NATIONAL UNIVERSITY OF LESOTHO

Department of Chemistry and Chemical Technology

DETECTION AND ESTIMATION OF AFLATOXINS IN GROUNDNUT AND GROUNDNUT MATERIALS

A project document submitted to the department of Chemistry and Chemical Technology in partial fulfillment of the requirements for the degree Bachelor of Chemical Technology (B.Chem.Tech.)

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Student number: 200700878

Supervisor: Prof. G.C Alemayehu

May 2011

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Declaration

This project has been composed by me, and has never been accepted in any previous application for a degree. The whole work, of which this is a record, has been done by me; and all sources of information have been acknowledged by means of references.

Nkhabu Rethabile Alina

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Acknowledgements

I am grateful to my supervisor, Prof. G.C Alemayehu, who, despite his tight diurnal schedule, assisted me in producing this project document.

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ABSTRACT

The aflatoxins are well suited for analysis by Thin Layer Chromatography (TLC) since most of the compounds fluoresce under long wave ultraviolet (UV) light. The TLC technique serves as both purification and quantitation step. Before the TLC analysis, the peanut butter and crushed groundnut (peanuts) samples were separately de-fatted with light petroleum (boiling point 40 to 60^0C, ant the toxins within the samples were extracted from the de-fatted samples with methanol, and the extracts were purified by repetition solvent partition technique. The solvents used in the former technique were water-methanol mixture and chloroform, and aflatoxins have high affinity for chloroform. The obtained extracts were diluted to desirable concentrations and were then used for TLC analysis using silica gel Kieselgel G chromatoplate, and the mobile phase as the methanol-chloroform combinations. The quantitation of the results obtained was by fluorescence using fluorescent lamp (365 nm).

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1. INTRODUCTION

1.1 General

1.1.1 Definition

Many agricultural commodities are vulnerable to attack by a group of fungi which have ability to produce toxic metabolites called mycotoxins. This term is derived from the “mikes” (Greek word for fungus) and “torsion” (poison). A mycotoxin is a toxic secondary metabolite (product of the primary metabolic product, not required for growth) produced by organisms of the fungus kingdom, known as molds [Berger et al: 2007]. Most fungi are aerobic and are usually found in extremely small quantities due to the minute size of their spores.

Among various mycotoxins, Aflatoxin contamination of agricultural commodities has gained global significance due to its importance to international trade. The reason for being, a subject for concern in agriculture on a global scale and in human and livestock health, is that when present in agricultural commodities such as groundnuts and other food items, aflatoxins reduce their quality and value, and can cause serious animal and human health problems [Webster: 1998].

1.1.2 Occurrence and Types

Aflatoxins are naturally occurring mycotoxins that are produced by many species of *Aspergillus*, a fungus, most notably *Aspergillus flavus (A.flavus)* and *Aspergillus parasiticus (A.parasiticus),* that cause a toxic response in vertebrates when introduced in low concentrations by a natural mean [Blumel D: 2005]. A.flavus, which is species mostly found in Africa and Asia, is common and widespread in nature, and produces the major aflatoxins of concern which are designated B1, B2, G1 and G2, while *A.parasiticus* is predominant in America and produces aflatoxins G1 and G2 only. While the presence of *A.flavus* does not always indicate harmful levels of aflatoxin it does mean that the potential for aflatoxin production is absent.

There are at least thirteen different types of aflatoxins produced in nature, with aflatoxin B1 considered as the most toxic followed by aflatoxins G1, B2 and G2 in order of decreasing potency.

1.1.3 Biosynthesis of Aflatoxins:

Aflatoxins being toxic complex organic substances can be produced in nature or laboratory cultures. In nature, aflatoxins are formed through a polyketide pathway involving a series of enzymatically catalyzed reactions [Farid W and Reddy S: 2004]. However in laboratory cultures, aflatoxins are biosynthesized after active growth has ceased, as is typical for secondary metabolites. The relative proportionality of the four major aflatoxins synthesized by aspergillus give a reflection of the genetic constitution of the product strain, and the parameters in association with fungal growth. In addition, derivative aflatoxins are produced as metabolic or environmental products.

1.1.3a Biosynthesis of Aflatoxin B1

Aflatoxin B1 is derived from both fatty acid synthase (FAS) and polyketide synthase (PKS). The biosynthesis begins with the synthesis of hexanoate by the FAS, which then becomes the starter unit for the iterative type 1 PKS. The PKS adds seven malonyl-Cobaltacenate extenders to the hexanoate to form the C20 polyacetate compound, which is relatively unstable. Thus, this compound is stabilized by the enzyme norsolorinic acid synthase (NAS) until all the acetate subunits have been added [Blumel D: 2005]. At which point the NAS folds the polyacetate in a particular way to induce cyclization to form the anthraquinone norsolorinic acid.

