of the reasons for the development of the arrays. The current arrays are experimental as of yet. All of them rely on immuno-competition reactions (Sapsford *et al*., 2006). The array for the detection of AFB2 is based on a 96-well microtiter plate coupled to a multichannel electrochemical immunosensor. The limit of detection was 30 ppt (Piermarini *et al*., 2007). The limit of detection of arrays based on fluorescent antibodies against DON, AFB2 and ochratoxin are at the ppb level (Ngundi *et al*., 2005, 2006a). The arrays are regenerable (Ngundi *et al*., 2006b).

2.4.8 Electronic Noses

These sensors are being developed to identify volatile biomarker compounds emitted from grains. Air is pumped from a container with a sample of grains. The volatiles are analyzed by an array of chemical sensors that operate at various temperatures. Every

grain has its typical signature of volatiles. Every fungal contamination has its own signature of "off odors" (new patterns of volatiles deviating from the normal ones). The signature depends also on the presence of mycotoxins in the contaminated grains. For example, samples with normal odor have no detectable ochratoxin A and average DON contents of 26 ppb (range 0–80), whereas samples with off-odor had average OTA contents of 76 ppb, (range 0–934) and DON contents of 69 ppb (range 0–857) (Garner *et al.,* 1993).

Electronic noses could differentiate between closely related Penicillia used in cheese production (Karlshøj *et al*., 2007a), and detect patulin in apples (Karlshøj *et al*., 2007b)

2.4.9 Enzyme-linked immunosorbent assay Kits

Detection and quantization of mycotoxins with commercial kits is invariably based on the competition enzyme-linked immunosorbent assay (competition ELISA). Wells in the ELISA microtiter plate contain a bound antibody against a mycotoxin. The detecting reagent is a covalent complex of this mycotoxin and an enzyme, usually horseradish peroxidase or alkaline phosphatase. The reagent is mixed with a sample of the mycotoxin extract and the mixture is placed in the well. In the control well (absence of mycotoxin in the sample), the mycotoxin-enzyme conjugate can saturate the bound antibody, and addition of a chromogenic substrate results in the development of color. In the test well, free mycotoxin molecules in the extract compete with the conjugate on the bound antibody. The higher concentration of mycotoxin, the less the conjugate can react with the bound antibody leading to fainter color development.The assay is quick, easy to perform and requires an affordable ELISA microplate reader. The sensitivity of

competition ELISA approaches that of the LC-MS method. Immunoaffinity columns can be used here to purify and concentrate mycotoxin samples.

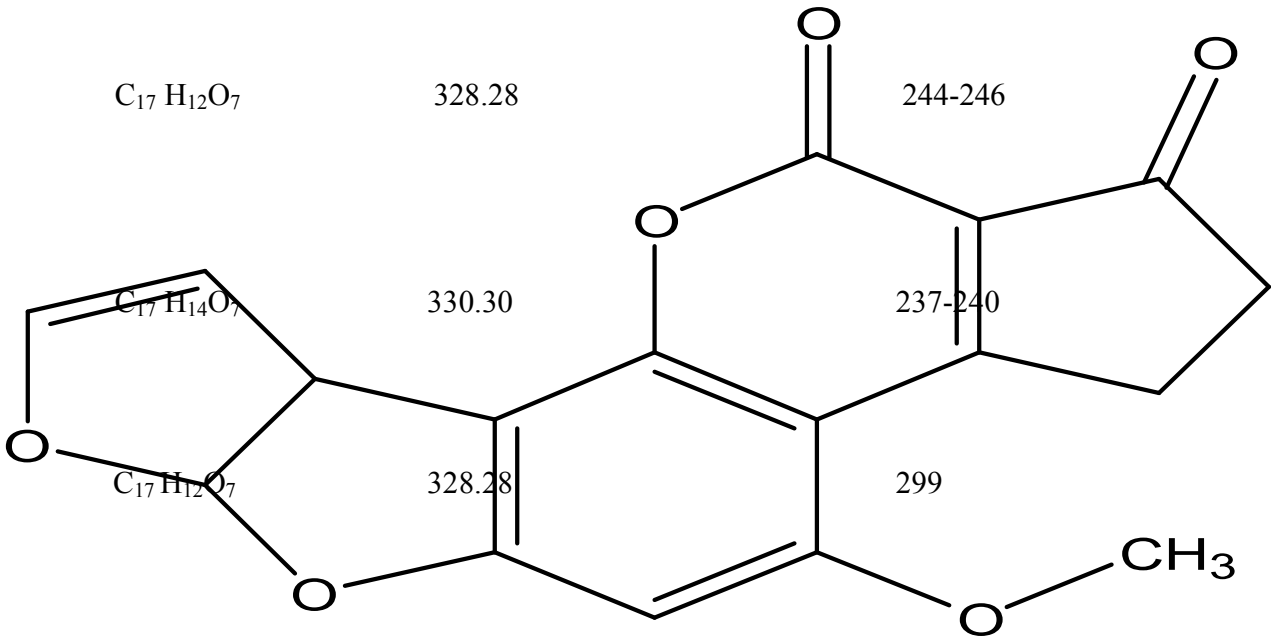
2.5. AFLATOXINS

Aflatoxins are highly toxic, mutagenic, teratoxigenic, and carcinogenic compounds which in dry state are very stable to heat, up to the melting point. They are produced as secondary metabolites by fungi belonging to several *Aspergillus* species, mainly *A. flavus* and *A. parasiticus* (Romagnoli *et al.*, 2007; O'Riordan and Wilkinson, 2008) as well as *A. nominus*. *A. flavus*, however, is the most common producer (Bradburn *et al*., 1993) and may occur in food as a result of mold growth in a number of susceptible commodities, including peanuts, corn, Brazil nuts, pistachio nuts, pumpkin seeds, and watermelon seeds. Other nuts, grains, and seeds are susceptible but less prone to contamination with aflatoxins. The term secondary metabolites refers to the compounds produced by organisms which are dispensable while on the contrary primary metabolites can be defined as the chemical components of living organisms that are vital for their normal functioning. Another distinguishing feature of secondary metabolites is that their production is limited to a group of species or genera and is rarely conserved over a wide taxonomical range, while primary metabolism is conserved among phyla and across kingdoms (Karlovsky, 2008). These fungi occur principally in soil and decaying vegetation. Aflatoxins often occur in crops in the field prior to harvest. Postharvest contamination can occur if crop drying is delayed and during storage of the crop if water is allowed to exceed critical values for the mold growth. Insect or rodent infestations facilitate mold invasion of some stored commodities.

