SudanUniversity of Science & Technology

College of Graduate Studies

Determination of Aflatoxin in Peanut and Sesame Cakes by Enzyme-Linked Immunosorbent Assay (ELISA)

تقدير الافلاتوكسين في امباز الفول السوداني وامباز السمسم باستخدام التحاليل المناعيةالمرتبطة بالانزيم

A Thesis Submitted inPartial Fulfillment of theRequirements of Master Degree in Chemistry

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June .2015

Dedication

To

The sweetest gift in my life, my mother.

My family

My colleagues and friends

I dedicate my work

Acknowledgements

All thanks to almighty Allah the sustainer of theuniverses.

I would like to express my gratitude to my supervisor Dr.ELMugdad Ahmed Ali for standing next to me during this study and providing me with the scientific surroundings to carry out this work.

My gratitude is ever so overwhelming towards staff of Arab Poultry Production & Processing Company for their help, especially Dr.Babiker Ahmed and my real friendNuhaBagadi.

I see myself privileged to have the opportunity to collaborate with so many brilliant people. I would like to thank Mr. Mohamed A. for providing mewith test samples.

I am ever so grateful to my dearest mother, Um AL Hassan, for everything. I have been blessed to have family who has supported, encouraged and inspired me endlessly throughout my life.

Abstract

Food security and safety is a basic human need and is considered as a hot issue for national and international organizations in the recent years. Chemical as well microbial food hazards are the most important concern nowadays, and among microbial food and feed hazards, mycotoxin attracted world’s attention towards fungal invasion to food elements.

Several oilseed cakes and meals are produced in Sudan on large amount as by-product of industry of vegetableoil for human consumption. In Sudan, groundnut, sesame, cotton seed and sunflower are the most common oilseeds. These cakes and meals are rich in protein content and usually used for livestock feed . Sorghum, peanut cake ,sesame meal and wheat bran are considered the main source of protein and energy for poultry in Sudan. Although groundnut meal is used commercially as the main source of protein for poultry in Sudan, it has anti-nutritional properties and highly susceptible for aflatoxin contamination.

Aflatoxin, which is produced by Aspergillusflavus and AspergillusParasiticusfungi metabolism, is one of the compounds in the mycotoxin group. The main types of aflatoxinsare AFB1, AFB2, AFG1 and AFG2.Aflatoxin can cause serious biological effectswhen it is consumed regularly at high levels . Some of the effects are inhibition of protein synthesis; inhibition of enzyme induction,inhibition of lipid synthesis and transport, immunosuppression, fatty degeneration and liver cancer.

Enzyme linked immunosorbent assay (ELISA) technique(thesimple ,sensitive, safe reagents and veryaccurate) was used to assess the levels of aflatoxin in peanut and sesame cakes. Randomandhomogenous 40 samples, collected from different markets inKordufan and Darfurin western Sudan during March and April 2013 were extracted with 70% (v/v) methanol. The extracted samples and horse radish proxidase-conjugated aflatoxinwere mixed and addedto the antibody coated microwell. On removal of non-specific reactants, tetra methyl benzidine chromogenic substrate was added and the microwellsmeasuredoptically by microplate reader at 650nm. The results showed that aflatoxin contents of peanut cakes ranged from 20.80µg/kg to 88.30µg/kg. The sesame cakes samples contained between 0.2µg/kgand 5.8 µg/kg aflatoxin.100 % and 90% aflatoxin contamination was found in thetwenty samples of peanut cake and eighteen samples of sesame cake respectivelyinvestigated. With a safe limit of 20 µg/kg set by food and drug administration agency, only the peanut cake samples were higher than allowable levels.Aflatoxin levels were consistently high in peanutcakes indicating that nuts are the most commonly contaminated food commodity.

المستخلص

الامن الغذائي وسلامة الغذاءهو حاجة انسانية اساسية كما تعتبر قضية ساخنة للمنظمات الوطنية والدولية في الماضي القريب . الكيماويات والميكروبات التي تسبب الاخطار الغذائية اصبحت الشاغل الاهم والسموم الفطرية جذبت اهتمام العالم نحو الغزو الفطري للعناصر الغذائية .

يتم إنتاج العديد من امبازو وجبات البذور الزيتية في السودان بكميات كبيرة كمنتج ثانوي من صناعة الزيوت النباتية للاستهلاك البشري. الفول السوداني، السمسم، بذور القطن وعباد الشمس هي البذور الزيتية الأكثر شيوعا في السودان . الامبازغني بالبروتين وعادة ما يستخدم لتغذية الماشية ، ويعتبر امباز الفول السوداني وامباز السمسم وردة القمح المصدر الرئيسي للبروتين والطاقة للدواجن في السودان. على الرغم من أن استخدام امبازالفول السوداني تجاريا كمصدر رئيسي للبروتين للدواجن لديه خصائص مضادة للتغذية وعرضة للتلوث بالافلاتوكسين.

الافلاتوكسين هو ناتج ثانوي لعملية الهضم في الفطريات . وهو احد الميكوتوكسينات وله عدة انوع منها AFB1, AFB2, AFG1وAFG2وهو يسبب امراض خطيرة جدا منها سرطان الكبد. تم استخدام جهاز تحليل المناعات المرتبطة بالانزيم لقياس مستوي الافلاتوكسين في امبازالفول السودانيوامبازالسمسم.تم جمع 40 عينة عشوائية ومتجانسة من ولايتي كردوفان ودارفور في غرب السودان خلال شهري مارس وابريل 2013.وتمت عملية الاستخلاص باستخدام كحول الميثانول (v/v) 70%. وتم خلط العينات المستخلصة بالانزيم المرتبط وونقلت الى الطبق المغطي بالاجسام المناعية ثم ازيلت الاجسام غيرالمرتبطة بالغسيل بالماء المقطروتمت اضاقة الكاشف الملون للرابطة antigen-antibody وتم قياس مستوى الأفلاتوكسينبقراءة الكثافة الضوئية(الامتصاص) بجهاز القراءة ELISA (للبساطة والدقة العالية والكواشف الامنة) وكان مستوبالتلوث بالافلاتوكسين في عينات امباز الفول السوداني20.8-88.3ppb.اما امباز السمسم فقد كان مستوى الافلاتوكسين ضمن الحدود المسموح به(20 ppb)0.2 to 5.8 ppb وهذا يدل على التلوث العالي لامباز الفول السوداني.

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List of Abbreviations

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
ALP	Alkaline phosphate
AOAC	Association of official analytical chemists
ASAE	A merican Society of Agriculural Engineers
BS	Black Sesame
BSA	Bovine serum albumin
DALYS	Disability-adjusted life yeas
DGF	Defatted Groundnut Flour
DMSO	Dimethyl Sulphonic acid
ELISA	Enzyme linked Immunosorbent Assay
FDA	Food and Drug Administration
FAO	Food and Agriculture Organization
HIV	Human Immuno deficiency Virus
HRP	Horseradish peroxidase
IARC	International Agency research on Cancer
IBD	Integrated Breeding Plat form

ICA	immunuaffinity column assay
IgG	Goat anti-rabbit
RIA	Radioimmunoassay
RSDR	The relative standard deviation for reproducibility
SSA	Sub-Saharan Africa
TFA	Triflouroacetic acid
WHO	World Health Organization
WS	White Sesame

CHAPTER ONE

Introduction & literature review

1.1Peanut plant

Peanuts are annual herbaceous, self-pollinating legume crops which belong to the section Arachis, series amphiploidies, familyLeguminosea(Gregory*et al.*,1973) and subfamily Papilionaceae. The distinguishing morphological features of this genus are aerial flowers that give rise to subterranean fruits. There are about 70 species, most of them diploid while two are allotetraploids. Five species have been cultivated, but only A. hypogaea has been domesticated and grown extensively for seeds and oil (Stalker, 1992).

The peanut was probably first domesticated and cultivated in the valleys of Paraguay in South America, Mexico and Central America (Fekria*et al*., 2010).

Figure .1 Peanutplant

Peanut plant (figure .1) is a legume whose fruit develops below the ground(figure .2).The flowers are formed and fertilized above ground, but downward growth of thepegs ensures that the fruit (pods and seeds) develops in the soil

(Waliyar&Reddy,2009). This facilitates the penetration of plant tissues with toxigenic fungi such asAspergillusflavus (Beasley, 2011).

Figure. 2 Peanut leaves and freshly dug pods

1.1.1 Global PeanutProduction

Peanuts are currently grown on over 22.2 million hectares worldwide with a totalproduction of over 35 million tons. India and China are the world's largest producersof groundnuts.

Millions of small-holder farmers in Sub-Saharan Africa (SSA) grow groundnut as a foodand cash crop, accounting for over 9 million hectares of cultivated farmland. Nigeria,Sudan and Senegal are Sub-Saharan Africa's leading producers, and the crop also doeswell in southern Mali and adjacent regions of Burkina Faso and Ghana.Although SSA has 40% of the world total of land under groundnut, its output amounts toonly 25% of total world production due to low yield (0.95 tonnes/ha as compared to 1.8tonnes/ha in Asia) (Integrated breeding platform, 2013).It is a major source of edible oil and protein meal and considered highly valuable for human and animal nutrition especially in the developing world. Presently, because of an increased awareness of the protein shortage existing in the world, the use of groundnuts as a food and cash crop has increased substantially. Peanuts animportant cash crop in the Sudan. In 2007, Sudan produced about 460.000 tons of peanut ,and ranked number nine in the world in the total world production of peanut (FAO, 2008).It comes first in the Arab countries, both in area and production of groundnut. Peanut is grown mainly for its oil, protein, plant residue and seed cake. More than half of the world peanut production is crushed for expulsion of oil, which is diverted mainly as edible oil (Fekria*et al.*, 2010)

Peanuts are known by many other local names such as earthnuts, ground nuts, goober peas, monkey nuts, pygmy nuts and pig nuts. Despite its name and appearance, the peanut is not a nut, but rather a legume. It is the 13th most important food crop and 4th most important oilseed crop of theworld(Reddy *et al*., 2011) after soybean, cottonseed and rapeseed. Worldwide, there are over100 countries growing peanuts and developing countries constitute 97% of Globalarea and 94% of the global production (Ntare*et al.*, 2007). Peanut seeds are a nutritional source of vitamin E, niacin, falacin, calcium, phosphorus,magnesium, zinc, iron, riboflavin, thiamine and potassium. Peanut kernels are consumed directly asraw, roasted ,boiled or also oil extracted from the kernel is used as culinary oil. It is also used asanimal feed (oil pressings, seeds, green material and straw) and industrial raw material (oil cakes andfertilizer). These multiple uses of peanut plant make it an excellent cash crop for domestic markets aswell as for foreign trade in several developing and developed countries.The crop is affected by several diseases like leaf spots, collar rot, rust, bud necrosis, stem necrosis etc,Apart from these, aflatoxin is one of the major problems, produced in the infected peanut seeds byAspergillusflavus and Aspergillusparasiticus, particularly at the end of seasonunder drought conditions (Surendranatha,Sudhakar&Eswara,2011)

1.1.2Usesof Peanut

Peanuts have many uses. All parts of the peanut plant can be used. The peanut, grown primarily for human consumption, has several uses as whole seeds or is processed to make peanut butter, oil, and other products. The seed contains 25 to 32% protein (average of 25% digestible protein) and 42 to 52% oil. A pound of peanuts is high in food energy and provides approximately the same energy value as 2 pounds of beef, 1.5 pounds of Cheddar cheese, 9 pints of milk, or 36 medium-size eggs. They can be eaten raw, used in recipes, made into solvents and oils, medicines, textile materials, and peanut butter (Arun, 2014).