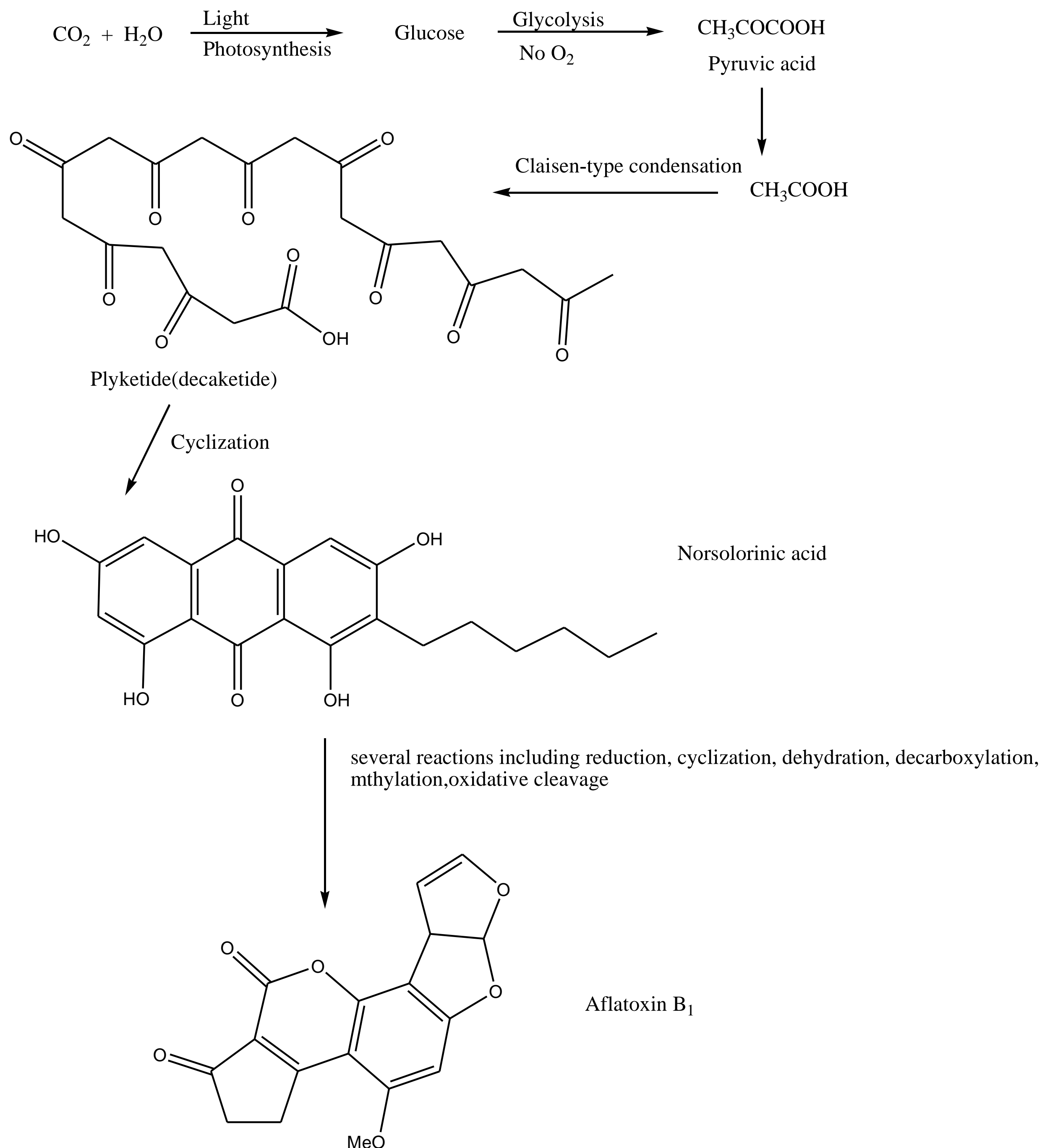
The anthraquinone is a ketone on the norsolorinic acid side chain, whose reduction reaction is catalyzed by NAS to yield averantin. Averantin is converted to averufin through two different enzymes, a hydroxylase and an alcohol dehydrogenase. These will oxygenate and cyclize the side chain of averantin to form the ketal in averufin. From this point, the biosynthesis of aflatoxin B1 becomes more complicated because most of the enzymes have not been characterized and there may be several more intermediates still unknown [Stoloff: 1999].

However, what is known is that averufin is oxidized by a specific oxidase in a Baeyer Villiger oxidation. This opens the ether rings and upon rearrangement, versiconal acetate is formed. The acetal in versicolorin acetate is formed from the cyclization of the side chain in the versiconal, which is catalyzed by Verb synthase, reduces versicolorin to form the dihydrobisfuran. Then the conversion of versicolorin acetate to demethylsterigmatocystin is catalyzed by a particular oxidase and a reductase. These enzymes both utilize molecular oxygen ant two NaDPH

(nitinamide adeninedinucleotide phosphate, cofactor) molecules to dehydrate one of the hydroxyl groups to the anthraquinone and open the quinine with the molecular oxygen [Garren: 1998].