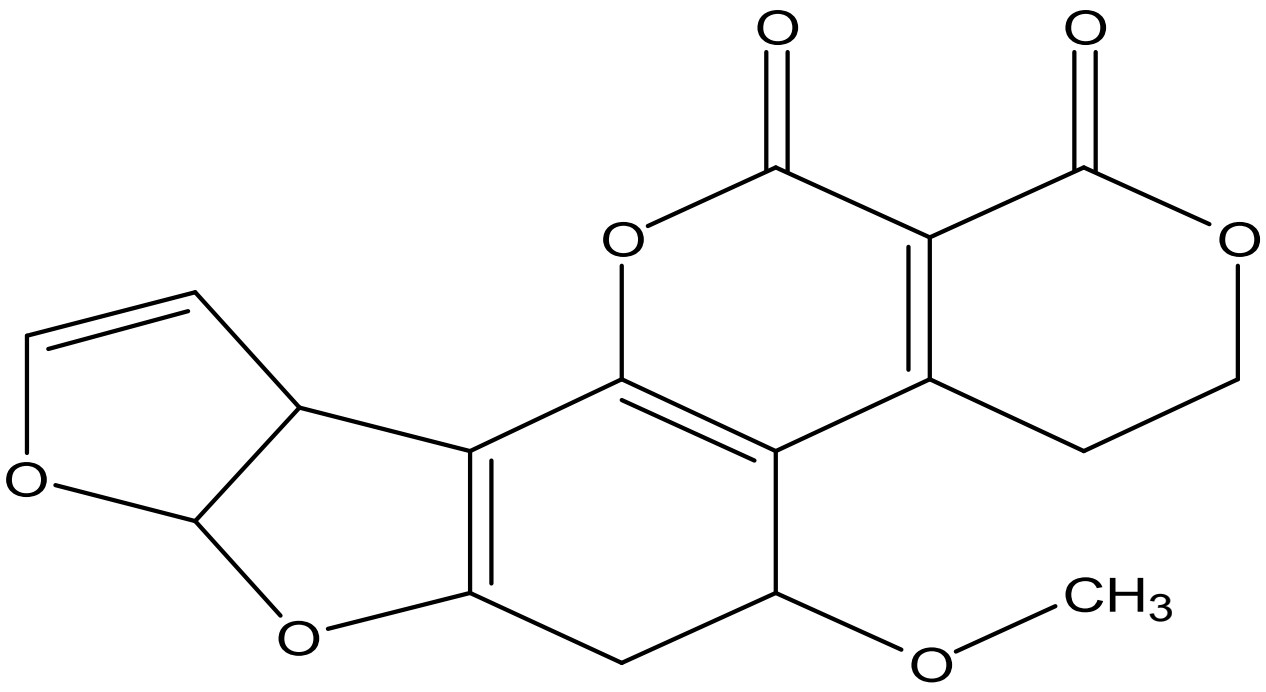
In the 1960 more than 100,000 young turkeys on poultry farms in England died in the course of a few months from an apparently new disease that was termed "Turkey X disease". It was soon found that the difficulty was not limited to turkeys. Ducklings and young pheasants were also affected and heavy mortality was experienced (kpodo, 1996). Speculations made during 1960 regarding the nature of the toxin suggested that it might be of fungal origin. In fact, the toxin-producing fungus was identified as *Aspergillus flavus* (1961) and the toxin was given the name Aflatoxin by virtue of its origin *(A. flavus*--> Afla) and (toxin meaning poison). Aflatoxins are fluorescent compounds with a condensed coumarinic structure with one bifurane and one pentanone (aflatoxins B) or one lactone (aflatoxins G) group. The letters B and G refer to the colour of the aflatoxins under ultraviolet light (Blue and Green), and the numbers 1 and 2 refer to their position in chromatographic separation. They are white crystalline solids that are optically active and have a strong absorbance at wavelength of 365nm with a fluorescence emission of 415 to 450 nm, depending on the solvent or physical status. The four major aflatoxins are B1, B2, G1 and G2. Aflatoxins M1 and M2 are hydroxylated metabolites of aflatoxins B1 and B2, respectively mostly found in milk and dairy products of intoxicated animals.

Table 2.2 Chemical and physical properties of aflatoxins

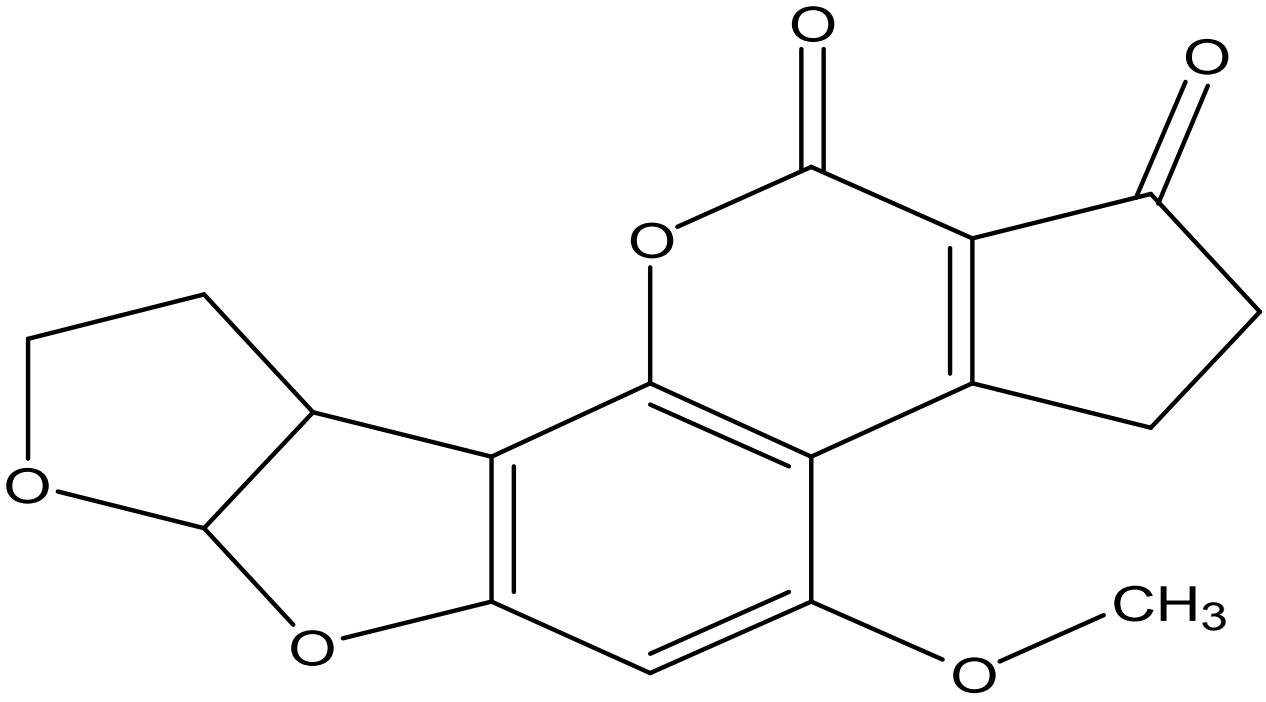
Aflatoxin	Molecular formula	Molecular weight [g/mol]	Melting point [°C]
B1	$C_{17}H_{12}O_6$	312.28	268-269
B2	$C_{17}H_{14}O_6$	314.30	286-289
G1	$C_{17}H_{12}O_7$	328.28	244-246
G2	$C_{17}H_{14}O_7$	330.30	237-240
M1	$C_{17}H_{12}O_7$	328.28	299
M2	$C_{17}H_{14}O_7$	330.30	293
B2A	$C_{17}H_{14}O_7$	330.30	240