Peanuts have a variety of industrial end uses. Paint, varnish, lubricating oil, leather dressings, furniture polish, insecticides, and nitroglycerin are made from peanut oil. Soap is made from saponified oil, and many cosmetics contain peanut oil and its derivatives. The protein portion is used in the manufacture of some textile fibers. Peanut shells are used in the manufacture of plastic, wallboard, abrasives, fuel, cellulose used in rayon and paper (ASAE ,1983).

1.1.3 Peanut Oil Extraction

Extraction Hydraulic pressing, expeller, and/or solvent extraction are the three general methods for extracting oil from the seed. When hydraulic pressing is used, it is followed by hot solvent extraction for nearly total recovery of the oil.

Expeller extraction relies on friction and pressure within the expeller, which causes the meal to heat, thus facilitating the oil extraction process. This process removes approximately 50% of the peanut oil. The remaining oil is extracted using hexane, which is later removed through an evaporation–condensation system. Solvent extraction involves petroleum hydrocarbons or other solvents. Solvent extraction is accomplished in closed systems where oil is removed and solvent reclaimed for reuse. The efficiency of extraction with hexane, 95% Ethanol, or absolute ethanol on peanut grits has been reported .Extracted oil is refined by deacidificationwith sodium hydroxide to neutralize the free-fatty acids, washing with water at about 82^0C to remove the sodium hydroxide, and then bleaching with bleaching clay at about 100^0 C under reduced pressure. The refined oil is then deodorized by heating under vacuum and blowing superheated steam through the oil. Deacidification and deodorization of peanut oil and other edible oils by dense carbon dioxide extraction has been investigated. The purpose of the refining process is to remove nontriacylglycerol components, including free fatty acids, nonhydratablephosphoacylglycerols, sterols, pigments, glucosides, waxes, hydrocarbons, and other compounds that may be detrimental to the flavor or oxidative stability of the refined oil.(Fereidoon,2005)

1.1.4 Peanutcake

The byproduct of peanut oil production is peanut cake, and depending on the methods used, the oil content remaining in the meal ranges from about 7% to 1%. Human consumption of peanut meal is negligible except in India and Argentina . The primary use of peanut meal is animal feed. When peanut meal is used for human or animal consumption, careful consideration should be given to the quality of the meal.Various oil seeds,ediblenuts, grains, and their derived products are subject to mycotoxin contamination, and these mycotoxins may have a detrimental effect on both human and animal health (Fekria*et al.*,2010).

Mycotoxins are generally associated with the protein fraction and are not found in refined oil because of the processing procedures. Unrefined or lightly refined oil may contain mycotoxins because of the fine residue particles contained therein meal from edible-grade peanuts with low oil content may be processed into flour for human consumption. When poor-quality grades are used, poor extraction efficiencies or lack of hygienic conditions exist, and the residue should be used as a fertilizer(FAO, 1995).

Peanut cake(Figure.3)is an excellent livestock feed because of its high protein content. The cake contains 45-60 protein, 22-30% carbohydrate, 3.8-7.5% crude fiber ,4-6% minerals and 1-7% oil . Defatted peanut flour (DGF) produced from cake blends easily and enhances or enriches the nutritive value of wheat and other flour. The DGF is an underutilized by-product of peanut processing that has potential to be used in food system as low fat peanut concentrate for extending comminuted meat products, production of beverages, fermented products, composite flours and protein supplementation of bakery products and weaning foods. It is also used in shaving cream, metal polish, bleach, ink, soap, shampoo, explosives, paint, rubber, axle grease,paper, wallboard, fireplace logs, cat litter and medicine. Despite the fact that DGF has an excellent potential in food formulations because of the high protein content, its uses remain limited. (Fekria*et al.,* 2010)

Figure.3 Peanut cake

1.2. Sesame plant

Sesame (Sesamunindicum. L., synonymous with Sesamunorientale L.),also knownasbenniseed(Africa),benne(SouthernUnitedStates), gingelly(India),gengelin(Brazil), sim-sim, semsem (Hebrew), and tila (Sanskrit), It is the world’s oldestoil crop. It belongs to the Tubiflorae order, Pedaliaceae family, which comprisesof 16 genera and some 60 species..Sesame grows in tropical and subtropical areas about 40 N latitude to 40Slatitude(figure.4). Sesame indicum L. is the commonly cultivated species of sesame. Ithas 26 somatic chromosomes (2n=26). Sesame is an annual. The fruit of sesame is a capsule (2–5 cm long and 0.5–2 cm in diameter), and itis erect, oblong, brown or purple in color, rectangular in section, deeply groovedwith a short, triangular beak .The capsules may have four, six or eightrows of seeds in each capsule. Most of the sesame capsules have fourrows of seeds, with a total of 70 seeds per capsule. The capsules with a widerdiameter will usually have higher rows of seeds and the total number of seeds percapsule can be as high as 100 ~ 200(Fereidoon,2005).

Figure .4 Sesame plant

Sesame seeds are small (3 ~ 4 mm long and 1.5–2 mm wide), flat, ovate (slightlyThinneratthehilumthanattheoppositeend),smooth,orreticulate.Thecolorvari esfrom white, yellow, gray, red, brown, to blackdepending on the cultivar harvested. The most traded variety of sesame is off-white colored(Figure5). It is generally believedthat the light-colored seeds with thin coats are higher inquality and oil content thanthe dark-colored seeds(Fereidoon,2005).

Figure .5 Sesame colors

The crop is harvested when the leaves, stems and capsules begin to turn yellow and the lowerleaves starts shedding. To prevent the shedding of grains, the crop is not allowed to becomedead ripe in the field. The ripe plantsare cut, carried to the threshing-yard, stacked for a weekin the sun with the cut-ends downwards and well shaken or beaten to take out the grains fromthe dry capsules. Winnowing and cleaning complete the process (Lokeshwar, 1997).

1.2. 1. Uses of Sesame

Sesame seed is a common ingredient in various cuisines. It is used whole in cooking for its rich nutty flavour. Sesame seeds are sometimes added to breads.

The oil is used widely in the some injectable drug formulations. The lignans such as sesamin, episesamin, sesaminol and sesamolin are major constituents of sesame oil and all have chemically methylenedioxyphenyl group (Gokbulut, 2010). It ranks ninth among the topthirteen oilseed crops which make up 90% of the world production of edible oil.(Adeola*et al.*, 2010).

The oil is also useful in the industrial preparation of perfumery, cosmetics (skin conditioningagents and moisturizers, hair preparations, bath oils, hand products and make-up), pharmaceuticals (vehicle for drug delivery), insecticides and paints andvarnishes (Chemonics International Inc., 2002).

Sesame seed has higher oil content(around 50%) than most of the known oil seeds (Hwang, 2005) The seed has 40-60 per cent of oil with almost equallevels of oleic (range 33-50%, typically 41%) and linoleic acids (range 33-50%, typically 43%) and some palmitic acid (range 7-12%, typically 9%) and stearic acid (range 3-6%, typically 6%) (Gunstone, 2004).

Oil can be classified in the oleiclinoleic acid group. The dominant saturated acids were palmitic (up to 8.58%) and stearic (up to 5.44%) (Nzikou*et al.*, 2009). An analytical comparison of the biochemical composition of Black Sesame (BS) and White Sesame (WS) produced in China .Mohammed&Hamza(2008) reported some physical and chemical characteristics of oils extracted from sesame seed(table .1).

Table.1Physical and chemical characteristicsof White Sesame and Black Sesame

physical and chemical characteristics	WS	BS
Colour	White	Black
Iodine value (gI2 /100g)	103	116
Oil content (%)	48	50
Specific gravity (g/cm3)	0.915	0.923
Acid value (mg KOH/g)	0.5	0.45
Peroxide value (Meq KOH/g)	8	7.45
Saponification value (mg KOH/g)	189	191

1.2.2 Processing of Sesame Seeds for Extraction of Oil

The nutrient composition of sesame meal varies widely depending on the variety used, the degree of decortication and the processing method employed .The hulls are separated from the kernel in decorticating machines or by soaking and rubbing the seed. Removal of the hull results in a reduction in fibre content of approximately 50% and increases the protein content, digestibility and palatability of the meal .The hull of the sesame seed accounts for 15 to 29 per cent of the whole seed. Occasionally, the seed is milled without decortication to improve the efficiency of oil extraction. The decorticated or undecorticated sesame seeds are processed for extraction of oil by ghani, rotary, expeller or solvent extraction methods(Yasothai, 2014).

1.2.3. Sesame Oil Extraction

The tradition way of extracting sesame oil from unroasted sesame seeds in India is done by ghani, which is basically a large pestle and mortar. The ghani is driven bybullocks. Sesame seeds are cleaned and dehulled before used in the ghani. Inmany parts of India, water or brown sugar is added to sesame seed in the ghani tofacilitate oil extraction. Sesame oil is removed from the ghani after millingand allowed to settle, skimmed, and sometimes strained through a cloth beforesale. The bullock-driven ghani is replaced by power-driven mills in most of theIndian villages in order to improve the efficiency of oil production(Fereidoon,2005).

The modern commercial methods of oil extraction from oilseeds include batch hydraulic pressing: Oil seeds are expressed by hydraulic pressure to yieldoilcontinuous mechanical pressing: Oil seeds are squeezed through a taperingoutlet and oil is expressed by the increasing pressure; and solventextraction: Oil seeds are extracted with solvent followed by removal of solventto yield oil. These methods are also employed in the extraction of sesame seedswith some modification.

Forunroasted sesameseeds, the commercial extractionof oiliscarried out usinga continuous screw-press or hydraulic press. Sesame seeds are small; they areusually cooked prior to oil extraction. Sesame oil is generally extracted inthreestages. The first stage is cold press; the cold-pressed oil obtained after filtrationis ready to use and has very good quality. It is light in color and agreeable intaste and odor. The second stage pressing is conducted with sesame residue underhigh pressure; it yields highly colored oil that needs refining before used foredible purpose. The residue left after the second stage pressing is extracted forthe third time under similar conditions as the second stage. Sesame oil obtained from the third stage pressing has very low quality and is used for nonedible purposes.

Alternatively, unroasted sesame seeds are pressed once followed by solventextraction to recover the oil from residue. The oxidative stability of sesame oilwas found to be dependent on the extraction method and seed pretreatment.Extraction of the sesame seeds after effective seed crushing with polar solvent,heptane-isopropanol (3:1, v/v), would yield a more stable oil from whole sesameseeds because more antioxidative substances and phospholipids could be extracted.Phospholipids may act as synergists to antioxidants(Fereidoon,2005).

The extraction of sesame oil from roasted sesame seed is generally Performedwith pressing. Solvent extraction is not used because the desirable roasted flavormay be removed during evaporation of solvent. In commercial production, continuousscrew-press or hydraulic press is employed. The hydraulic press can bevertical or horizontal. The continuous screw may be operated twice in order toincrease the oil yield. Proper cooking (100^0C, 7 min) and addition of water(12.5%) after roasting can also raise the oil yield(Fereidoon,2005).

1.2.4 Sesame oil cake

The sesame seed contains about 50% oil and 20-25% protein. The residue sesame oil cake contains on an average 32% crude protein, 8-10% oil, total oil and albuminoids of 40-42% and rich in essential amino acids namely methionine and cystine. Sesame oil cake has different colors (figure.6). It has been used as an excellent protein supplement for dairy cattle. Its usage as a protein supplement for poultry is verylimited. Hence, it was proposed to evaluate the feeding value of sesame oil cake on breast meat composition in commercial broilers. (Boorman,1999).