The next steps become even more complicated. Upon forming the aldehyde in the ring opening step, the ring is oxidized to form carboxylic acid and subsequently a decarboxylation reaction occurs to close the ring system in demethylsterigmatocystin. The next two steps in the biosynthetic pathway is the methylation by adenosylmethionine (SAM) of the two hydroxyl groups on the xanthone part of demethylsterigmatocystin by two different methytransefases. This yields o-methylsterigmatocystin. In the final steps there is an oxidative cleavage of the aromatic ring and loss of the carbon in o-methylsterigmatocystin, which is catalyzed by an oxidotereductase. Then the final recyclization occurs to form aflatoxin B_1.

Generally, the biosynthesis of aflatoxin B_1 can be shown by the reaction steps below,



1.1.4 Effects on Human and Animal Life

Aflatoxins are potent molecules with many biological effects and are listed as probable human carcinogen by the International Agency for Research on cancer. Aflatoxins cause aflatoxicosis-

disease due to accumulation of aflatoxin in the mammal's body, especially in the liver. They are toxigenic, carcinogenic, mutagenic, immune suppressive and teratogenic in various animal species. Aflatoxin B1 is usually the most abundant naturally occurring member of the family, and most studies on the pharmacological activity of aflatoxin have been conducted with this congener [Lillard E: 2001]. Aflatoxin B1 is the most potent hepatocarcinogenic agent known, although the liver by no means is the only organ susceptible to aflatoxin carcinogenesis.

Although the susceptibility of animals to aflatoxins varies from species to species, the chance of human exposure to acute levels of aflatoxin is remote in well-developed countries. In undeveloped countries, the expression of aflatoxin-related diseases is influenced by factors such as age, nutrition, sex and possibility of concurrent exposure to other toxins. The main target organ in mammals is the liver, so aflatoxicosis is primarily a hepatic disease [Stack M.:2002]. Effects on the lungs, myocardium and kidneys have also been observed and aflatoxin can accumulate in the brain. Conditions increasing the likelihood of aflatoxicosis in humans include limited availability of food, environmental conditions that favour mold growth on foodstuffs, and lack of regulatory systems of aflatoxin monitoring and control.

Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is not easy to avoid because fungal growth in food is not east to prevent. Although heavily contaminated food supplies are not permitted in the market place in developed counties, concern for possible adverse effects due to long-term exposure to low levels of aflatoxins in food supply still remains. The evidence that prove that aflatoxin ingestion through contaminated foodstuffs is its cause of the major etiological factors in human hepatocellular carcinoma (HHC) in China and Sub-Saharan Africa [Roberts: 2001]. This is because these countries lie in regions where the temperatures and relative humidity often favour mold growth.

Another evidence showing that humans are exposed to aflatoxins by consuming contaminated foodstuffs is a classic case that occurred in Malaysia in 1990. Reports indicate that approximately 40 people were affected and 13 children died after eating noodles highly contaminated with aflatoxin and boric acid. High levels of aflatoxins were found on autopsy in the liver, lung, kidney, heart, brain and spleen [Waliyar and Reddy: 1999]. It is also considered that aflatoxins may play a role in a number of diseases, including kwashiorkor and hepatisis.

In the temperate developed areas of the world, acute poisoning in animals is rare. The outbreak of so called "Turkey-X disease" which caused the deaths of about 100,000 turkeys and other poultry in the UK in 1960 was reported to have been caused by extremely high concentrations of aflatoxins in imported groundnut meal. Affected birds were reported to have symptoms such as loss of appetite, and became lethargic and died within 7 days from the onset of the symptoms. Young animals are particularly susceptible to aflatoxin poisoning, especially males than females. For most species, the LD_{50} (lethal dose) is between 0.5-and10 mg/kg body weight.

1.1.5 Contaminated Commodities

The most pronounced aflatoxin contamination has been encountered in many agricultural commodities. The fungi that produce aflatoxin grow on crops such as peanuts (especially), wheat, beans, rice, corn, cottonseed, which are major crops regularly displaying high levels of aflatoxin contamination [Blumel D: 2005]. The groundnut which is mostly contaminated is the most important oilseed crop in developing countries and a valuable source of protein for human and animal nutrition. Since *A.flavus* and *A.parasiticus* are nearly ubiquitous in the natural environment, numerous other grains, legume, other oilseeds, spice crops, as well as coffee and cocoa have been reported to be contaminated with aflatoxins. Milk, eggs, and meat products are sometimes contaminated because of the animal consumption of aflatoxins-contaminated feed [Longmont C: 2004].