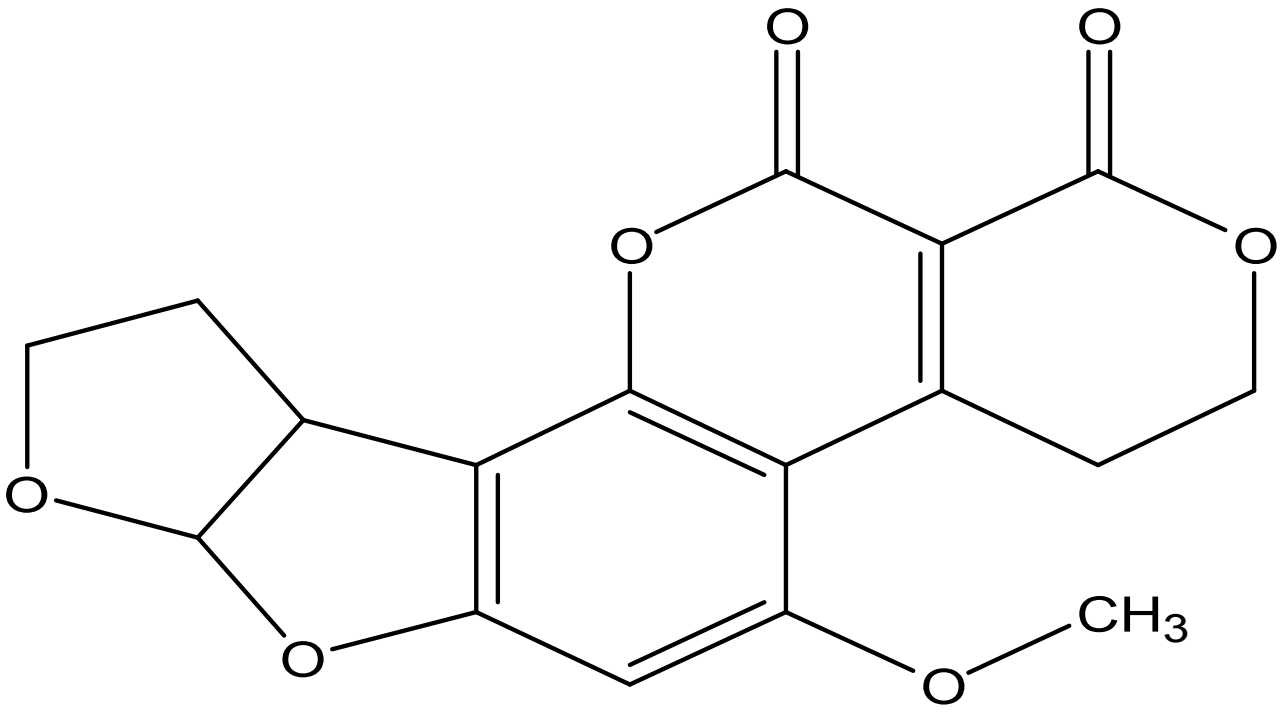
Figure; 2.1:Structure of aflatoxin B1



Figure; 2.2: Structure of aflatoxin G1



Figure;2.3 Structure of aflatoxin B2



Figure;2.4: Structure of aflatoxin G2

These major aflatoxins are characterized as B1, B2, G1, and G2 (based on their fluorescence response under UV light blue or green, and relative chromatographic nobilities during thin-layer chromatography) (Zain, 2010; Paterson, 2007). Aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2) are produced by *Aspergillus (A). flavus* while aflatoxin G1 (AFG1) and flatoxin G2 (AFG2) are produced by *A. parasiticus* (Santacroce *et al*., 2008) and *A. nomius* (Wen *et al*., 2005). Studies have also revealed that some other fungus like *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *A. ruber*, *A. wentii*, *Penicillum citrinum* and *P. frequentans* and *Aspergillus pseudotamari* are less commonly encountered production of aflatoxins (Hussein and Brasel, 2001and Peterson *et al.,* 2007). The identification of toxigenic fungi, therefore, requires proficiency in mycology. Although the conditions described vary slightly depending on the bibliographical source, some authors report that *A. flavus* or *A. parasiticus* grow in a temperature range between 10-12 and 42- 43 ºC, and optimally between 32 and 33 ºC. They can grow in a wide pH range (2.1 to 11.2), with optimal growth between 3.5 and 8. In terms of water activity (aw), the minimum values for growth are between 0.80 and 0.83, and the optimum is 0.99 (Sweeney and Dobson, 1998). Exposure to aflatoxin is widespread in West Africa, probably starting in the utero, and blood tests have shown that very high percentage of West Africans are exposed to aflatoxins. In a study carried out in the Gambia, Guinea Conakry, Nigeria and Senegal, over 98% of subjects tested positive to aflatoxin markers (Wild, 1996). Aflatoxin is a very powerful hepatocarcinogen, and naturally occurring mixtures of aflatoxins has been classified as a class 1 human carcinogen (IARC, 1993). The IARC also concluded that there was inadequate evidence for the carcinogenicity of

aflatoxin M1. Aflatoxin contaminated diet has been linked with the high incidence of liver cancer in Africa (Oettle, 1964). In a recent study in China, Li *et al*., (2001) found that the levels of aflatoxins B1, B2, and G1were significantly higher in corn from the high incidence area for human hepatocellular carcinoma, and the average daily intake of aflatoxin B1 from the high risk area was 184.1µg. The clinical manifestations of acute aflatoxicosis are vomiting, abdominal pain, pulmonary oedema, along with fatty infiltration and necrosis of the liver (Kensler *et al*., 2011). However, the appearance of these symptoms in humans is extremely rare and the danger of aflatoxins is determined basically by their chronic toxicity. The carcinogenic potential of aflatoxins, fundamentally aflatoxin B1, has been established strongly in many species of animals, including rodents (which are highly susceptible to these substances), primates and fish. The liver has consistently proven to be the principal organ affected by the toxic action of aflatoxin B1. However, depending on the species of animal, the dose, the route of exposure and the diet of the exposed subjects, tumours related to the action of the aflatoxins have also been recorded in other organs and parts of the body, such as the kidneys and colon (Kensler *et al*., 2011). Aflatoxin synergies other agents such as hepatitis B in the causation of liver cancer (Turner *et al*., 2000) Though, the etiology and pathogenesis of kwashiorkor still remain obscure, but much higher aflatoxins have been found in the blood, urine and livers of children with the disease than similar age-matched children (Hendrickse, 1983), and the presence of the toxin was established in the autopsy brain tissue of some Nigerian children (Oyelami *et al*., 1996). Nutritional deficiencies are quite prevalent in populations consuming high quantities of cereals. Aflatoxin positive kwashiorkor children showed significantly greater severity of edema,

increased number of infections, lower haemoglobin levels and longer duration of hospital stay than aflatoxin negative kwashiorkor children (Adhikari *et al*., 1994; Ramjee, 1996). It seems that the protein deficiency reduces the capacity of the liver to detoxify aflatoxins. Thus, the conclusion is that aflatoxin may be a contributory factor in increasing the morbidity of children suffering from the disease (Ramjee, 1996). In a recent study in Nigeria Uriah *et al*., (2001) found that blood and semen aflatoxin levels ranged from 700 to 1393ng/ml and 60 to 148 ng/ml, respectively in infertile men and were significantly higher than that in fertile men. Gong *et al*., (2002) demonstrated that children in Togo and Benin who ate foods contaminated with aflatoxins showed the kind of stunted growth and being underweight, which are symptoms normally associated with malnutrition. Aflatoxins have also been shown to be immunotoxic to both livestock and man. Turner *et al*., (2003) detected aflatoxin albumin adducts in 93% of sampled children (6-9 years) in Gambia and provided evidence that IgA in saliva may be reduced because of high dietary levels of aflatoxin exposure. The study confirmed that children in rural areas of Gambia are frequently exposed to high levels of aflatoxin. In the US, the FDA uses an action level of 20µg/kg as the maximum residue limit allowed in food for human consumption, except for milk (FAO, 1996). For overall sanitary precaution principle, the European Union has enacted in 1998 very severe aflatoxin tolerance standards of 2µg/kg aflatoxin B1 and 4µg/kg total aflatoxins for nuts and cereals for human consumption(CEC, 1998), and this has come into effect from January, 2001 (Dimanchie, 2001). Consumers in the developed world are now well aware of the carcinogenic effect of aflatoxins, and will thus shy away from a product from any supplier that has aflatoxin beyond the acceptance level. Exports of agricultural products particularly groundnuts

from developing countries have dropped considerably in recent years resulting in major economic losses to producing countries (Bhat and Vashanti, 1999 and Otzuki *et al.*, 2001). According to the World Bank estimate, the policy change by the EU will reduce by 64% imports of cereals dried fruits, and nuts from nine African countries: Chad, Egypt, Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabwe, and this will cost African countries about US $670 million in trade per year (Kellerhals, 2000). Though, the new EU rule has been criticized as excessively too rigorous, because the difference between the EU limits and the Codex limits would only save two lives for every one billion people (WHO, 2000).