Figure.6 Different colors of Sesame cake

1.3 Aflatoxins

Humans are continuously exposed to varying amounts of chemicals that have been shown to have carcinogenic or mutagenic properties in environmental systems.Exposure can occur exogenously when these agents are present in food, air or water, andalso endogenously when they are products of metabolism or pathophysiologic states suchas inflammation. Great attention is focused on environmental health in the past twodecades as a consequence of the increasing awareness over the quality of life due tomajor environment pollutants that affect it. Studies have shown that exposure toenvironmental chemical carcinogens have contributed significantly to cause humancancers, when exposures are related to life style factors such as diet (Wogan*et al.,* 2004).

The contamination of food is part of the global problem of environmental pollution. Foodstuffs have been found contaminated with substances having carcinogenic, mutagenic, teratogenic and allergenic properties. As these substances canbe supplied with food throughout the entire life-time of a person, it is necessary to dealwith the chronic action of trace amounts of such substances. Hence the systematicdetermination of the foreign substances in nutritional products and feedstock plays animportant role. The determination of trace impurities presents considerable difficultiesowing to the fact that food is a complex system containing thousands of major and minorcompounds (Nilufer&Boyacio, 2002).

Aflatoxins are well recognized as a cause of liver cancer, but they have additional important toxic effects. In farm and laboratory animals, chronic exposure to aflatoxins compromises immunity and interferes with protein metabolism and multiple micronutrients that are critical to health. These effects have not been widely studied in humans, but the available information indicates that at least some of the effects observed in animals also occur in humans. The prevalence and level of human exposure to aflatoxins on a global scale have been reviewed, and the resulting conclusion was that ≈4.5 billion persons living in developing countries are chronically exposed to largely uncontrolled amounts of the toxin. A limited amount of information shows that, at least in those locations where it has been studied, the existing aflatoxin exposure results in changes in nutrition and immunity. The aflatoxin exposure and the toxic affects of aflatoxins on immunity and nutrition combine to negatively affect health factors (including HIV infection) that account for >40% of the burden of disease in developing countries where a short lifespan is prevalent. Food systems and economics render developed-country approaches to the management of aflatoxins impractical in developing-country settings, but the strategy of using food additives to protect farm animals from the toxin may also provide effective and economical new approaches to protecting human populations. (Jonathan *et al.*,2004)

Aflatoxin is a common contaminant of foods, particularly in the staple diets of many developing countries. This toxin is produced by fungal action during production, harvest, storage, and food processing, and it is considered by the US Food and Drug Administration (FDA) to be an unavoidable contaminant of foods. The FDA's goal has been to minimize contamination; this goal was realized by implementing regulations that required special attention to the management of the problem. However, the methods used to ensure minimal contamination in developed countries cannot realistically be used in developing countries, because of the characteristics of the food systems and the technological infrastructure in those countries; therefore, aflatoxins are uncontrolled in these situations. The result is a "divide" in the prevalence of aflatoxicosis exposure between people living in developed and developing countries (Jonathan *et al.*,2004).

The World Health Organization (WHO) does not recognize aflatoxins as a high-priority problem because of their analysis of factors contributing to the burden of disease across the world. For developing countries where a short lifespan is prevalent, the priority issues identified by the WHO and their importance as factors contributing to the burden of disease [as measured by disability-adjusted life years (DALYs)] are presented in table 2.(Jonathan *et al.*,2004).

Table: 2 Disability-adjusted life years (ALAYs)for developing countries

Risk factor	Percentage of DALYs%
Underweight	14.9
Unsafe sex	10.2
Unsafe water	5.5
Indoor smoke from solid fuels	3.7
Zinc deficiency	3.2
Iron deficiency	3.1
Vitamin A deficiency	3.0
Blood pressure	2.5
Tobacco use	2.0
Cholesterol	1.9

World Health Organization priority health risks and associated burden of disease in disability-adjusted life years (DALYs) for developing countries where short lifespan is prevalenthas not identified aflatoxins as a high-priority risk. By application of the logic (derived from the current medical focus on the carcinogenicity of aflatoxin) that aflatoxin is reflected in the incidence of liver cancer alone, the priorities defined in Table:2 are justified. However, review of literature shows that, because of the immunologic and nutritional effects of aflatoxin, there is a reasonable probability that the 6 top WHO risk factors (which account for 43.6% of the DALYs in countries where short lifespan is prevalent), as well as the risks of liver cancer, are modulated by aflatoxin(Jonathan *et al.,*2004)*)*.

1.3.1 Occurrence of Aflatoxins

Aflatoxins are a group of heterocyclic, oxygen-containing mycotoxins thatpossess the bisdifuran ring system. It was discovered some 43 years ago in Englandfollowing a poisoning outbreak causing 100,000 turkeys death (Cespedez&Diaz, 1997). The aflatoxins are the most widely distributed fungal toxinsin food. The occurrence of the aflatoxins in food products demonstrated that the highlevels of aflatoxins are significant concern both for food traders and food consumers(Tozzi*et al.,* 2003). Aflatoxin is a by-product of moldgrowth in a wide range of agriculture commodities such as peanuts,maize and maize based ,cottonseeds, cocoa, coffee beans, medical herbs, spices, melonseeds and also in human food such as rice , groundnut , peanut products , corn , vegetableoil , beer , dried fruits, milk and dairy products (Arrus*et al.,* 2004). Meat andmeatproducts are also contaminated with aflatoxins when farm animals are fed withaflatoxin contaminated feed (Chiavaro*et al*., 2001).

The molds that are major producers of aflatoxin are Aspergillusflavusand Aspergillusparasiticus. Aspergillusflavus, which is ubiquitous, produces B aflatoxinswhileAspergillusparasiticus, which produces both B and G aflatoxins, has more limited distribution(Garcia-Villanova et al., 2004). A picture of Aspergillusflavus seen under an electronmicroscope is shown in Figure :7

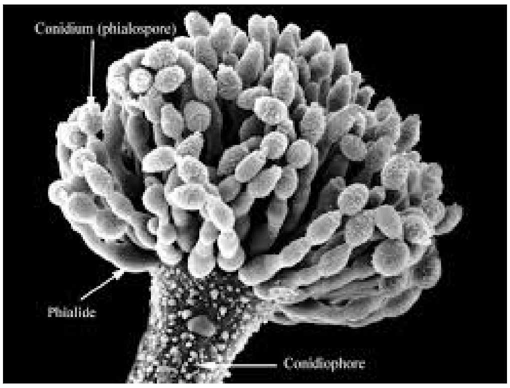

Figure7.Aspergillusflavus seen under an electron microscope

Black olive is one of the substrate for Aspergillusparasiticus growth andaflatoxin B1 production as reported by(Leontopoulos*et al*., 2003). Biosynthesis ofaflatoxins by this fungi depends on the environmental condition such as temperatureand humidity during crop growth and storage (Tarin*et al*.,2004). The optimum temperatures for aflatoxins growth are27.84^{0}C and 27.30^{0}C at pH=5.9 and 5.5 respectively.

Before harvest, the risk for the development of aflatoxins is greatest duringmajor drought (Turner *et al*., 2005). When soil moisture is below normal andtemperature is high, the number of Aspergillus spores in the air increases. These sporesinfect crops through areas of damage caused by insects and inclement weather. Once infected, plant stress occurs, which favor the production of aflatoxins. Fungal growthand aflatoxins contamination are the sequence of interactions among the fungus, thehost and the environment. The appropriate combination of water stress, hightemperature stress and insect damage of the host plant are major determining factors inmold infestation and toxin production (Koehler*et al.*, 1985). Additional factors such as heat treatment, modified-atmospherepackaging or the presence of preservative, also contribute in increasing growth rate ofthe aflatoxins.

Farmers have minimal control over some of these environmental factors. However appropriate pre-harvest and post-harvest management and good agriculturalpractice, including crop rotation, irrigation, timed planting and harvesting and the useof pesticides are the best methods for preventing or controlling aflatoxinscontamination (Turner *et al.,* 2005). Timely harvesting could reduce crop moisture to apoint where the formation of the mould would not occur. For example harvesting cornearly when moisture is above 20 percent and then quickly drying it to a moisture levelof at least 15 percent will keep the Aspergillusflavus from completing its life cycle,resulting in lower aflatoxin concentration. Aflatoxins are to be found in agriculturalproducts as a consequence of unprosperous storage conditions where humidity of 70- 90% and a minimum temperature of about 10^0 C. Commodities that have been driedto about 12 to 0.5 % moisture are generally considered stable, and immune to any riskof additional aflatoxins development. Moreover, the minimum damage of shells duringmechanized harvesting of crop reduces significantly the mould contamination.

Biocontrol of aflatoxin contamination is another way to reduce this contamination. Thenatural ability of many microorganisms including bacteria, actinomycetes, yeasts,moulds and algae has been a source for bacteriological breakdown of mycotoxins. Themost active organism such as Flavobacteriumaurantiacum which in aqueous solutioncan take up and metaboliseaflatoxins B1, G1 and M1 (Smith &Moss, 1985).

Production of aflatoxins is greatly inhibited by propionic acid as revealed by(Molina &Gianuzzi,2002) when they studied the production of aflatoxins in solidmedium at different temperature, pH and concentration of propionic acid.

Other chemicals that can inhibit the growth of this fungus are ammonia, coppersulphate and benzoic acid(Gowda *et al.*, 2004).

1.3.2 Chemistry of aflatoxins

Aflatoxins can be classified into two broad groups according tochemical structure whichare difurocoumarocyclopentenoneseries and difurocoumarolactone(Heathcote, 1984).They are highly substituted coumarin derivatives that contain a fused dihydrofuranmoietyThe chemical structure of coumarin is shown in Figure :8

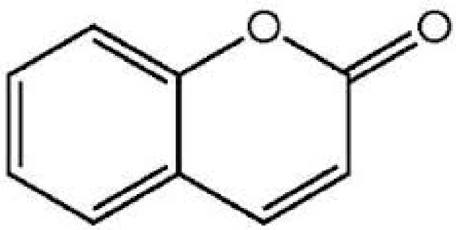

Figure 8 : Chemical structure of coumarin

There are six major compounds of aflatoxin such as aflatoxin B1 (AFB1),aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1) andaflatoxin M2 (AFM2)(Goldblatt, 1972). The former four are naturally found aflatoxins and the AFM1 andAFM2 are produced by biological metabolism of AFB1 and AFB2 from contaminated feedused by animals.They are odorless, tasteless andcolorless. The scientific name for these aflatoxin compounds are listed in Table 3

Table 3: Scientific name for aflatoxin compounds

Aflatoxin B1 (AFB1)	2,3,6a,9a-tetrahydro-4-methoxycyclopenta[c] furo[3’,2’:4,5]furo[2,3-h][l] benzopyran-1,11-dione
Aflatoxin B2 (AFB2)	2,3,6a,8,9,9a-Hexahydro-4-methoxycyclopenta[c] furo[3’,2’:4,5]furo[2,3-h][l] benzopyran-1,11-dione
Aflatoxin G1 (AFG1)	3,4, 7a,10a-tetrahydro-5-methoxy-1H, 12H furo[3’,2’:4,5]furo[2,3-h]pyrano[3,4-c]l]- benzopyran
Aflatoxin G2 (AFG2)	3,4,7a,8,9,10, 10a-Hexahydro-5-methoxy-1H,12H- furo[3’,2’:4,5]furo[2,3-h]pyrano[3,4-c][l]- benzopyra
AflatoxinM1 (AFM1)	4-Hydroxy AFB1
AflatoxinM2	4-Hydroxy AFB2