1.1.6 Factors Influencing Growth

The occurance of aflatoxins in agricultural commodities is influenced by certain environmental factors. This is because the mold that produce aflatoxins occurs in soil, decaying vegetation, hay and grains undergoing microbiological deterioration and invades all types of organic substances whenever and wherever the conditions are favorable for its growth. Favorable conditions include high moisture content (humidity- at least 7%) and high temperatures, therefore the extent of contamination will vary with geographic location, agricultural and agronomic practices and the susceptibility of agricultural commodities to fungus in the field, before they are harvested, during storage, and/or processing period. After harvest, contamination can occur if crop drying is delayed and during storage of the crop if the crop is kept too moist [Garren: 1998]. Insects or rodent infestations facilitate mold invasion of some stored commodities.

Harvest is often the most stressful time to farmers and processors due to contamination of their crops. Groundnut contamination with aflatoxin is of great concern among the agricultural commodities exposed to aflatoxin contamination. Since groundnut flowers are formed and fertilized above ground but downward growth of the pegs ensures that the fruit (pods and seeds) develop in the soil, shells of immature and mature pads are commonly infected by *A.flavus* but very little seed infection occur at pre-harvest stage [Blumel D: 2005]. Hence the stage at which the crop is harvested can therefore influence aflatoxin contamination.

If groundnuts are affected during pre-harvest stage, then contamination will occur during post-harvest stage. After harvest stage, if the groundnuts pods infected with A.flavus are kept for drying, they will have exposure to rapidly changing environmental conditions, which result in shift in the dominant and subdominant fungal species on and within the pads. When groundnuts with high moisture content are lofted and dried in wind, there may be considerable invasion of seeds by *A.flavus* and other fungi already established in the shell. Therefore the stage at which the groundnut (or any agricultural commodities contaminated with aflatoxin) is dried can also influence aflatoxin contamination.

During storage, the main factors which can influence the growth of A.flavus are mainly moisture (relative humidity), temperature, time and gaseous composition of the atmosphere. *A.flavus* infection and aflatoxin contamination in groundnuts are likely to increase during storage until the moisture content drops below 9%. However, natural accumulation of carbon dioxide and a decrease in levels of oxygen in closed storage can reduce development of the fungi, and aeration is necessary during storage to reduce aflatoxin contamination. Important factors that contribute to *A.flavus* growth and aflatoxin contamination of groundnuts during storage are high relative humidity and temperatures, rain water leakage, condensation and insect infestations [Weil: 2002].

1.1.7 Legislation

Given the potential for prevention, aflatoxins as human carcinogens and their known activity as toxins in animal feed, many international regulatory agencies monitor aflatoxins entering the food chain. Aflatoxins are natural contaminants of groundnuts and other agricultural commodities, hence exposure of humans to some levels of aflatoxins may be difficult to avoid. Therefore sensible regulating measures are needed for foods and feeds as economic losses could

occur if limits are unnecessarily strict for exportation of food stuffs. Many European countries have reasonably set a limit of 3µg/kg of aflatoxins. The maximum permissible levels range from 20µg/kg to 50µg/kg (in whole feedings for animals) [Reddy and Frarid: 2000].

There are several defense mechanisms against aflatoxin contamination in commodities. Once a fungus grows on a plant and produces its aflatoxin, a real concern is that these poisons are completely heat stable, so neither cooking nor freezing destroys the toxin. They remain on the food indefinitely. Prevention is the first line of defense against aflatoxins entering the food chain. Moisture, temperature, and composition of the substrate are the chief factors affecting fungal growth and toxin production. Therefore, alteration of these conditions can reduce fungal growth. Detoxification is the last line of defense [Blumel D: 2005]. Several commercially feasible methods of ammoniation have been developed for reducing levels of aflatoxin contamination in animal feed.

1.2 Structure and physical properties

1.2.1 Structure

The common structural feature of the four major aflatoxins is a dihydrodifurano or tetrahydrofurano group fused to a substituted coumarin group. Aflatoxins are normally referred to the group of difuranocoumarins and classified in two broad groups according to their chemical structure; the difurocoumarocyclopentenone series which includes aflatoxin B_1, B_2, M_1 and M_2 (major metabolites of aflatoxin B_1 and B_2 respectively), and aflatoxicol, and the difurocoumarolactone series which includes aflatoxin G_1, G_2, M_{1a}, M_{2b} and $B_{3.}$ The structures of the major aflatoxins are shown below [Reddy and Frarid: 2000],