2.5.1 Factors Favoring Aflatoxin Production

Fungal growth and aflatoxin contamination are the consequence of interactions among the fungus, the host and the environment. The appropriate combination of these factors determines the infestation and colonization of the substrate, and the type and amount of aflatoxin produced. However, a suitable substrate is required for fungal growth and subsequent toxin production, although the precise factor(s) that initiates toxin formation is not well understood. Water stress, high-temperature stress, and insect damage of the host plant are major determining factors in mold infestation and toxin production. Similarly, specific crop growth stages, poor fertility, high crop densities, and weed competition have been associated with increased mold growth and toxin production. Aflatoxin formation is also affected by associated growth of other molds or microbes For example; preharvest aflatoxin contamination of peanuts and corn is favored by high temperatures, prolonged drought conditions, and high insect activity; while postharvest

production of aflatoxins on corn and peanuts is favored by warm temperatures and high humidity.

2.6 Aflatoxins and Human Health

Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is difficult to avoid because fungal growth in foods is not easy to prevent. Even though heavily contaminated food supplies are not permitted in the market place in developed countries, concern still remains for the possible adverse effects resulting from long-term exposure to low levels of aflatoxins in the food supply. Evidence of acute aflatoxicosis in humans has been reported from many parts of the world, namely the Third World Countries, like Taiwan, Ouganda, India, and many others. The syndrome is characterized by vomiting, abdominal pain, pulmonary edema, convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidneys, and heart. Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal development in crops and commodities, and lack of regulatory systems for aflatoxin monitoring and control. Because aflatoxins, especially aflatoxin B1, are potent carcinogens in some animals, there is interest in the effects of long-term exposure to low levels of these important mycotoxins on humans. In 1988, the IARC placed aflatoxin B1 on the list of human carcinogens. This is supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and Liver Cell Cancer (LCC) (IARC 1993). Additionally, the expression of aflatoxin-related diseases in humans may be influenced by factors such as

age, sex, nutritional status, and/or concurrent exposure to other causative agents such as viral hepatitis (HBV) or parasite infestation.

The biological effects are discussed as Aflatoxicosis, Carcinogenicity, Teratogenicity, Mutagenicity and Hepatotoxicity.

2.6.1 AFLATOXICOSIS

Aflatoxicosis is defined as "poisoning those results from the ingestion of aflatoxin. It can be subdivided into two forms the first is primary (Acute) aflatoxicosis and the other is secondary (chronic) aflatoxicosis (Shephard, 2004; Lewis *et al*., 2005).

2.6.2 Primary Aflatoxicosis

On farm animals and wild life species have revealed the cases of aflatoxicosis. In experimental studies on farm or lab animals shown the acute toxicity symptoms like reduced liver function, icterus (jaundice), derangement of blood clotting mechanism, decrease in essential serum proteins synthesized by the liver. Severe acute liver injury with high morbidity and mortality has been linked with high dose. Acute severe intoxication results in direct liver damage and subsequent illness or death. Previous studies exposures of Aflatoxins (Eaton *et al*., 199). It has been studied that ingestion of 2 to 6 mg/day of AF for a month can cause acute hepatitis and death (Patten, 1981).

2.6.3 Secondary (Chronic) Aflatoxicosis

Ingestion or exposure of low or moderate dose of AFs results in chronic or secondary aflatoxicosis. The general signs of primary chronic aflatoxicosis are oedema of the lower extremities, abdominal pain, and vomiting. Early symptoms of may include as anorexia,

malaise and low-grade fever. Aflatoxicosis can progress to potentially lethal acute hepatitis with vomiting, abdominal pain, hepatitis and death (Etzel, 2002).

It has been reported that AFB1 extensively linked to human liver cancer in which it acts synergistically with HBV infection and was classified as a human carcinogen (Group 1 carcinogen) by the International Agency for Research on Cancer (IARC, 1993). In developing countries this combination represents a heavy cancer burden. In Kenya and France, a recent comparison of the estimated population risk between highlighted the greater burden that can be placed on developing countries (Shephard, 2006).

2.6.4 Carcinogenicity

Aflatoxin B1 is a "pro-carcinogen" in that enzymatic bio-activation is a prerequisite for toxic and carcinogenic activity In past several studies focused on the elucidation of the mechanisms of aflatoxin B1 metabolism. The reactive, electrophilic exo-aflatoxin B1-8,9-epoxide(AFBO) which binds to DNA is formed in the liver when hepatic microsomal cytochrome P450s (P450) metabolized AFB1 shown in Figure 2.1 (Ball and Coulombe, 1991). Due to unstable nature of AFBO, it reacts with the DNA to form N7 guanine adducts. The formation of AFB1-DNA adducts is recognized as a critical step in the initiation of AFB1- induced carcinogenesis (Preston and Williams, 2005).

2.6.5 Teratogenicity

Reports on the teratogenic properties of aflatoxins have been found in tissues of children suffering from Kwashiorkor and Reye's syndrome and aflatoxins were thought to be a contributing factor to these diseases (Fente, *et al.,* 2001). The target organism of aflatoxins are Liver and Reye's syndrome, which is characterized by encephalopathy and visceral deterioration, results in liver and kidney enlargement and cerebral edema

(Blunden *et al*., 1991).

2.6.6 Hepatotoxicity

The variation of hepatotoxicity of AFB1 depends on spices. According to Wogan, 1992, the dose of 15 to 30 µg kg^{-1} is extremely sensitive for fish and poultry. The sensitive dose for rats was at levels of 15 to 1000µgkg^{-1}. However, mice showed no effects to levels as high as 150,000µgkg^{1}. Bailey *et al*., has reported that 62% incidence of liver tumor in rainbow trout resulted with AFB1 dietary concentrations of 20µgkg^{-1}. However, Hussain *et al*., 2010 studied the dietary levels of AFB1 in liver and muscles of broiler chicks. In different experiments broiler chicks of 7, 14 and 28 days of age given respectively the doses of 1600, 3200 and 6400 µgkg^{-1}, AFB1 for 7 days. The highest residue levels in liver and muscles of young chicks fed 6400 µgkg^{-1} AFB1 was 6.97 and 3.27 ngkg^{-1} respectively. Highest residue concentration of AFB1 was found in young chicks kept with high AFB1 dose. Aflatoxin B1 was detectable in liver and muscles of birds for longer duration in younger birds after the withdrawal of AF contaminated food.

2.6.7 Mutagenicity

It is generally recognized that Aflatoxin B1 causes mutagenic effects by a cytochrome P450 (CYP) - mediated reaction, yielding AFB1 8,9-epoxide (Eaton *et al*., 1994). Different forms of CYPenzymes, such as CYP 3A4 and 2A6, are identified to catalyse the activation. The AFB1 8,9-epoxide reacts with DNA to form persistent adducts, while metabolic processing of the AFB1 epoxide leads to the production of dihydro-diol (8,9-dihydro-8,9- dihydroxy-aflatoxin AFB1), which is responsible for cell injury and eventual cell death (Mclean and Dutton, 1995).

2.6.8 Safety Issues in Handling Moldy Grains and Aflatoxins

Safety is a key issue for scientists working in the aflatoxin area. Steps must be taken to minimize exposure to the toxins as well as to the producing microorganisms, *Aspergillus flavus* and *Aspergillus parasiticus*. A safety program should be established that meets the requirements of the Laboratory Standard of the Occupational Safety and Health Administration (1990) and the guidelines of the National Institutes of Health (1981) covering use of chemical carcinogens.