Aflatoxins have closely similar structures and form a unique group of highly oxygenated,naturally occurring heterocyclic compounds. The chemical structures of these aflatoxinsare shown in Figures 9 and 10

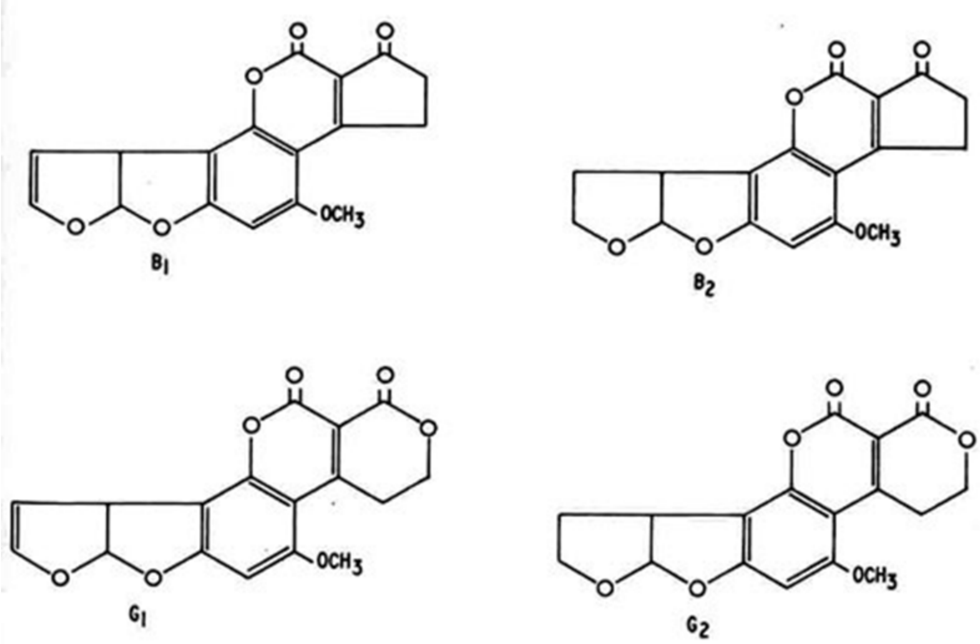

Fig. 1 Structures of aflatoxins B_1, B_2, G_1, and G_2.

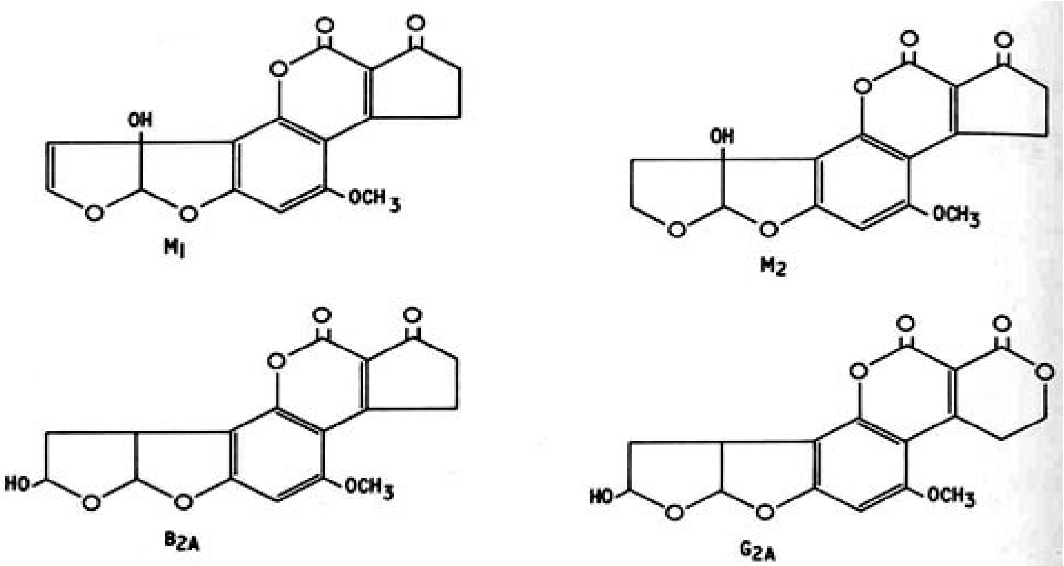

Fig. 2 Structures of aflatoxins M_1, M_2, B_{2A}, and G_{2A}.

The G series of aflatoxin differs chemically from B series by the presence of a β-lactonering instead of a cyclopentanone ring. Also a double bond that may undergo reductionreaction is found in the form of vinyl ether at the terminal furan ring in AFB1 and AFG1but not in AFB2 and AFG2. However this small difference in structure at the C-2 and C-3double bond is associated with a very significant change in activity, whereas AFB1 andAFG1 are carcinogenic and considerably more toxic than AFB2 and AFG2.The dihydrofuran moiety inthestructure is said to be of primary importance in producing biological effects.

Hydroxylation of the bridge carbon of the furan rings for AFM1 does not significantlyalter the effects of the compounds. The absolute configuration of AFB2 and AFG2follows from the fact that it is derived from the reduction of AFB1 and AFG1respectively.

AFB is the aflatoxin which produces a blue color under ultraviolet while AFGproduces the green color. AFM produces a blue-violet fluorescence while AFM2produces a violet fluorescence (Goldblatt, 1972). Thenatural fluorescence of aflatoxins arises from their oxygenatedpentaheterocyclicstructure. The fluorescence capacity of AFB2 and AFG2 is ten times larger than that ofAFB1 and AFG1, probably owing to the structural difference, namely double bond onthe furanic ring. Such a double bond seems to be very important for the photophysicalproperties of these derivatives measured just after spectroscopic studies (Cepeda*et al.*,1996). The excitation of the natural fluorescence of AFB1 and AFG1 can be promotedin many different ways such as post-column iodination ,post-column bromination, use of cyclodextrin compound and trifluoroacetic acid, TFA (Nieduetzki*et al*., 1994).

AFB1 and AFG1 form hemiacetals, AFB2a and AFG2a when reacted withacidic solution such as triflouroacetic acid (TFA) as represented in Figure

:11(Joshua,1993). The hydroxyaflatoxins are unstable and tend to decompose to yellowproductsin the presence of air, light and alkali. Their UV and visible spectra are similar to thoseof the major aflatoxins.

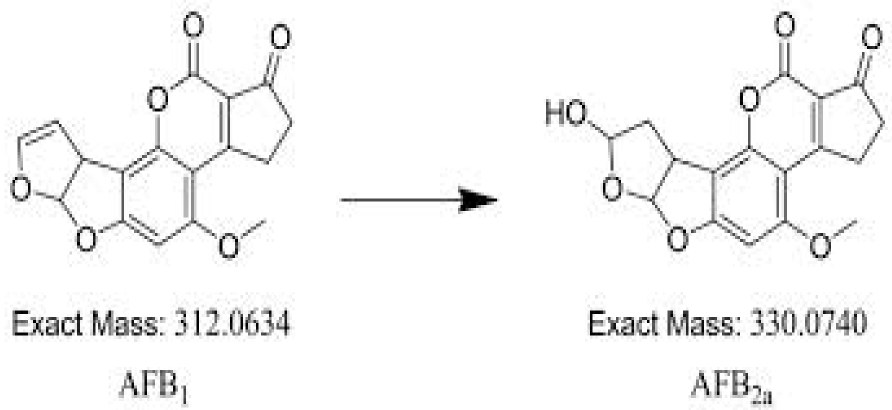

Figure 11: Hydration of AFB1 by TFA produces AFB2a (Joshua, 1993).

The close relationship between AFB1, AFG1, AFB2a and AFG2a was shown by thesimilarities in their IR and UV spectra. The main difference between AFB2a andAFG2a with AFB1 and AFG1 are found in the IR spectra, where an additional band at3620 cm^{-1} indicates the presence of a hydroxyl group in AFB2a and AFG2a. Theabsence of bands at 3100, 1067 and 722 cm^{-1} (which arise in AFB1 and AFG1 from thevinyl ether group) indicates that the compounds are hydroxyl derivatives of AFB2 andAFG2. Some chemical and physical properties of aflatoxin compounds are listed inTable 4:(Weast&Astle, 1987).

Table 4:Chemical and physical properties of aflatoxin compounds

	AFB1	AFB2	AFG1	AFG2
Molecular formula	$C_{17}H_{12}O_6$	$C_{17}H_{14}O_6$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$
Molecular weight	312	314	328	330
Crystals	Pale yellow	Pale yellow	Colorless	Colorless
Melting point(° C)	268.9	286.9	244.6	237.40
Fluorescenceunder UV light	Blue	Blue	Green	Green
Solubility	Soluble in water and polar organic solvent. Normal solventsare: methanol, water: acetonitrile (9:1), trifluoroacetic acid, methanol: 0.1N HCl (4:1), DMSO and acetone			
Other properties	Odorless, colorless and tasteless in solution form. Incompatible with strong acids, strong oxidising agents andstrong bases. Soluble in water, DMSO, 95% acetone orethanol for 24 hours under ambient temperature			

Many researches have studied the stability of aflatoxins.For example, AFB1was not found in fruit samples after being irradiated with 5.0 Kg or more of gamma irradiationas reported by(Aziz &Moussa ,2002). (Gonzalez *et al*., 1998) have studiedthe effect of electrolysis, ultra-violet irradiation and temperature on the decompositionof AFB1 and AFG1. UV irradiation caused an intense effect on aflatoxins where after60 min of radiation, AFG1 suffers practically with no more decomposition. However,when the aflatoxin solutions were placed in a 90° C bath for 3 min, a decrease of 20%from total amount of both AFB1 and AFG1 was obtained. A greater extent ofdecomposition (50%) was found for treatment at 100° C during longer time. Levi(1980) reported that experimental roasting under conditions simulating those of thetypical roasting operation (20 min at 200 ± 5° C) destroyed about 80% of AFB1 addedto green coffee. It was also found that AFB2 decomposed to a larger extent than AFG1indicating a lower stability against prolonged heat treatment. During foodfermentation which involved other fungi such as Rhizopusoryzae and R. oligosporus,cyclopentanone moiety of AFB1 was reduced resulting in the formation of aflatoxicolA which is non-toxic compound. It retains the blue-fluorescingproperty of AFB1 under UV light. It has been considered to be one of the mostimportant B1 metabolites because there is a correlation between the presence of thismetabolite in animal tissues and body fluids with toxicity of AFB1 in different animals(Lau a& Chu, 1983). Aflatoxin solution prepared in water, dimethyl sulphonic acid(DMSO), 95% acetone or ethanol is stable for 24 hours under ambient conditions.

AFM1 is relatively stable during pesteuring, sterilisation, preparation and storage ofvarious dairy products (Gurbay*et al*., 2004).

1.3.3 Aflatoxins and health

Aflatoxins have received greater attention than any other mycotoxins.There arepotent toxin and were considered as human carcinogen by The International Agency forResearch on Cancer (IARC) as reported in World Health Organisation (WHO)'smonograph (1987). These mycotoxins are known to cause diseases in man and animalscalled aflatoxicosis (Eaton &Groopman, 1994). Human exposure to aflatoxins isprincipally through ingestion of contaminated foods (Versantroort et al., 2005).Inhalation of the toxins may also occur occasionally due to the occupational exposure.After intake of contaminated feedstuffs aflatoxins cause some undesirable effect inanimals, which can range from vomiting, weight loss and acute necrosis to varioustypes of carcinoma, leading in many cases to death. Even at low concentration, aflatoxins diminish the immunefunction of animals against infection. Epidemiological studies have shown acorrelation between liver cancer and the prevalence of aflatoxins in the food supply.(Pestka&Bondy, 1990).