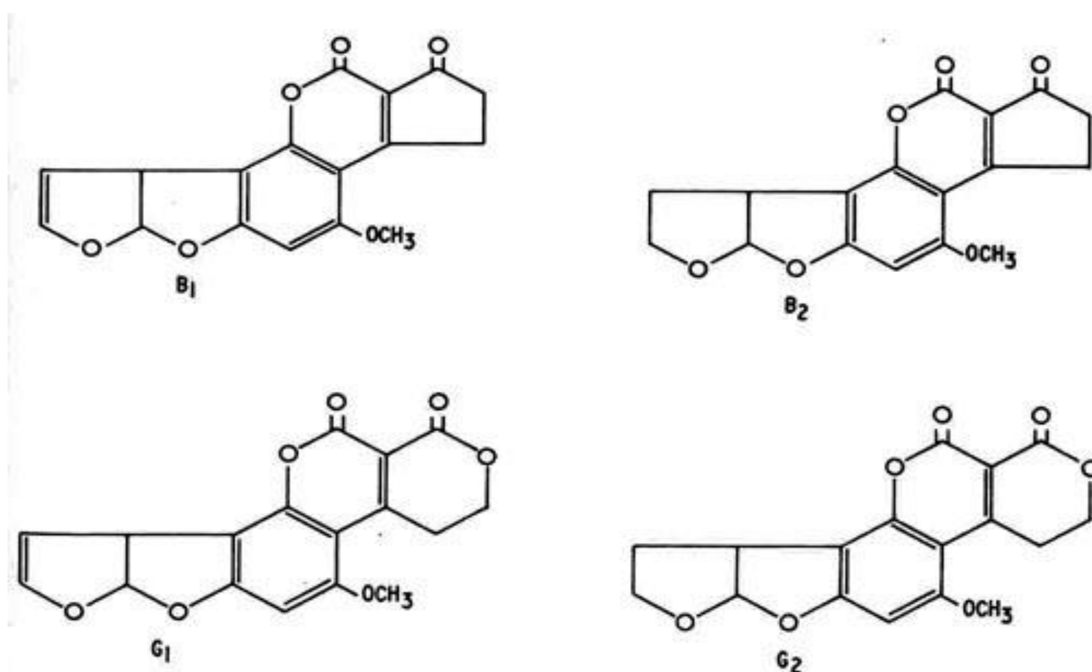


Fig. 1 **Structures of aflatoxins B_1, B_2, G_1, and G_2.**

Aflatoxin B_2 and G_2 are the dihydro derivatives of the parent compounds B_1 and $G_{1,}$ respectively. Structurally, the dihydrofuan moiety, containing double bond, and the constituents linked to the coumarin moiety are of great importance in producing biological effects.

1.2.1 Properties

Generally the major aflatoxins have the following physical properties as shown in the table below.

Table 1: General Properties of major Aflatoxins

Aflatoxin	Molecular formula	Molecular weight (g/mol)	Melting point (^{0}C)
B_1	$C_{17}H_{12}O_6$	312.3	268-269
B_2	$C_{17}H1_4O_6$	314.3	286-289
G_1	$C_{17}H_{12}O_7$	328.3	244-246
G_2	$C_{17}H_{14}O_7$	330.3	237-240

Aflatoxins are crystalline substances, freely soluble in moderately polar solvents such as chloroform, methanol and dimethyl sulfoxide, and dissolve in water to the extent of 10-20 mg/litre. Crystalline aflatoxins are extremely stable in the absence of light and particularly ultraviolet-visible (UV) radiation, even at temperatures in excess of $100^{o}C$. A solution prepared in chloroform or benzene is stable for years if kept cold and in the dark.

1.3 Chemical Reactions of Aflatoxins

1.3.1 Heat

The studies of the reactions of aflatoxins to various physical conditions and reagents have been extensively performed because of the possible application of such reactions to the detoxification of aflatoxins contaminated materials. Heat is one of the physical conditions that lead to reactions of aflatoxins. In dry state, aflatoxins are very stable to heat up to their melting points. However, in humid conditions and at elevated temperatures there is destruction of aflatoxins over a long period of time. Such destruction can occur either with aflatoxin in oilseed meals, aflatoxin in roasted peanuts or aflatoxin in aqueous solution at pH 7 [Reddy and Frarid: 2000].

1.3.2 in Basic and Acidic Conditions

The lactone ring in aflatoxins makes them susceptible of alkaline hydrolysis. In alkali solutions, hydrolysis of the lactone moiety occurs. Since recrystallization following acidification of a basic solution containing aflatoxins may occur, the hydrolysis appears to be reversible. At high temperatures under alkali conditions (or ammonia and various amines), ring opening followed by decarboxylation occur, and reaction may further lead to the loss of the methoxy group from the aromatic ring. On the other hand, in the presence of mineral acid, aflatoxin B_1 and G_1 are converted into aflatoxin B_{2A} and due to acid-catalyzed addition of water across the double bond in the furan ring. Aflatoxin B2A can alternatively be formed by reaction of aflatoxin B1 with water catalyzed by light, as shown below,