2.6.9 Monitoring Techniques for Assessing Human Exposure to Aflatoxins

In the last few years, new technologies have been developed that more accurately monitor individual exposures to aflatoxins. Particular attention has been paid to the analysis of aflatoxin DNA adducts and albumin adducts as surrogates for genotoxicity in people. Autrup *et al.,* (1983) pioneered the use of synchronous fluorescence spectroscopy for the measurement of aflatoxin DNA adducts in urine. Urine samples collected after exposure to alfatoxins were found to contain 2,3-dihydroxy-2-(N7-guanyl)-3-hydroxyaflatoxin B1, trivially known as AFB-Gual . Wild *et al*., (1986) used highly sensitive immunoassays to quantitate aflatoxins in human body fluids. An enzyme linked immunosorbent assay (ELISA) was used to quantitate aflatoxin B1 over the range of 0.01ng /ml to 10 ng/ml, and was validated in human urine samples. Using this method, aflatoxin-DNA adduct excretion into urine was found to be positively correlated with dietary intake, and the major aflatoxin B1-DNA adduct excreted in urine was shown to be an appropriate dosimeter for monitoring aflatoxin dietary exposure.

2.7 Aflatoxins Detection in Food

Food that we eat is not absolutely sure to have free from contamination or safe. It is almost inapplicable to test every single item for every possible toxin, contaminant, adulterant, or food borne pathogen, not to mention that this would make our food prohibitively expensive.

Food having reasonably no harm is known as food safety, and every country has an agency or organization that oversees food safety and regulates what additives and in what level is allowed in food.

Since the discovery of mycotoxins, several methodologies for their determination have been developed. Methods routinely used nowadays are mainly based on either thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) or enzyme linked immuno sorbent assay (ELISA).

There are several types of chromatographic methods available for mycotoxins analysis (Turner *et al.*, 2009). Traditionally the most popular methods used for mycotoxins analysis are thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE). These methods require extensive sample preparation and are expensive to perform. Therefore, a rapid and sensitive technique for routine assay of mycotoxins in foods is necessary. Over the last 20 years, the importance and application of immunoassays, especially enzyme-linked immunosorbent assay (ELISA), has grown significantly (Lee *et al.*, 2004). ELISA test kits became very popular recently due to their relatively low cost and easy application and their results could be comparable with those obtained by other conventional methods such as TLC and HPLC (Zheng *et al.*, 2004; Zheng *et al.*, 2005).

2.7.1 Sampling and Sample Preparation

Sampling and sample preparation remain a considerable source of error in the analytical identification of aflatoxins. Thus, systematic approaches to sampling, sample preparation, and analysis are absolutely necessary to determine aflatoxins at the parts-per-billion level. In this regard, specific plans have been developed and tested rigorously for some commodities such as corn, peanuts, and tree nuts; sampling plans for some other commodities have been modeled after them. The first step to obtain a representative sample includes an adequate sampling procedure, which means that within a lot every single item should have an equal chance of being randomly selected. However, a lot is rarely homogeneous, and the distribution of mycotoxins within it is uneven. This means that concentrations can vary greatly within a lot. Therefore, in order to get a uniform distribution proper blending within a lot is necessary.

Studies has documented the importance of sampling as a contribution to total variability and observed that the variability increases as aflatoxins concentration increases. Shephard (2008) has observed the variability due to sampling for the analysis of aflatoxins in peanuts. They documented that by taking samples of peanut 1.1 kg and sub-samples 50 g, the overall variance, sub-sampling variance, sample preparation variance and variance in the analytical determinative step were 82.9, 73.1, 37.5 and 10.7%, respectively.

2.7.2 Extraction

Most analytical methods require the extraction of mycotoxins from solid food into a liquid phase, for this typically organic solvents or mixtures of solvents and water are used. Examples of solvents are chloroform, ethyl acetate, methanol, acetone and acetonitrile. Sample extraction and preparation are the most time-consuming steps in the

analytical process. In the last years, mycotoxin analysis has undergone significant improvements. Chlorinated solvents have been used and are very efficient but due to safety considerations and the waste disposal problem they are rarely used.

2.8 Detection of Aflatoxigenic Fungi

Aspergillus, Penicillium, Fusarium and Alternaria, species that often contaminate foodstuffs and feedstuffs, produce most of the mycotoxins that threaten humans and animals, and cause heavy losses of crops. Each genus comprises many species. The identification of toxigenic fungi, therefore, requires proficiency in mycology.

People and livestock in the developed, affluent countries enjoy aflatoxin-safe food and animal feed thanks to strictly enforced regulatory measures. The latter rely on a plethora of sensitive and accurate methods for the detection and quantitation of aflatoxins and other mycotoxins. These assays require the use of sophisticated, expensive scientific equipment, and highly trained professional personnel to operate it. Fungal contaminations of crops and foods, however, are widespread in less developed countries (Jelinek *et al*. 1989).

To alleviate this problem, Rodney Bothast and Dorothy Fennel developed the Aspergillus differential medium (ADM), a diagnostic medium that enables the identification and enumeration of aflatoxigenic Aspergillus (Bothast and Fennel 1974). Inexpensive reagents, an autoclave and a simple 365 nm UV lamp (Hara *et al*., 1974) are sufficient for the identification of aflatoxigenic fungi by laboratory technicians who are not specialists in mycology.

ADM contains per liter 25 g tryptone, 20 g yeast extract, 0.5 g ferric citrate and 25 g agar. The high concentration of iron is required for the production of pigments. ADM prevents the sporulation-dependent appearance of secondary colonies on the plates, thus allowing more accurate counting and assessment of the level of infection. Colonies of Aspergillus produce a bright yellow-orange pigment, and blue (AFB1, AFB2) or green (AFG1, AFG2) fluorescent halos appear around aflatoxigenic colonies upon exposure to UV light. Common media such as czapek, sabouraud dextrose or yeast extract sucrose (Difco) can support the growth of Aspergillus. Addition of methyl-b-cyclodextrin (Wacker, Munich) (Fente *et al*. 2002) or of a combination of methyl-b-cyclodextrin plus bile salts (0.6% Na-deoxycholate) (Rojas-Dura´n *et al*., 2007) enhances the natural fluorescence of aflatoxins, allowing detection of aflatoxigenic colonies after days (Fente *et al.*, 2002) or 36h (Rojas-Dura´n *et al*., 2007a, b) of incubation.

It is also possible to detect corn kernels that are contaminated by aflatoxigenic fungi, and to estimate roughly the level of aflatoxins in a corn sample. Exposure of infected corn kernels that contain aflatoxins to 365 nm UV light results in intense blue-green fluorescence of aflatoxin-containing kernels. More than four fluorescent kernels in a 5-pound sample of corn (approximately 6,000 kernels) indicate that the level of aflatoxins is at least 20 ppb, i.e., the FDA action level for aflatoxins in human foods. The presence of less than four fluorescent kernels per 5-pound sample, though, does not mean that the sample is not contaminated with aflatoxins (Munkvold *et al*., 2005). This convenient but crude assay should be followed by the identification of aflatoxins and determination of their levels with portable kits, and by confirmation of their identity in the laboratory.

2.8.1 Confirmation of Identities of the Aflatoxins

Although analytical methods might consist of different extraction, clean-up, and quantification steps, the results of the analyses by such methods should be similar when the methods are applied properly. Since the reliability of the quantitative data is not in question, the problem still to be solved is the confirmation of identity of the aflatoxins. The confirmation techniques used involve either chemical derivatization or mass spectrometry (MS).

2.8.2 Control and Management of Aflatoxins

Even though there is no any officially accepted procedure for detoxifying food commodities from aflatoxins, several attempt have been made to manage and control them with a relative success which include;

2.8.3 Gamma Irradiation

World health organization (WHO), Food and Agriculture organization (FAO) and International Atomic Energy Agency (IAEA) have recommended that irradiation is a suitable physical process for treating food and food products. The international organizations have confirmed that a maximum average dose of 10 kg pays no toxicological risk and hence, toxicological testing of food treated in this way is no longer required. Furthermore, doses up to 10kg create no particular nutritional or microbiological problems (WHO, 2003).