In views of occurrence and toxicity, AFB1 is extremely carcinogenic whileothers are considered as highly carcinogenic. It isimmunosuppressive and a potent liver toxin; less than 20 μg of this compound is lethalto duckling (Hussein &Brasel, 2001). AFB1 is biochemically binding to DNA,inhibit DNA, RNA, and protein syntheses, and effects DNA polymerase activity. Previous research results have demonstrated thecovalent binding of highly reactive metabolite of AFB1 to the N-7 atom of guanineresidues, resulting in major DNA adducts (Johnson *etal*.,2000).

The four main aflatoxins display decreasing potency in the order AFB1 > AFG1 > AFB2 > AFG2 .This order of toxicity indicates that thedouble bond in terminal furan of AFB1 structure is a critical point for determining thedegree of biological activity of this group of mycotoxins. Itappears that the aflatoxins themselves are not carcinogenic but rather some of theirmetabolites. For example, metabolite transformation of AFB1 bycytochrome P-450 enzyme produces aflatoxin Q1 (AFQ1), AFM1, AFB1-epoxide andaflatoxin P1 (AFP1). For hamiacetals, AFB2a and AFG2a,however, are relatively non-toxic despite the close similarity of their structure to those of AFB1 and AFG1 even at the highest dosages (1200 μg for AFB2a and 1600 μg forAFG2a)(Hall &Wild, 1994).

AFM1 is the main metabolic derivatives of aflatoxins in several animals speciesIt is found in the cow milk that which has consumed feed which is contaminated withAFB1 as reported by (Tuinstra,1990). The relative amount of AFM1excreted is related to the amount of AFB1 in the feed, and about 0.1% of AFB1ingested is excreted into milk as AFM1. There was a linear relationshipbetween the amount of AFM1 in milk and AFB1 in feeds consumed by animals asreported by(Dragacci,1995). AFM1 is produced by hydroxylation of AFB1 in the liverof lactating animals, including humans. It is also known as milk toxin which is muchless carcinogenic and mutagenic than AFB1. It has been classified by the InternationalAgency For Research Cancer (IARC) as a Group 2 carcinogen (IARC, 1993). It cangenerally be found in milk and milk products such as dry milk, butter, cheese,yogurt and ice cream. AFM2 is the analogous metabolic derivatives of AFB2.

Aflatoxins have been considered as one of the most dangerous contaminant infood and feed. The contaminated food will pose a potential health risk to human such asaflatoxicosis and cancer (Jeffrey &Williams, 2005). Aflatoxins consumption bylivestock and poultry results in a disease called aflatoxicosis whose clinical sign foranimals include gastro intestinal dysfunction, reduced reproductivity, reduced feedutilisation, anemia and jaundice. Humans are exposed to aflatoxins by ingestion,inhalation and dermal exposure as reported by (Etzel,2002). LD50 which is the amountof a materials, given all at once, causes the death of 50% (one half) of a group of testanimal for most animal and human for AFB1 is between0.5 to 10.0 mg kg-1 body weight (Smith &Moss, 1985). Clinicalfeatures were characterised by jaundice, vomiting, and anorexia and followed byascites, which appeared rapidly within a period of 2-3 weeks.

Besides causing health problems to humans, aflatoxin also can causeadverseeconomic effect whichlowers yield of food production and fiber crops andbecoming a major constraint of profitability for food crop producer countries.It has been estimated thatmycotoxin contamination may affect as much as 25% of the world's food crop eachyear resulting in significant economic loss for these countries. Aflatoxins also inflict losses to livestock and poultry producers from aflatoxincontaminatedfeeds including death and the more subtle effects of immune systemsuppression, reduced growth rates, and losses in feed efficiency (Rachaputi*et al*., 2001).

Due to the above reason, aflatoxin levels in animal feed and various human foodproducts is now monitored and tightly regulated by most countries. In Malaysia,the action level for total aflatoxins is 15ppb (Malaysian Food Act, 1983). TheEuropean Commission has set limits for the maximum levels of total aflatoxins andAFB1 allowed in groundnuts, nuts, dried fruit and their products. For foods ready forretail sale, these limits are 4 ppb for total aflatoxins and 2 ppb for AFB1, and for foodsthat are to be processed further the limits stand at 15 ppb for total aflatoxins and 8 ppbof AFB1, (European Commission Regulation, 2001). The Food and DrugAdministration (FDA) in USA has established an action level of 0.5 ppb of mycotoxin,AFM1, in milk for humans and 20 ppb for other aflatoxins in food other than milk.

In Brazil, the limits allowed for food destined for human consumption are 20µg/kg of total aflatoxin for corn in grain, flours, peanut and by-products, and 0.5 µg/l ofAFM1 in fluid milk (Sassahara*et al*., 2005). Australia has established a minimum levelof 15 ppb for aflatoxin in raw peanut and peanut product (Mackson*et al*., 1999). InGermany, regulatory levels of total aflatoxins are 4 ppb and 2 ppb for AFB1. Forhuman dietary products, such as infant nutriment, there are stronger legal limits, 0.05ppb for AFB1 and the total aflatoxins (Reif&Metzger, 1995). The Dutch Food Actregulates that food and beverages may contain no more than 5 µg of AFB1 per kg(Scholten&Spanjer, 1996).

Regulatory level set by Hong Kong government is 20 ppb for peanuts andpeanut products and 15 ppb for all other foods (Risk Assessment Studies report, 2001).Because of all these reasons, systematic approaches to sampling, sample preparationand selecting appropriate and accurate method of analysis of aflatoxin are absolutelynecessary to determine aflatoxins at the part-of-billion (ppb) level as reported by Parkand Rua (1991).

1.3.4 Aflatoxins Measurement

Much research work has been devoted over the last 40 years for developing methods fordetection and determination of aflatoxins in foods and agriculture commodities (Holcomb, *et al*., 1992). This effort is continuing and keeping pace with the progress inanalytical chemistry. Methods for aflatoxins are required to meet the legislation, monitoringand survey work, and for research. Different highly efficient and sophisticated techniqueshave been developed in the recent years for the determination of aflatoxins in differentcommodities. Presently the most commonly used methods for detection of aflatoxins are:high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) andenzyme-linked immunosorbent assay (ELISA) (Lee *et al*., 2009) and fluorometeric method(Hansen, 1990). All analytical procedures include the steps: sampling, extraction, clean- up(purification) and determination (identification and quantification).

1.3.4.1Sample preparation techniques

Sampling and sample preparation is of utmost importance in the analytical identification ofaflatoxins. It certainly affects the final conclusion. For the determination of aflatoxins at theparts-per-billion level, the systematic approaches to sampling, sample preparation andanalysis are absolutely necessary. European Union has formed specific plans for certaincommodities e.g. corn and peanuts. The performance of sampling plans for aflatoxin ingranular feed products, such as shelled maize (Johansson, *et al*., 2000) and cotton seed(Whitaker *et al*., 1976) has been evaluated, while there has been little evaluation of samplingplans to detect aflatoxin in milk.

In case of sampling of solid commodities the entire primary sample must be ground andmixed so that the analytical test portion has the same concentration of toxin as the originalsample. In case of sampling of liquid commodities like milk, due to homogeneousdistribution of aflatoxins in liquid milk, there is less uncertainty in aflatoxin measurement inmilk. After proper sampling, there are the steps of extraction and clean-up which may be the sameor different step. Extraction of samples, together with effective clean-up step, isan essential step in the analysis of aflatoxins. The *analyte* dissolves into the extractionsolvent. The interfering compounds are removed by clean-up step. Common extractionsolvents for aflatoxins are acetonitrile-water and methanol-water.(Irineo,2011)

In addition to conventional technique of liquid-liquid extraction, there was need to developnew techniques due to its time consuming and tedious to apply nature. The new approacheshave been developed to lessen the problems. A number of clean-up columns, using differentprinciples such as solid phase extraction and immunoaffinity techniques, have beendeveloped. The new techniques are easy to use and easily available. The immunoaffinitycolumns enhance selectivity, as only the analyte is retained in the column which can be elutedeasily. On the other hand, in Mycosep columns the analyte is passed and all the otherinterfering contaminants are retained(Irineo,2011).

1.3.4.2 Detection techniques

After the extraction of the analyte (aflatoxin) from the sample and applying a clean-upprocedure to remove interferences, then comes identification and quantification which is the last step inthe analytical methodology. For the detection of aflatoxins, three main types of assays havebeen developed. These include biological, analytical and immunological methods. Thebiological methods were used when analytical and immunological methods were notavailable for routine analysis. Biological assays are non-specific and time consuming andare qualitative in nature.

1.3.4.2.1 Analytical methods

Many analytical methods have been developed and are available forestimation ofaflatoxinsin agricultural commodities. These include: thin-layer chromatography, high performancethin-layer chromatography,and high-performance liquid chromatography(Irineo,2011).

1.3.4.2.2 Immunological methods

Immunological methods are based on the affinities of the monoclonal or polyclonalantibodies for aflatoxins. Due to the advancement in biotechnology, highly specificantibody-based tests are now commercially available for measuring aflatoxins in foods inless than ten minutes. There are two major requirements for immunological methods. Firstrequirement is high quality antibodies and second is methodology to use the antibodies forthe estimation of aflatoxins. Being low molecular weight molecules, aflatoxins cannotstimulate the immune system for the production of antibodies. Such molecules of lowmolecular weight, which cannot evoke the immune system, are called haptens. Therefore,before immunization, aflatoxins must be conjugated to a carrier molecule which is a largermolecule like proteins. Bovine serum albumin (BSA) is most commonly used as a carrierprotein and hapten is conjugated with it. The three types of immunochemical methods are:immunuaffinity column assay (ICA), radioimmunoassay (RIA), and enzyme-linkedimmunosorbent assay (ELISA). Immunoaffinity columns are mainly used for clean-uppurposes and RIA has limited use in aflatoxins analysis. ELISA is most commonly used forthe estimation of aflatoxins(Irineo,2011).

Many rapid tests, using specific antibodies for isolation and detection of mycotoxins in foodhave been discussed and applied by various workers (Newsome, 1987; Groopman& Donahue,1988). Use of immunoaffinity cartridges is a more recent advanced quantitative extraction ofaflatoxin. Monoclonal antibodies specific for aflatoxin are immobilized on Sepharose® andpacked into small cartridges. The work of (Mortimer et al.,1987) is very important as it isthefirst published method for aflatoxin M1 with immunoaffinity columns. For the aflatoxindetermination, a milk sample is loaded onto the affinity column. The antigen i.e., aflatoxin isselectively complexed by the specific antibodies on the solid support to form antigen-antibodycomplex. Then, the column is washed with water to remove all other matrix components ofthe sample. A small volume of pure acetonitrile is used to elute the aflatoxin and the eluate isconcentrated and analyzed by HPLC coupled with fluorescence detection.