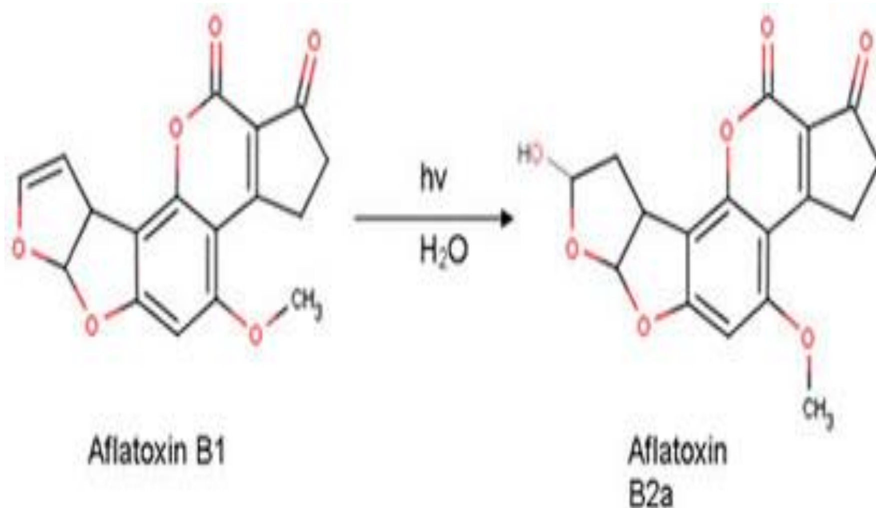


Figure 2: conversion of aflatoxin B_1 to B_{2A}

. In the presence of acetic anhydride and hydrochloric acid, the reaction proceeds further to give acetoxy derivative [Weil: 2002].

1.3.3 Reduction and Oxidation

Aflatoxins can also undergo oxidation and reduction reactions. Many oxidizing agents such as sodium perborate, potassium permanganate, hydrogen peroxide, chlorine, ozone and sodium hypochlorite react with aflatoxin and change the aflatoxin molecule in some way which is indicated by the loss of fluorescence. The uncertainty of these reaction mechanisms result in unidentified products in most areas [Guengerich et al: 2002].

Hydrogenation of aflatoxin B_1 and G_1 to yield aflatoxin B_2 and G_2 respectively is an example of reduction reaction. Further reduction of aflatoxin B_1 yields tetrahydroxyaflatoxin. Reduction of aflatoxin B_1 and B_2 with sodium borohydride yield aflatoxin RB_1 and RB_2 respectively [Guengerich et al: 2002]. These arise as a result of opening of the lactone ring followed by the reduction of the acid group and reduction of the ketone group in the cyclopentene ring.

The exo isomer of aflatoxin B_1 8,9-epoxide appears to be the only product of aflatoxin B_1 (AFB_1) involved in reaction with DNA. Although the epoxide hydrolyzes rapidly in water, DNA adducts are produced, as shown below [Johnson and Guengerich: 1997],

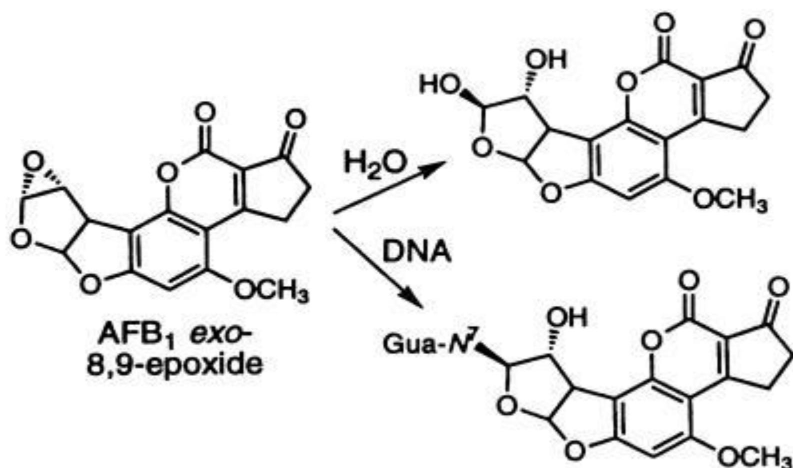


Figure 3: reaction of aflatoxin B_1 exo-8,9 epoxide with water and DNA.

1.4 Estimation of Aflatoxins in Food Samples

1.4.1 Sampling and Prepaation

Sampling is an important step in testing commodities for aflatoxin contamination. Aflatoxins occur in an extremely heterogeneous manner in food commodities, and unfortunately the highly skewed nature of the distribution of aflatoxin complicates the sampling procedure. For instance, toxicology often resides in only a few contaminated commodities. Additionally, there is extreme variation in the level of aflatoxin among contaminated commodities. Therefore, it is crucial that sampling is carried out in a way that ensures that the analytical sample is truly representative of the consignment [Friesen et al: 1999].