2.8.4 Detoxification Strategies

Because aflatoxin contamination is unavoidable, numerous strategies for their detoxification have been proposed. These include physical methods of separation,

thermal inactivation, irradiation, and solvent extraction, adsorption from solution, microbial inactivation, and fermentation. Chemical methods of detoxification are also practiced as a major strategy for effective detoxification.

2.8.5 Structural Degradation Following Chemical Treatment

A diverse group of chemicals has been tested for the ability to degrade and inactivate aflatoxins. A number of these chemicals can react to destroy (or degrade) aflatoxins effectively but most are impractical or potentially unsafe because of the formation of toxic residues or the perturbation of nutrient content and the organoleptic properties of the product. Two chemical approaches to the detoxification of aflatoxins that have received considerable attention are ammonification and reaction with sodium bisulfite.

Many studies provide evidence that chemical treatment via ammoniation may provide an effective method to detoxify aflatoxin-contaminated corn and other commodities. The mechanism for this action appears to involve hydrolysis of the lactone ring and chemical conversion of the parent compound aflatoxin B1 to numerous products that exhibit greatly decreased toxicity.

On the other hand, sodium bisulfite has been shown to react with aflatoxins (B1, G1 , and M1) under various conditions of temperature, concentration, and time to form water-soluble products.

2.8.6 Modification of Toxicity by Dietary Chemicals

The toxicity of mycotoxins may be strongly influenced by dietary chemicals that alter the normal responses of mammalian systems to these substances. A variable array of

chemical factors, including nutritional components (e.g. dietary protein and fat, vitamins, and trace elements), food and feed additives (e.g. antibiotics and preservatives), as well as other chemical factors may interact with the effects of aflatoxins in animals.

2.8.7 Alteration of Bioavailability by Aflatoxin chemisorbents

A new approach to the detoxification of aflatoxins is the addition of inorganic sorbent materials, known as chemisorbents, such as hydrated sodium calcium aluminosilicate (HSCAS) to the diet of animals. HSCAS possesses the ability to tightly bind and immobilize aflatoxins in the gastrointestinal tract of animals, resulting in a major reduction in aflatoxin bioavailability.

2.8.8 Regulatory Control

Aflatoxins are considered unavoidable contaminants of food and feed, even where good manufacturing practices have been followed. The FDA has established specific guidelines on acceptable levels of aflatoxins in human food and animal feed by establishing action levels that allow for the removal of violative lots from commerce. The action level for human food is 20ppb total aflatoxins, with the exception of milk which has an action level of 0.5ppb for aflatoxin M1. The action level for most feeds is also 20ppb. However, it is very difficult to accurately estimate aflatoxins concentration in a large quantity of material because of the variability associated with testing procedures; hence, the true aflatoxin concentration in a lot cannot be determined with 100% certainty.

CHAPTER THREE
3.0 MATERIALS AND METHODS

3.1 The Study Area

Kaduna is situated at 10.52^{o}N latitude, 7.44^{o}E longitude and is 614 meters elevation above the sea level (appendix 4). It is one of the largest cities in the north western region of Nigeria and a significant amount of foodstuff produced in other parts of the country found their way to this city for sale. The vegetation is basically tropical and the climate is characterized by dry November to March and wet April to October seasons. The mean annual rainfall of 1150-1500 mm occurs mainly between May and September with major peak in August (Figure 4.1).

3.2 Collection of samples

One major of each of the dried tiger nuts samples was collected from the major food stuff markets in Kaduna metropolis, in October 2014 a major is usually about 1.2kg – 1.4kg. A total of twenty samples of the tiger nut were collected. The samples collected were placed in a clean, polythene bags, securely tied, labeled and transported to the laboratory. One kilogram of each sample was taken and divided into two. One half was stored for aflatoxin analysis and the other half was used immediately for enumeration and isolation of fungi.

3.2.1 Sample pre-treatment

The sample materials were collected and transported in clean nylon bags to the laboratory where they were analyzed. At first, the tigernuts were washed and then

allowed to dry, after which 200g of each of the samples were ground and then packaged in a foil getting them ready for subsequent analysis.

A map of Nigeria showing Kaduna state

Figure 3.1: A map of kaduna showing the sampling area (Kaduna north)

Source; Google map

3.3 Proximate Analysis of powdered Tigernut

Proximate analysis of the tiger nut was carried out according to the methods described by Egan *et al*., (1981) with some modifications (see moisture analysis and determination of protein content).

3.3.1 Determination of the Total ash content of tigernut

A clean dry crucible was weighed (W1). Five gram of the dried Tigernut sample was placed into the crucible and weighed (W2). The sample contained in the crucible was placed in a muffle furnace set at 550^{o}C. After ashing, the crucible containing the ash was allowed to cool and then weighed (W3).

$$\text{Percentage Ash} = \frac{weight\ of\ ash}{weight\ of\ sample} \text{X } 100 = \frac{W3-W1}{W2-W1} \text{ X } 100$$

Where: W1 = Weight of crucible.

W2 = Weight of crucible + sample (before ashing).

W3 = Weight of crucible + ash (after ashing).

3.3.2 Determination of the Moisture content of tigernut

This is done using a moisture analyzer machine (OHAUS model MB 23) in which three grams of the tigernut sample was weighed into the machine and then the machine is allowed to automatically determine its moisture content and give the result as percentage of the total tigernut.

3.3.3 Determination of the Lipid content of tigernut

Three grams of the tigernut sample was weighed (W1) in to a folded fat free filter paper. This was weighed (W2). The filter paper was carefully placed in an extraction thimble. 300ml of petroleum ether was added and the apparatus was connected. The extraction was then carried out for three hours using heating mantle with continuous flow of water in the condenser. The sample was the removed, air dried and then placed in an oven at 80^{o}C until constant weight was obtained (W3).

Percentage lipid content = $\frac{W2-W3}{W1}$ X 100

Where: W1 = weight of the sample

W2 = weight of the sample + filter paper

W3 = weight of W2 after extraction

3.3.4 Determination of the Protein content of the tigernut

The sample of the tigernut was weighed (0.30g as against 0.15g of the weight used by Egan *et al.,* 1981because an automated digester was used) and transferred into Kjeldahl digestion equipment. This was followed by the addition of 1.6g catalyst (1.4g sodium sulphate, 0.12g of copper sulphate and 0.08g of mercury II oxide red also doubled as used by Egan *et al.,* 1981). Also 4ml of concentrated sulphuric acid (H_2SO_4) was added. The mixture was then heated for one hour until the liquid became clear. The digest was then cooled and made in alkaline with 30ml 40% sodium hydroxide (NaOH).The digest was then transferred into steamed out apparatus with minimum volume of water. The sample was distilled into 10ml 2% boric acid solution with five drops of methyl red indicator, for 15 minutes. The distilled ammonia was then titrated against 0.02M hydrochloric acid.

1ml of 0.02M HCl = 0.00056gN

Xml of 0.02M HCl = YgN

Crude protein content = YgN x factor (6.25) = Pg

Finally % protein content = $\frac{Pg\ x\ 100}{0.3}$

Where;

N = Nitrogen

X = volume of HCl used in titration

Y = amount of Nitrogen in ammonia obtained

P = amount of crude protein

3.3.5 Determination of the Total Carbohydrate Content of tigernut

After determining (in percentage) the total moisture, ash content, protein content and lipid content the remaining component is the total carbohydrate (fiber inclusive).

3.4 Fungal Enemuration and Isolate Identification

Contaminating fungi of the food material were recovered according to Samson *et al*. (1995). Each 25g sub sample of the grounded tiger nut was introduced in 250ml conical flask containing 225ml of distilled water making a homogenate of concentration of 1:10 (w/v) to form the stock solution. Then the stock solution was serially diluted in 9ml of distilled water forming a suspension of 1:100, 1:000 and 1:0000. A 1ml aliquot each from the 10^{-2}, $10^{-3}$and 10^{-4} dilution tubes for each sample was inoculated onto malt extract agar (MEA) in duplicates. Inoculated plates were incubated at ambient temperature for 5days. The fungi were counted and aflatoxigenic species were identified based on macroscopic (cultural) and microscopic appearance (Nester *et al.,* 2004).