Many collaborative studies were done to develop the immunological methods; especially for aflatoxin M1. Immunoaffinity-based methods for aflatoxin M1 were modified and subsequently published and studied collaboratively under the auspices of the International Dairy Federation and AOAC international by groups of mainly European laboratories that could determine aflatoxin M1 in milk at concentrations equal to 0.05 µg/ L. The collaborative study of (Tuinstra*et al.*, 1993) led to International Dairy Federation Standard 171. Another collaborative study (Dragacci*et al.*, 2001) was conducted to evaluate the effectiveness of an immunoaffinity column clean-up liquid chromatographic for determination of aflatoxin M1 in milk at proposed European regulatory limits. The procedure included centrifugation, filtration, and application of the test portion to an immunoaffinity column. Then the column was washed with water and aflatoxin was eluted with pure acetonitrile. Aflatoxinwas separated by reversed-phase liquid chromatography and detection was made with fluorescence detector. Liquid milk samples (frozen), both naturally contaminated with aflatoxin M1 and blank samples for spiking, were sent to 12 collaborators in 12 different European countries. Test portions of milk samples were spiked at 0.05ng aflatoxin M1per mL. After the removal of two non-compliant sets of results, the mean recovery of aflatoxin M1 was 74%. The relative standard deviation for repeatability (RSDr) ranged from 8 to 18%, based on results of spiked samples (blind pairs at 1 level) and naturally contaminated samples (blind pairs at 3 levels). The relative standard deviation for reproducibility (RSDR) ranged from 21 to 31%. As evidenced by HORRAT values at the low level of aflatoxin M1 contamination, the method was acceptable within and between laboratory precision data for liquid milk. The collaborative study resulted in approval of AOAC Official Method 2000.08 (AOAC Official Method 2000.08, 2005).

1.3.4.2.2.1 Radioimmunoassay (RIA).

The radioimmunoassay technique relies on the principle of competitive binding betweena radioactive-labeled antigen and a nonradioactive antigen. The radioactive-labeled antigen competes with unlabelled nonradioactive antigen for a fixed number of antibody or antigen binding sites on the same antibody.A known quantity of labeled antigen and unknown amount of unlabeled antigen from standards competitively react with a known and limiting amount of the antibody . The amounts of labeled antigen are inversely proportional to the amount of unlabeled antigen in the sample, Radioimmunoassay was the first immunoassay technique to be developed and was applied in the detection of insulin in human blood. Radioimmunoassay has also been used for analysis of aflatoxins in food samples. The use of solid phase radioimmunoassay technique in the determination of aflatoxin B1 in peanut andadetectionlimitof1µg/kg was achieved. Similarly, radioimmunoassays have been used for the qualitative and quantitative determination of aflatoxin B1 levels andaflatoxinM1 levels . The major advantage of radioimmunoassay is the ability to perform multiple analyses simultaneously with high levels of sensitivity and specificity .However, RIAs also suffer from a number of disadvantages: it requires an antigen in a pure state, a radioactive isotope is used as a label and is associated with potential health hazards, and it has problems associated with the storage and disposing of the low-level radioactive waste . These disadvantages have limited the frequent use of RIA in the day today analysis of aflatoxins (Alex *et al,* 2014).

1.3.4.2.2.2 Enzyme-Linked Immunosorbent Assay (ELISA).

The potential health hazards related to the use of radioimmunoassay led to the lookout for a safer alternative and a suitable alternative to radioimmunoassay was to replace a radio active signal with a nonradioactive one. This was achieved by labeling either the antigens or the antibodies with enzymes instead of isotopes (Alex *et al,* 2014).

Two types of ELISA have been used for the analysis of aflatoxins: direct ELISA, and indirect ELISA. Both types are heterogeneous competitive assays. Direct competitive ELISA involves the use of an aflatoxin-enzyme conjugate, while indirect competitive ELISA involves the use of a protein-aflatoxin conjugate and a secondary antibody such as goat anti-rabbit IgG to which an enzyme has been conjugated. Although horseradish peroxidase (HRP) is the most commonly used enzyme for conjugation, other enzymes such as alkaline phosphatase have also been used (Anjaiah*et al*. 1989).

1.3.4.2.2.2.1 Direct Competitive ELISA

In this assay, a specific antibody is first coated to a solid phase such asamicroliterplate. The sample extract or standard toxin is generallyincubated simultaneously with enzyme conjugate or separately incubated in twosteps. After appropriate washings, the amount of enzyme bound to the plate isdetermined by incubation with a specific substrate solution. The resulting color isthen measured spectrophotometrically or by visual comparison with standards. Sincethis assay is based on competition for antibody binding sites, free toxin concentrationis inversely related to antibody-bound enzyme conjugate.(Chu *et al*., 1987)

Several direct competitive ELISA procedures have been reported forthe analysisof aflatoxins in groundnuts and groundnut products. Some of the ELISAprocedures took a rather long time to complete, and gave large coefficients of variationwithin each sample because the sample matrix often interfered with the assays .This problem could be overcome by dilution of the sample to arange which does not affect the assay or by using acontrol sample extract as diluent.(Chu *et al*., 1987).

In aflatoxin analysis, directcompetitive enzyme-linked immunosorbent assay is used. The enzyme-linkedimmunosorbent assay is detection and quantification of an antigen (aflatoxin) in a sample byusing an enzyme labeled toxin and antibodies specific to aflatoxin. The enzyme-linkedimmunosorbent assay is based on antigen-antibody reaction (Aycicek*et al*., 2005). Antigen isthat substance which can elicit production of antibodies when introduced into warm bloodedanimals. Whereas antibodies are glycoproteins which are produced as a result of an immuneresponse, after introduction of antigens, leading to the production of a specific antigen-antibodycomplex. In the direct competitiveenzyme-linked immunosorbent assay, specificantibodies for aflatoxin are coated on to the wells in the microtiter strip. The test samples oraflatoxin standards are added to the wells. After incubation and washing, enzyme conjugate(a conjugate of aflatoxin and bovine serum albumin is attached with an enzyme molecule, suchas, horseradish peroxidase or penicillinase or alkaline phosphatase) is added to the wells. Freeaflatoxin and aflatoxin enzyme conjugate compete for the aflatoxin antibody sites in the wells.Washing step removes any unbound enzyme conjugate. Then substrate/chromogen is addedto the wells and incubated. The bound enzyme conjugate converts the colorless chromogeninto a blue product. The stop solution is added which leads to color change from blue toyellow. Then measurement is made photometrically at 650 nm in an ELISA reader. Theabsorbance is inversely proportional to the aflatoxin concentration in the sample i.e., the lowerthe absorbance, the higher the aflatoxin concentration..The main instrument used in enzyme-linked immunosorbent assay is the ELISA reader. It is basically a photometric instrument which gives the absorbance of the solution at the end of the process.

1.3.4.2.2.2.2 Indirect ELISA

A few indirect ELISA procedures have been reported for the analysis of aflatoxin inagricultural commodities. In this procedure, aflatoxin-protein conjugate (KLH-aflatoxin B1)is coated onto the microtiter plate. Sample or standard aflatoxin is added to the wellsfollowed by an aliquot of anti-aflatoxin antibody. The amount of antibody bound tothe plate is detected by the addition of goat anti-rabbit IgG conjugated to alkalinephosphatase (ALP) followed by reaction with p-nitrophenyl phosphate to give acolored product. The toxin is determined by comparing with a standard curve fromknown toxin concentrations. This procedure takes a long time (about 5.5 h).Ramakrishna &Mehan(1993) reported both direct andindirect competitive ELISAs for aflatoxin B1 in groundnuts. In these assays, methanoi-water-KCl(70+30 v/v, 0.5%) extracts of groundnuts were diluted to 1:10 withPBS-Tween buffer and then assayed.

The sensitivity of indirect ELISA is comparable to that of direct ELISA. Becauseonly small amounts of antibody are required for indirect ELISA, this method is usednot only for toxin analysis, but also to monitor the antibody titers of hybridomaculture fluids for the screening of monoclonal antibody-producing cells.

Direct ELISA is usually preferred for aflatoxinsince itutilizes a single conjugated protein, requires one less incubation step and one lesswashing step, and shows less variability than indirect ELISA(Irineo,2011).

1.4 Literature Review

Younis& Kamal ,(2003)assessed the level of Aflatoxin contamination in400 Sudanese peanut and peanut product samples by HPLC and TLC the highest aflatoxin level were detected in peanut butter (32-54 µg/kg) and the lowest in peanut kernels (3-8µg/kg) ,while peanut cakes and roasted peanuts showed moderate level of contamination (7-10 µg/kg) ,and (4-12 µg/kg) respectively).

Ram *et al*.,(1986) detected aflatoxin B1 in maize and cotton seed by ELISA and found that the toxin is in the range of 7 to 422 µg/kg and 7 to 3258 µg/kg respectively.

Anjaiah et al .,(1989) used competitive direct ELISA for estimation of aflatoxin B1 in naturally contaminated groundnut seed samples and concluded that this assay is more rapid and less expensive than physicochemical methods. Moreover, it can be used to detect as low as 50 pg of aflatoxin B1.

Candlish*et al*., (1987) estimated the aflatoxin content in groundnut kernels, groundnut butter and maize by enzyme immunosorbent assay (EIA) and thin layer chromatography (TLC) and observed the positive correlation between EIA and TLC.

Cole *et al*.,(1988) estimated the aflatoxin content in 152 groundnut grade samples by ELISA and HPLC (High performance liquid chromatography) and found that 41 percent of the samples contained 26-2542 µg/kg aflatoxin. The results of ELISA and HPLC agreed in 98.6 percent of the composite lot analysis with the detection of 20 µg/kg or greater.

Mortimer *et al*.,(1988) analyzed the aflatoxin content in groundnut butters (129 samples) by ELISA and these results showed that 6.2 percent of samples contained aflatoxin over 10 µg per kg, 8 percent contained 2.5 to 10 µg per kg and in the remainder (86 percent) does not contain aflatoxin. These results concluded that ELISA is a faster than conventional approaches.

Chu*et al.*, (1988) analyzed aflatoxin content in groundnuts and groundnut products by Radio immunoassay (RIA), which had sensitivity in the range of 0.1-0.5 ng, whereas ELISA, had sensitivity in the rangeof 2.5-25pg per assay. Simple and quick immunoassay (ELISA) protocols for monitoring aflatoxinBl ingroundnuts was developed that require less than 1h to complete and detect 5 to 10µg/ kg product.

Figuiera*et al.*, (1990) estimated the aflatoxinBl in groundnuts, Brazil nuts, Almonds, Hazelnuts andWalnuts by ELISA and recommended an alternate method to the already adopted TLC method.

Azer&Cooper, (1991) used ELISA system and HPLC method simultaneously to analyse 178 samplesof foodstuffs for total aflatoxins. High correlation coefficient values obtained between results of twomethods with nuts, nut products, groundnuts and poor correlation for cereals and grain samples.

Patey*et al*., (1992) used ELISA technique forthe quantification of aflatoxin content in peanut butter.

Aldao*et a*l., (1995) quantified the aflatoxin B1 by an indirect ELISA in groundnut samples and observedthe cross reactivity of antibodies with aflatoxin B2, G1 and G2.

Sylos*et al*., (1996) estimated aflatoxincontent in 10 samples of groundnut and 9 samples of maize by ELISA and mini column chromatographyand detected >20 µg/ kg toxin in 50 percent groundnut seeds and none in maize samples and also ELISAhas taken less time to complete than mini column chromatography.

Zhang *et al*., (1997) collected 246samples of corn from different areas and analyzed for aflatoxins and fuminosins by ELISA and moretoxins were detected in areas of high oesophageal cancer (HEC) and low toxins were detected in HEClow risk areas.

Reddy *et al.*, (2001) estimated AFB1 in different grades of chilli samples by indirect ELISAand showed that 59 percent of samples were contaminated with AFB1and 18 percent contained the toxinat non permissible levels. Maximum percentage of chilli pods showing AFB1 levels higher than 30 µg perkg in grade 3.

1.5 Problem statement

All the studiesmentioned in literature reviewdidn’t estimate total aflatoxin in peanut and sesame cakes .Aim of this study is to determinate total aflatoxin (AFB1,AFB2,AFG1and AFG2) in Sudanese peanut and sesame cakes by immunological method usingdirectenzyme-linked immunosorbentassay technique.ELISA methods potentially have advantages over the otherprocedures because of its simplicity sensitivity,safety of reagents and highaccuracy.