Firstly, the samples have to be prepared before the analysis. Proper grinding and sub-division of the sample is obviously essential before performing analysis of aflatoxins. Before the analysis, the aflatoxins have to be extracted from the sample after grinding, usually with an organic solvent, the mostly used being methanol. Extraction with methanol provides sensitive and selective results for a wide range of foods and animal feed. Initially, in most sampling techniques, the aflatoxins are first extracted with light petroleum (boiling range 40°-60^oC). Light petroleum is an excellent defatting agent and removes most of the fat from the sample. The extraction is performed using a soxhlet extractor (figure 3) [Coker: 2001],

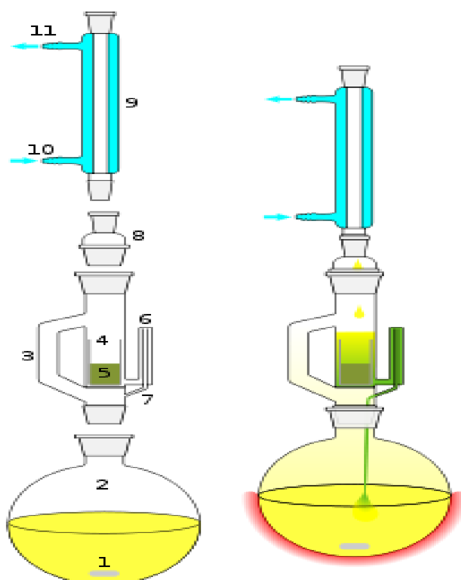


Figure 4: a schematic representation of a soxhlet extractor.

in which the numbers represent; 1 a stirring bar; 2 a boiling flask; 3 a distillation path;4 a thimble; 5 a solid; 6 a siphon top; 7 a siphon exit; 8 an expansion adaptor; 9 a condenser; 10 cooling water in, and 11 cooling water out. A soxhlet extractor is a piece of laboratory apparatus originally designated for the extraction of a lipid from a solid material.

1.4.2 Analysis Methods

Many different analytical and immunochemical methods are available for the estimation of aflatoxin levels in various agricultural commodities. Numerous physicochemical methods (analytical methods) have been developed for the analysis of aflatoxins in agricultural commodities. All analytical methods for aflatoxins basically involve the same steps- sampling, extraction, clean-up, separation and quantification [Mehan: 1997].

On the other hand, immunochemical methods use immune response, with the quantification of the reaction by competitive binding of either radio-labeled aflatoxin or enzyme linked aflatoxin. The accuracy of the immunoassay of aflatoxins in naturally contaminated samples is affected by the specificity of the antibody used and by the presence of structurally related analogs of the mycotoxin in the sample that may react with the antibody.

Since the first analytical methods for aflatoxins were published in 1964, thin layer chromatography (TLC) has been the only technique capable of detecting and quantitating aflatoxins at low levels. In the 1970s and early 1980s various high performance liquid chromatography (HPLC) and radio immunoassay (RIA) based methods were developed, and being more sensitive than TLC methods, were used whenever a high degree of accuracy was required.

The TLC technique serves as both purification and quantification step. Successful analyses of any commodity depend on the selection of appropriate methods for preparation of extract. Also crucial to successful analysis is TLC itself. It is not sufficiently appreciated that good quantitation requires efficient chromatography, that is, separation of the analysis from each other and from other extractives [Farid W and Reddy S: 2009]. Various analytical procedures use TLC plates coated with silica gel (Kieselgel G), and solvent systems such as chloroform: methanol and chloroform: acetone for TLC development.

1.4.3 Quantitation

The detection method for aflatoxins is by fluorescence technique. The estimation of aflatoxins visually or by densitometry by measuring the intensity of fluorescence of the aflatoxin spots is the most widely used technique for their quantitation. Basically, there are two techniques used in visual estimation one technique is the method which involve serial dilution of unknown extracts and standards to a point of undetectable fluorescence (about 0.1-0.5ng for aflatoxin B and G). however, this technique depends on the intensity of the ultraviolet (UV) light, the silica gel and the residual solvent in the silica gel, as well as the compactness of the spot, the quantity and type of extract interferences, the darkness of the room and the usual acuity of the observer [Wilson :2002].

The other technique for visual estimation involves comparison with the fluorescence intensity of extract spots with those of standard aflatoxin spots. For viewing the TLC spots, a fluorescent lamp with long-wave UV light (365nm) is a suitable starter unit. On the TLC plates, the four common substances (aflatoxin $B_{1,}$ B_2, $G_{1,}$ and G_2) are distinguished on the basis of their color, B standing for blue and G for green, with subscripts relating to the relative chromatographic mobility [Blumel D: 2005]. That is, the naturally occurring aflatoxins are identified in

physicochemical assays as intensely blue (aflatoxins B_1 and B_2) or green (aflatoxins G_1 and G_2) fluorescent compounds under long-wave UV light.