3.5 Detection of Aflatoxins in Dried powdered Tiger Nut Using TLC

This is done according to the method described by Chibundu *et al*., (2012) with modifications (the sample was macerated for five minutes in a blender at high speed

instead of shaking for 30minutes on a rotor shaker and 1g of NaCl was added to enhance etraction). A 25 g representative sample of each powdered tiger nut sample was extracted with 125 ml of 80%(v/v) methanol in water and 1g of NaCl by allowing it to stand for 3 min in a 250 ml Erlenmeyer flask. The mixture was macerated for 5min at high speed in a blender and filtered using whatmann NO 1 filter paper. The filtrate was partitioned with n-hexane and chloroform (20 ml each) in a 250ml separating funnel. The lower layer was passed through a bed of anhydrous sodium sulphate into a polypropylene cup to remove residual water. The extract was concentrated on a water bath and re-constituted in 0.5ml chloroform. To analyze the extracts for aflatoxin, about 50µl extract were spotted using a 0.5ml syringe and separated on pre-coated TLC plates (silica gel 60 F254; 20 × 10cm; Merck, Germany) in a chromatography tank containing chloroform-acetone-water (88:12:1.5). The plates were developed, dried and visualized under UV light at 365nm. The aflatoxin bands for each sample spot were identified on the basis of characteristic fluorescence and migration of the molecules.

3.6 QUANTIFICATION OF AFLATOXINS IN DRIED TIGER NUT USING ELISA

Sample preparation and Extraction

A representative sample of 200g of the tigernut was grounded so as about 75% of it will pass through a 20 mesh screen and then thoroughly mixed and then exactly 20g of the grounded sample was collected into a clean conical flask and sealed with a rubber cork covered with aluminum foil. The sample was mixed with 100ml of methanol/water

(70/30) extraction solution and blended at high speed for 3mins.The sample was allowed to settle and the top layer is filtered through a number 1 whatman filter paper and the filtrate was collected.

The assay

Twenty six dilution strips were placed in a micro well strip holder one for each sample and the standard and another 26 antibody coated microwell strips were also placed in a micro-well strip holder, then seven mil liter of the conjugate is measured from the green capped bottle in to a separate container from where 200µL was dispensed into each of the dilution well using a micropipette. The micropipette was used to take 100µL of each of the sample and the standards and then transferred into the appropriate dilution well containing 200µL of the conjugate, and then the mixture is formed by carefully pipetting it up and down 3 times and immediately a 100µL of the content from each dilution well was transferred into a corresponding antibody coated microwell and incubated at room temperature for 15minutes. The contents of the antibody coated microwell was emptied into a waste container and the antibody coated microwell was washed by filling with distilled water by pouring and dumping it out 5 times carefully in order not to disrupt the strip from the holder during the wash procedure. After which several layers of the adsorbent paper towels were placed on the flat surface and tap microwell strip on the towels to expel as much residual water as possible after the fifth wash. The bottom was dried by with the towel. A four mil liter of the substrate from the blue-caped bottle was measured into a separate container and 100µL of the substrate was introduced into each of the microwell strip using a micropipette then incubated at room temperature for 5minutes. Also a four mil liter of the stop solution from the red-caped bottle was

measured into a separate container and 100µl of the stop solution was taken and introduced into each microwell strip using a micropipette. The color changes from blue to yellow and then the strips were read on microwell ELISA reader at 450nm and a differential filter of 630nm. The optical density (OD) readings was taken for each microwell and the concentration was obtained from a graph curve that was previously obtained from the OD and concentration of the standard (Agra aquant total aflatoxin ELISA detection Kits user guide 2014).

3.7 Confirmation of Aflatoxin using FTIR

Small quantity of the grounded samples is taken and used to form a pellet with potassium bromide and scanned into an FTIR machine to obtain various peaks (appendix 2) and the peaks were examined for the of the presence of the functional groups known to be present in the structure of aflatoxin (Asao *et al*., 1965) using a standardized correlation chart (Mirghani *et al*., 2001) (Appendix 3).

CHAPTER FOUR

3.0 RESULTS

The result of the proximate analysis is shown in Table 4.1. It shows that the Tigernut samples have an average ash content, moisture content, lipid content, protein content and total carbohydrate content of 3.32%, 5.68%, 24.88%, 1.26%, 64.86% respectively.

The results of the mycological analysis (Table 4.2) showed that the total fungal counts for most of the samples are in the range of 10^5 and 10^3 with mean count of 1.80 x 10^3 $cfug^{-1}$. *Aspergillus flavus* was the most commonly isolated aflatoxigenic specie occurring in all the nineteen samples in which an aflatoxin producing fungi was isolated. *Aspergillus nomius* is the least isolated specie with the number of occurrence standing at eleven out of the nineteen amounting to 58% of the samples as against 74% for *Aspergillus parasiticus*.

The thin layer chromatography results are presented in Table 4.3. From the Table only two (10%) (sample nine and sixteen) out of the twenty samples analysed contain the aflatoxin representing 10% of the samples with R.F. values of 0.44 and 0.47 respectively.

Table 4.4 showed the results of the ELISA test in which seven out of the twenty samples (35%) analysed contain the total aflatoxin with concentration ranging from 0.02 to 23.3 making it one of the most sensitive and rapid method of aflatoxin detection in tiger nut .

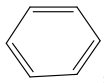
The result for the FTIR method of detection after the interpretation of peaks of the absorption bands (Appendix 2) shows the presence of the functional groups such as  , C=O, C-O, and C-H, found in the structure of the four major aflatoxins in seven of the samples confirming 6 results of the ELISA as seen in Table 4.5

Table 4.1: Average Proximate Composition of the Tigernut

Composition	percentage
Total ash content	3.32%
Total moisture content	5.68%
Total protein content	1.26%
Total lipid content	24.88%
Total carbohydrate	64.86%

Table 4.2: Occurrence of fungi in tiger nut

Sample	Fungal count	*A. flavus*	*A. parasiticus*	*A. nomius*
1	$3.3x10^3$	-	-	-
2	$3.6 x10^3$	+	+	+
3	$5.1 x10^4$	+	+	+
4	$3.2 x10^3$	+	+	-
5	$3.2 x10^3$	+	-	-
6	$3.7 x10^3$	+	+	+
7	$2.8 x10^3$	+	-	-
8	$4.0 x10^3$	+	-	-
9	$3.5 x10^4$	+	+	+
10	$1.2 x10^4$	+	+	+
11	$1.7 x10^5$	+	+	-
12	$1.3 x10^4$	+	+	+
13	$8.0 x10^3$	+	+	+
14	$8.9 x10^3$	+	+	-
15	$1.0 x10^4$	+	+	+
16	$1.4 x10^4$	+	+	-

17	5.1 x10^3	+	+	+
18	3.2 x10^3	+	-	+
19	7.0 x10^3	+	-	-
20	2.6 x10^4	+	+	+

Average 1.80 x 10^3 $cfug^{-1}$

Table 4.3: Occurrence of aflatoxin in tiger nut using TLC technique

Sample	Fluorescence	Occurrence	R F
1	Nil	-	
2	Nil	-	
3	Nil	-	
4	Nil	-	
5	Nil	-	
6	Nil	-	
7	Nil	-	
8	Nil	-	
9	Blue	+	0.44
10	Nil	-	
11	Nil	-	
12	Nil	-	
13	Nil	-	
14	Nil	-	

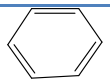
15	Nil	-	
16	Blue	+	0.47
17	Nil	-	
18	Nil	-	
19	Nil	-	
20	Nil	-	