1.6Objectives of Study:

1. Determination aflatoxin contamination in peanut and sesame cakes by ELISA technique.
2. Comparison ofaflatoxin contamination in peanut and sesame cake from Kordufan and Darfur states.

CHAPTER TWO

Materials and methods

2.1Materials

2.1.1 Microwellreader with a 650 nm filter (ELISA INSTRUMENT) TECAN sunrise

2 .1.2 ELISA aflatoxinkit (Neogen kit)

B. Agri-Screen and VeratoxNeogen'smicrowellmycotoxin tests are competitive direct enzyme-linked immunosorbent assays (CD-ELISAs). Each test kit contains (Figure 12)

a) 48 antibody-coated microwells
b) 48 red-marked mixing well.
c) 4 yellow –labeled bottles of 1.5 ml each 0, 5,15and 50 ppb aflatoxin controls.
d) 1 blue- labeled bottle of 7 ml aflatoxin HRP conjugate solution
e) 1 green-labeled bottle of 24 ml K-Blue Substrate solution
f) 1 red-labeled bottle of 32 ml Red Stop Solution.

Figure 12

2.1.3 Other chemicals and equipments

1. 70% methanol.

2. Graduatedcylinder .

3. Sample collection tubes.

4. Agri-grind grinder.

5. Scale capable of weighing 2000 gram.

6. Filter paper.

7. 100 µL pipettor.

8. 12- channel pipettors.

9. Tips.

10. Microwell holder.

11. Timer.

12. Wash bottle.

13. 2 reagent boats for use with 12-channel pipttor.

14. Paper towels.

15. Distilled water.

16. Beaker (500 ml)

17. Flask (500 ml)

18. Funnel

2.2. Method(AOAC,2000).

2.2.1. Sample Preparation and Extraction

Random sampling procure was adopted .Peanut and sesame cakes were collected from oil mills in Kordufan and Darfur states .Forty samples of peanut and sesame cakes (2 kg for each samples) were taken randomly from a batch of40 sacks grounded and thoroughly mixed.

The extraction solvent used in the Vertex test method is a methanol/water (distilled) mixture consisting of 70 percent methanol and 30 percent water.70 % methanol solution prepared.

Fifty grams of each 40 samples of peanut cake and sesame cake were transferred to a beaker (500 ml) and 250 ml of 70 % methanol solution added andstirred vigorously for3 minutes the mixture filtered and the filtrate collected as sample.

2.2.2 The Procedure:

All reagents (Neogen kit) allowed to warm to room temperature 18-30^0 C for 20 minutes . One red-marked mixing well were removed for each sample plus 4 red-marked well for controls, and placed in the well holder. An equal number of antibody-coated wells were removed. Each reagent mixed by swirling the reagent bottle .100 µL of conjugate placed from the blue –labelled bottle in each red marked mixing well. 100 µL of controls and samples, transferred to the red-marked mixing wells. A 12 –channel pipettor used to mix the liquid in the wells by pipetting it up and down 3 times.100 µL transferred to the antibody-coated wells. Mixed by sliding the microwell holder back and forth on a flat surface for 20 seconds without splashing reagents from the wells. Incubate for 2 minutes at room temperature 18- 30^0 C. Each antibody well filled with distilled water and dumped them out. This step repeated 5 times, and then the wells turned upside down and tapped out on a paper towel until the remaining water has been removed. The needed volume of substrate pipetted from the green-labeled bottle into the green-labeled reagent boat and, with new tips, 100 µL of substrate pipetted into the wells and mixed by sliding back and forth on a flat surface for 20 seconds. Incubate for 3 minutes. Red stop solution pipetted from the red-labeled bottle into the red-labeled reagent boat. 100 µL red stop, added to each well and mixed by sliding back and forth on a flat surface. Microwells read in amicrowellreader (ELISA) using a 650 nm filter.After read absorbance of standards and samples the results calculated by NeogenVeratox software.

CHAPTER THREE

Results and discussion

3.1 **Levels of aflatoxin contamination in peanut cakes**

Tables 5 and 6, figures13 and 14 show the aflatoxin contamination levels in peanut cakes samples collected from Kordufanand Darfur respectively.

Table (5)Aflatoxin detected in peanut cake collected from Kordufan

Standards +Samples	Absorbance	Result ppb.
Standard 0ppb	1.304	0.0
Standard 5ppb	0.818	5.1
Standard 15ppb	0.493	14.5
Standard 50ppb	0.193	50.8
Peanut cake (1)	0.216	45.7
peanut cake (2)	0.173	59.8
Peanut cake (3)	0.325	27.0
Peanut cake (4)	0.141	75.9
Peanut cake (5)	0.179	57.4
Peanut cake (6)	0.390	20.8
Peanut cake (7)	0.254	37.3
Peanut cake (8)	0.199	50.5
Peanut cake (9)	0.158	66.5
Peanut cake (10)	0.184	55.5

Correlation Coefficient= 0.9997

SLOPE = -2.2424

Y- INT = 2.1057

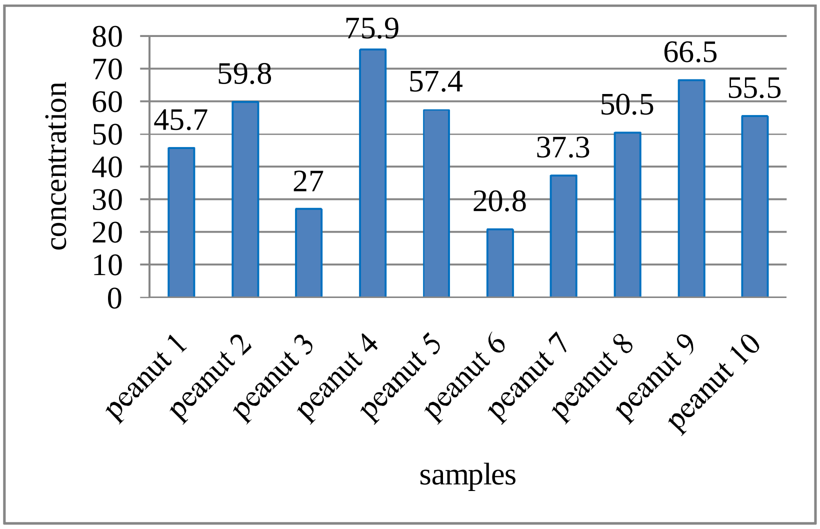

Figure .13Concentration of aflatoxin in peanut cake collected from Kordufan

Table (6): Aflatoxin detected in peanut cake collected from Darfur

Standards +Samples	Absorbance	Result ppb.
Standard 0ppb	1.403	0.0
Standard 5ppb	1.004	5.0
Standard 15ppb	0.610	15.2
Standard 50ppb	0.253	49.7
Peanut cake (1)	0.312	38.8
Peanut cake (2)	0.172	76.3
Peanut cake (3)	0.285	43.2
Peanut cake (4)	0.150	88.3
Peanut cake (5)	0.225	56.8
Peanut cake (6)	0.199	65.1
Peanut cake (7)	0.297	41.1
Peanut cake (8)	0.186	70.1
Peanut cake (9)	0.232	54.8
Peanut cake (10)	0.376	30.7

Correlation Coefficient = 0.9999Slope= -2.4362Y-ITN = 2.6178

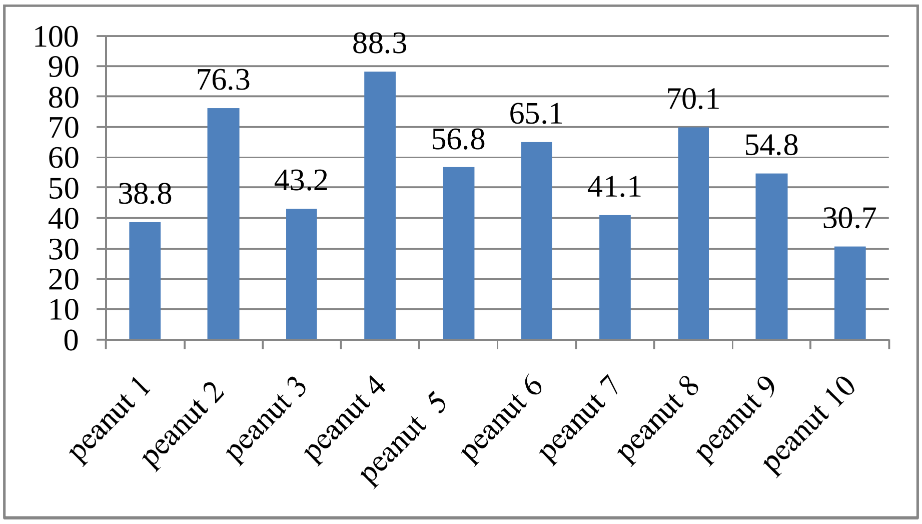

Figure. 14Concentration of aflatoxin in peanut cake collected from Darfur

The Correlation coefficient of peanut cakes collected from Kordufan andDarfur are 0.9997 and 0.9999 respectively.

3.2 Levels of aflatoxin contamination in sesame cakes

Tables (7) and (8), figures15 and 16shows the aflatoxin contamination levels in sesame cakes samples collected from KordufanandDarfur state.

Table (7): Aflatoxin detected in sesamecakes collected from Kordufan

Standards +Samples	Absorbance	Result ppb.
Standard 0ppb	1.036	0.0
Standard 5ppb	0.755	5.0
Standard 15ppb	0.459	15.2
Standard 50ppb	0.185	49.7
Sesame cake (1)	0.941	1.5
Sesame cake (2)	1.006	0.5
Sesame cake (3)	1.118	0.0
Sesame cake (4)	0.990	0.7
Sesame cake (5)	0.868	2.7
Sesame cake (6)	0.996	0.6
Sesame cake (7)	1.055	0.0
Sesame cake (8)	1.025	0.2
Sesame cake (9)	1.010	0.4
Sesame cake (10)	0.810	3.8

Correlation Coefficient =1.0000

Slope = -2.5139

Y-ITN 2.7394

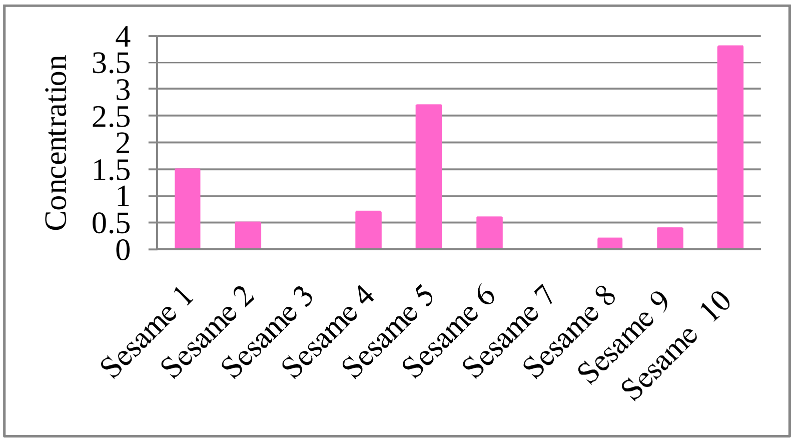

Figure.15 Concentration of aflatoxin in Sesame cake collected from Kordufan

Table (8): Aflatoxin detected in sesame cake collected from Darfur

Standards +Samples	Absorbance	Result ppb.
Standard 0ppb	1.638	0.0
Standard 5ppb	1.160	4.9
Standard 15ppb	0.704	15.5
Standard 50ppb	0.308	49.3
Sesame cake (1)	1.364	2.4
Sesame cake (2)	1.399	3.1
Sesame cake (3)	1.251	3.7
Sesame cake (4)	1.327	2.8
Sesame cake (5)	1.145	5.1
Sesame cake (6)	1.221	4.1
Sesame cake (7)	1.099	5.8
Sesame cake (8)	1.340	2.7
Sesame cake (9)	1.280	3.4
Sesame cake (10)	1.300	3.1

Correlation Coefficient =0.9997SLOPE -2.3479Y-INT 2.5109

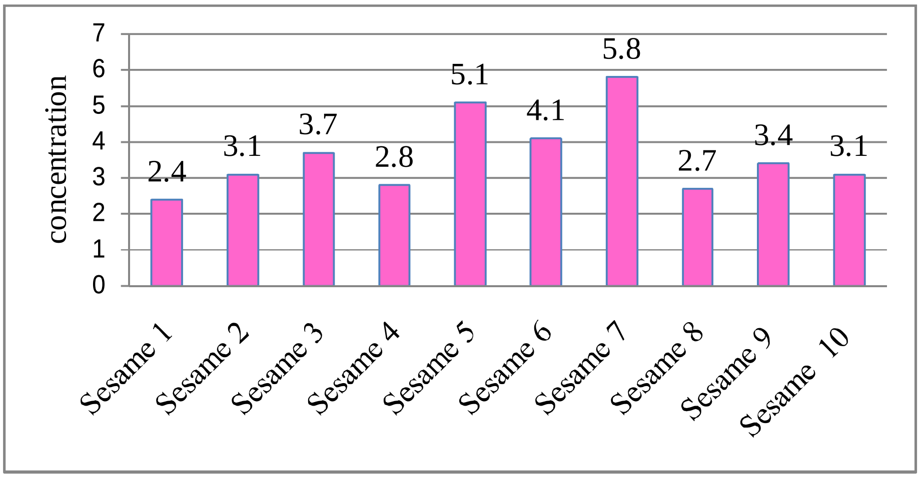

Figure.16 Concentration of aflatoxin in Sesame cake collected from Darfur

The Correlation coefficient of sesame cakes collected from kordufan andDrfur are1.0000 and 0.9997 respectively.

All values of the Correlation coefficient are acceptable (1.0000 is perfect. Any value over 0.9800 is acceptable .Anything under 0.9800 indicates potential problem with the standard curve and test should be evaluated) . The standards are back calculated against themselves. SLOPE AND Y-Intercept (Y-INT): These parameters indicate the position of the calculated line to the standard levels and used as an indicator of how consistently the test runs. (AOAC, 2000).

Among the 40 samples investigated, 38 were contaminated with aflatoxin (95% incidence). Twenty samples of peanut cake out of twenty samples (100% incidence) were contaminated with aflatoxin indicating that nuts were the most commonly contaminated food commodity, the levels of aflatoxin in peanut cake ranged from 20.8µg/kg to 88.3 µg/kg (figure 19). Eighteen samples of sesame cake out oftwenty (90%) were contaminated with aflatoxin, contained 0.2µg/kg to 5.8 µg/kg total aflatoxin contents (figure 19).

Food and Drugs Administration allowable levels of Aflatoxin in food and feedis 20 µg/kg for humans in all food except milk and in all animal species in feed expect corn (100µg/kg) and cotton seed meal (300µg/kg) . According to the FDA all samples of sesame cakes from Kordufan and Darfur were with the allowable level range (figure 18).All samples of peanut cakes from Kordufan and Darfur werehigher than allowable range. (Figure17).

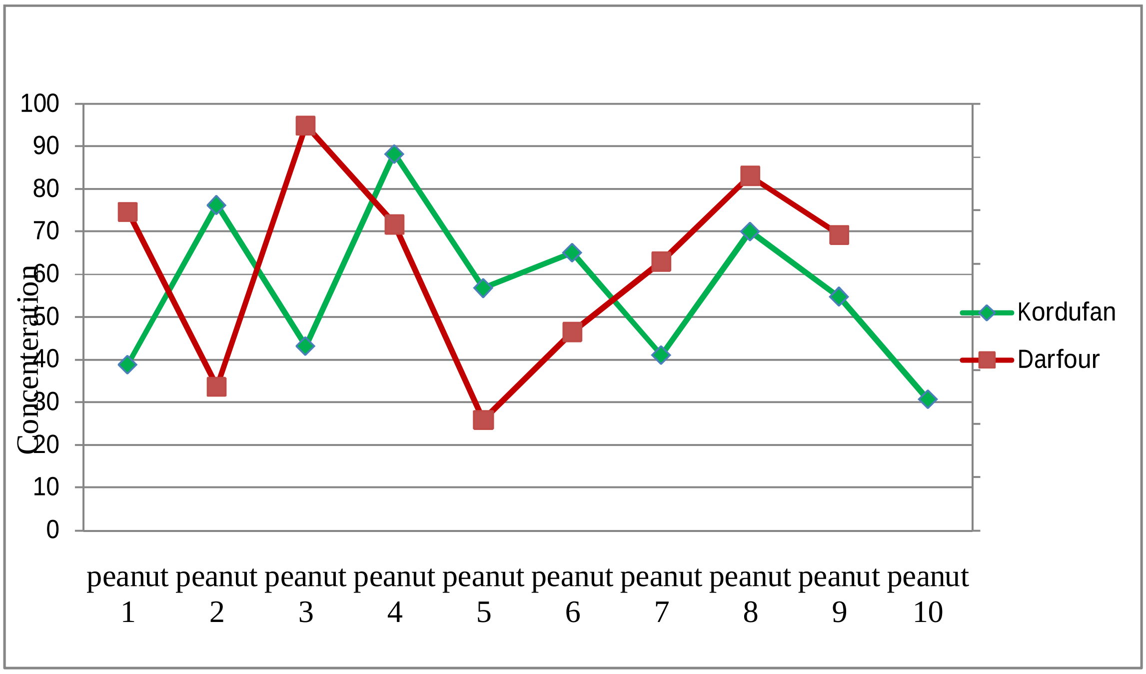

Figure.17 Comparison between peanut cakes from Kordufan and Darfur

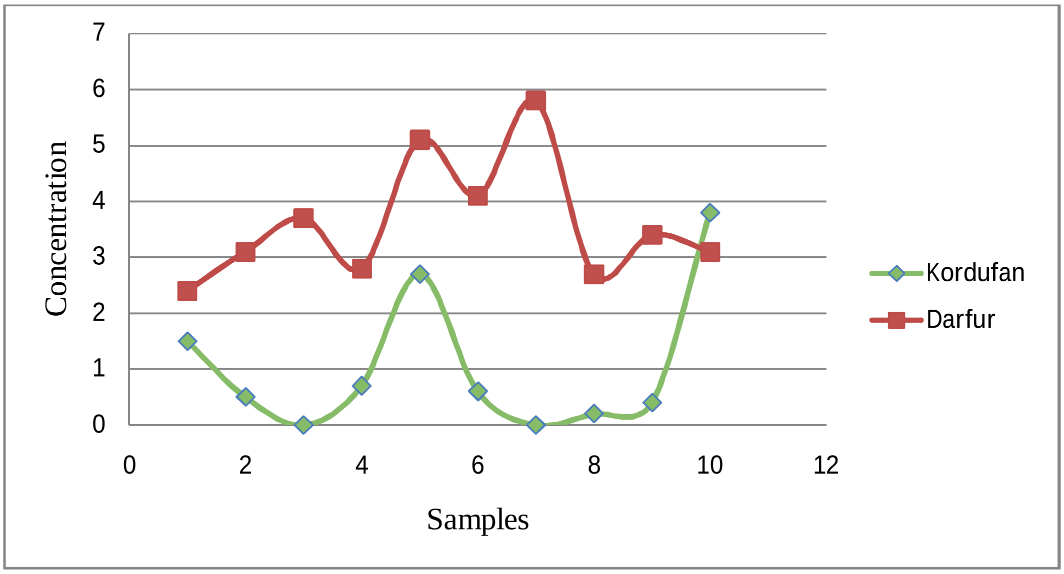

Figure.18Comparison between Sesame cake from Kordufanand Sesamecakesfrom Darfur

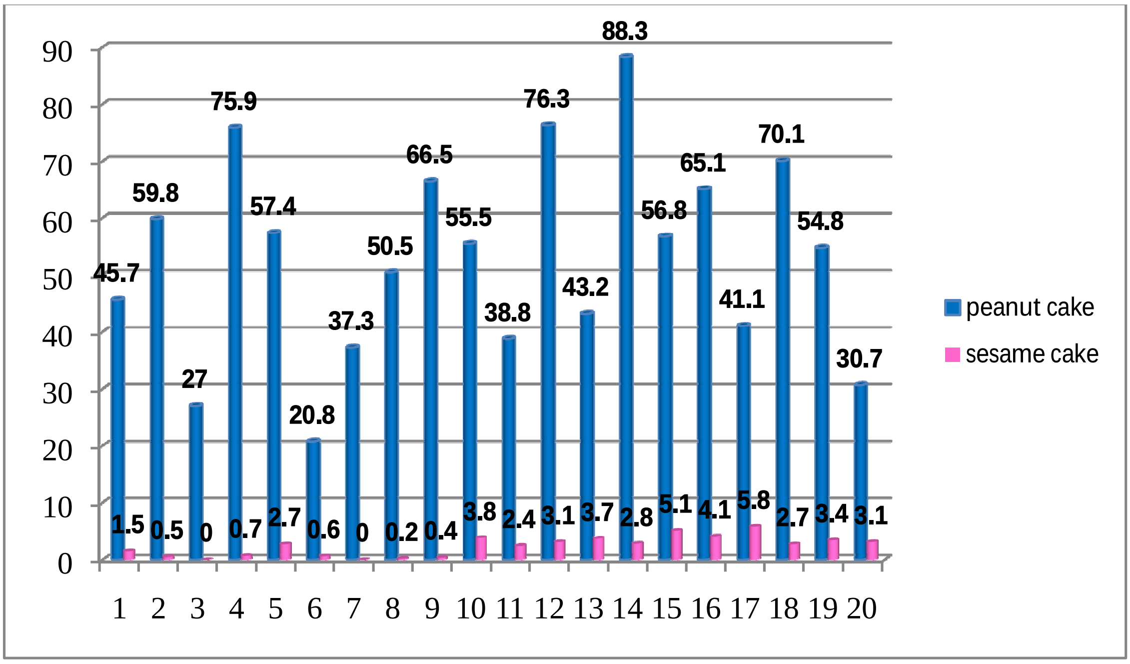

Figure.19 Comparison between peanut cakesand sesame cakes

3.3 Conclusion

Immunological method (ELISA) technique was used to determine aflatoxin levels in the Sudanese sesame and peanut cakes collected from Kordufan and Darfur.The results of this study showedthehigh levels of contaminationin 20 peanut cakesamples (100%) and the concentrationrangesfrom 20.80µg/kg - 88.30 µg/kg .The results also show the acceptable levels in 20 samples of sesame cakes and that the concentration ranges from 0.2µg/kg -5.8 µg/kg..

ELISA technique could be applied in the monitoring ofaflatoxin contamination in a large number of samples in acost and time effective manner.

3.4Recommendation

1. Moreregular monitoring of peanut cake.
2. Sudanese Standard and Metrology Organization (SSMO) has to give more attention and set their own levels of aflatoxin in food and feed.
3. Similar studies are needed for other Sudan states.

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