Table 4.4: Occurrence of aflatoxin in tiger nut samples based on ELISA technique

Sample	OD	%OD	Concentration	Occurrence
1	0.865	106	00.0	-
2	0.836	107	00.0	-
3	0.746	90	00.2	+
4	0.839	102	00.0	-
5	0.853	103	00.0	-
6	0.818	100	00.0	-
7	1.002	121	00.0	-
8	0.895	110	00.0	-
9	0.011	1	23.3	+
10	0.933	114	00.0	-
11	0.652	80	02.7	+
12	0.707	87	00.9	+

13	0.722	89	00.4	+
14	0.786	96	00.0	-
15	0.809	99	00.0	-
16	0.013	2	23.2	+
17	0.909	111	00.0	-
18	0.877	107	00.0	-
19	0.909	111	00.0	-
20	0.622	85	01.4	+

Table 4.5: Occurrence of aflatoxin functional group in tiger nut samples using FTIR

Sample		C=O	C-O	C-H	Inference
1	-	+	+	+	Absent
2	-	+	+	+	Absent
3	+	+	+	+	Present
4	-	+	+	+	Absent
5	-	-	+	+	Absent
6	-	+	+	+	Absent
7	-	+	+	+	Absent
8	-	+	+	-	Absent
9	+	+	+	+	Present
10	+	+	+	-	Absent
11	+	+	+	+	Present

12	+	+	+	+	Present
13	-	+	+	-	Absent
14	-	+	+	+	Absent
15	-	+	+	+	Absent
16	+	+	+	+	Present
17	+	+	+	+	Present
18	+	+	-	-	Absent
19	+	+	+	+	Present
20	+	-	-	+	Absent

CHAPTER FIVE

5.0 DISCUSSION

Proximate analysis of the Tigernut showed that it contained about 5.68% of moisture

Which is enough for fungal growth, the Tigernut has high lipid content and carbohydrate which is similar to the report on the proximate composition of Tigernut (Tigernut traders 2013). The study showed low protein content and a high ash content when compared to the (Tigernut traders 2013) probably because of the difference on variety of the Tigernut.

The result of the mycological analysis as seen in Table 4.1showed that the fungal count for most of the samples which fall in the range of $10^3$and 10^4 with an average value of 1.80 x 10^3cfu/g are still within the maximum acceptable limit recommended by the international commission on microbiological specifications for food (ICMSF) of less than

10^5 except for sample eleven which have the fungal load above 10^5 $cfug^{-1}$ this is probably because the storage time and condition such as the moisture content of the tiger nut sample favors the proliferation of the fungi that is why Ogundimu (2010) reported that the average fungal load is around 10^5 $cfug^{-1}$ in Ogun state, Nigeria which has a relatively higher humidity than Kaduna. This explains why the stored tiger nut will contain more moisture making it more favorable for the fungal growth. According to Musa and Hamza (2013), the fungal population of the processed tiger nut drink in Kaduna is between the range of 1.80×10^2 and 7.20×10^2 $cfug^{-1}$ this is probably due to the fact that processing reduced the fungal load. Three aflatoxin producing species were isolated from the samples analysed and *Aspergillus flavus* is the most common occurring in all the samples except for sample one this is because it can survive a very low water activity compared to even *Aspergillus nomius* which is very similar to it (Kurtzman *et al*., 1987). The presence of *Aspergillus nomius* in some samples is also of great health concern because the organism causes a disease known as fungal keratitis if it finds its way into the eye through hand contamination.

The thin layer chromatography technique showed that only two (sample nine and sixteen) out of the twenty samples analysed contain the aflatoxin with R.F. values (retention factor) of 0.44 and 0.47 respectively as seen in Table 4.2 representing 10% of the samples This is because the aflatoxin even if present in some other samples is below the detection limit of the thin layer chromatography procedure for aflatoxin detection which is 3.125µg/kg. This is particularly not worrisome as regard to the TLC detection procedure because even if the aflatoxin is present it is surely below the approved maximum quantity of aflatoxin in ready to eat food which stands at 4µg/kg making 90% of the samples

analysed safe for human and other animals consumption. Isabel *et al.,* (2005) worked on tiger nut drink popularly known as horchata in Spain where only 1 out of 22 or about 4.5% of the samples analysed contain aflatoxin. This is also suggesting that processing could reduce the level of contamination not only by the fungi but even by the aflatoxin. It is important to note the detection of aflatoxin using this technique in a ready to eat food like tiger nut is of great health concern because that alone is indicating that the food contain the aflatoxin in a concentration that at least is very close to the approved maximum level by CODEX alimenterius of 4µg/kg. Prolong exposure to such food may predispose the consumer to high risk of cancer (Cullen and Newberne 1994).

The ELISA test result shows that the number of samples containing the aflatoxin is higher when compared to the two positive samples (10%) obtained using the TLC procedure although only two of the seven positive samples contain aflatoxin in a concentration above the maximum required limit in ready to eat food placed by CODEX alimentarius which is also the limit used in Nigeria by NAFDAC. This means the procedure is also supporting the notion that TLC procedure can be used as a chief and easy tool for preliminary aflatoxin detection in a situation where a more complex procedure is not readily available. The result shows a higher contamination when compared to the work of Natividad *et al.,* (2010) who analysed 37 samples of tiger nut in Spain using liquid chromatography fluorescence detection and a method based on matrix solid phase dispersion extraction and found only three of the samples to becontaminated by aflatoxin in Spain. The presence of high concentration of more than 23µg/kg in two of the samples is indicative of the fact that those samples are stored in a condition that favored the production of the toxin.

In general terms, the samples analysed through the various procedures shows that seven out of twenty samples contained the aflatoxin however, the result also reveals that even though thin layer chromatography detection procedure is not as sensitive as the other techniques it is still enough to screen samples that are consumed raw since it proves that the false negative result shown by the tin layer chromatographic technique are actually in a concentration that is below the minimum acceptable standard by the national agency for food and drug administration making it the food in question at least safe for consumption

The result of the work further shows that the FTIR detection procedure even as sensitive as it is cannot be used as a sole detection technique for the determination of an aflatoxigenic safety of ready to eat food because the procedure only determines the presence of the aflatoxin functional groups in the sample and does not gives any idea on the concentration or at least the minimum concentration of the toxin in the sample analysed unlike the TLC procedure which even though cannot detect a very small concentration of aflatoxin it has a minimum detection limits. Therefore, once the toxin is determined using the procedure it can safely be concluded that the food in question contained aflatoxin concentration, that is at least close to the minimum acceptable limit approved by the relevant agencies such as the national agency for food and drug administration and codex allimenterrious (2004) which stands at 4µg/kg in Nigeria and most of Europe respectively. However, certain countries in the world have a higher tolerable limit for aflatoxin such as china and India for example have 20µg/kg and 30µg/kg respectively making the ELISA procedure to be the best option as it can determine the exact concentration of the sample. Comparing the result of the fungal count

and the ELISA procedure also shows that those samples that have a high fungal load are the most likely to be contaminated by the toxin

5.1 CONCLUSION

From the result obtained it can be concluded that the most of the tiger nut samples examined are safe for human consumption because even if the aflatoxin is present the concentration is still below the maximum acceptable limits by the national food and drugs administration and control and the FAO. However, the presence of samples with high level of aflatoxin contamination suggest that there is need improve storage condition of tiger nut so that the fungal proliferation and aflatoxin production can be reduced to the barest minimum (acceptable limits).

5.2 RECOMMENDATION

- There should be proper storage of the tigernuts to avoid insects and rodent infestation.

- There should be good agricultural practice to minimize the field mold colonization of the tigernut.
- There should be careful and extensive drying of the tigernut to minimize the moisture content.
- Tigernut that appears moldy should be removed from the lots to prevent further invasion.

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