Besides quantitation, confirmation of identity of aflatoxins is necessary. Some compounds which behave like aflatoxins may appear on TLC plates. To eliminate such false positives, the identity of the toxin in positive samples needs to be confirmed. Because initial identification of unknowns in a chromatogram is based on the similarities of their R_f values with those of standard aflatoxins, additional proof of identification is required. Several techniques that can be used for confirmation of identity of aflatoxins include rechromatography with several different solvents and the use of the spray agents, especially mineral acids sprays; the acid changes the fluorescence of the aflatoxin (B_1 and B_2) from blue to yellow[Farid W and Reddy S: 2009].

Many factors can adversely affect TLC separation and quantitation. These factors include the TLC plate, reference standards, spotting on the TLC plate, development of the chromatogram and mobile phase. For the TLC plate, silica gel is used almost exclusively for TLC of the aflatoxins. The commercially available silica gel varies greatly in their chromatographic properties, and these variations show up as difference in their ability to separate the four principle aflatoxins from each other and from interferences. Fortunately, commercially prepared TLC plates are improved in uniformity of layer thickness and thickness hence can separate the four aflatoxins quite well. Reference standards of aflatoxins are obtained from many sources and are often a source of error itself in analysis.

2. EXPERIMENTAL METHODS

2.1 Materials and Equipment

The reagents used in this study were: light petroleum (boiling range 40^0C to 60^0C) used for de-fatting the groundnut materials (peanut butter and crushed groundnuts); methanol used in the extraction process; chloroform used for separation of the methanoic extract; anhydrous sodium sulphate used for drying the extract; methanol (5%)-chloroform mixture used as mobile phase in TLC analysis; chromatographic silica gel- Kieselgel G used as the stationary phase adhered on the chromatoplate. All the reagents used were of analytical- reagent grade. The samples used in this study of detection and estimation of aflatoxins in groundnuts and groundnuts materials were peanut butter and groundnuts (peanuts).

The apparatus used in this study, most of which were glassware apparatus were: 250ml boiling flasks; soxhlet extractor (capacity, 100mL); extraction thimbles, in which the sample is placed for de-fatting and extraction; coil- condenser used for condensation during de-fatting and extraction;6-hole water bath (to accept 250ml flasks) used for heating; conical 250ml separating funnel; 8cm(diameter) filter funnels used for separating the extract from anhydrous sodium sulphate; 100ml beakers; calibrated flasks- capacity, 25ml and 50ml; micropipette- capacity, 50µl); disposable pipettes, chromatographic tank; and fluorescent lamp.

2.2 Sampling

Careful sampling is an essential preliminary to the method, and with whole groundnuts sampling is exceptionally difficult, since the toxicity may be associated with a very small proportion of affected kernels. The whole groundnuts (peanuts) were obtained from fruit&veg city store in Maseru town. The shells of the groundnuts were removed and the inner core (seeds) was crushed to small particles weighing 19.0287g. Sampling was not necessary for peanut butter sample.

2.3 Experimental Procedure

2.3.1 De-fatting and Toxin Extraction

The crushed groundnut sample was placed in the extraction thimble and was extracted with light petroleum in a soxhlet extractor (syphon-rate, 8 to 11 changes per hour) for four hours to removal of fats in the sample. The residual sample was dried off from the thimble. The de-fatted sample was then extracted with methanol (to extract the toxin) for four hours. The methanol extract, after concentrating to 50ml using the rotary evaporator, was transferred to the separating funnel, into which the distilled water, 30ml, used for rinsing the extraction flask was also added. The flask was washed with 25ml chloroform and the washing was transferred to the separating funnel. Since methanol is soluble in water while chloroform is not, only two layers were formed after thoroughly shaking the separator. Chloroform being denser was the bottom layer, which was run off into a beaker. The separation extraction was repeated three more times with 25ml portions of chloroform and the extracts were combined. 10g of anhydrous sodium sulphate was added to the former and left to dry for a long while. After drying, the sodium sulphate was separated from the extract by gravity filtration. The extract was then concentrated to 25ml using the rotary evaporator, that was solution A. 5ml of solution A was diluted with chloroform to 50ml to give solution B.

2.3.2 Thin Layer Chromatography of the toxin

The chromatoplate was cut in the dimensions of approximately 5×10 cm. 10µl of solution A and 5 and 20µl of solution B were spotted 1cm from one edge of a chromatoplate and about 2cm apart. The chromatoplate was then developed in subdued light in the chromatographic tank containing the mobile phase made up of 5 per cent methanol in chloroform until a solvent path-length of almost 10cm from the base-line had been obtained. The mobile phase was distributed through the tank by swirling, to improve the elution process.

2.3.3 Assessment of Toxicity levels

After the development of the chromatoplate, it was dried off the mobile phase. The dried, developed chromatoplate was examined at a distance of approximately 30 cm from the Philips fluorescent lamp and observed the absence or presence of the fluorescent spot and recorded the R_f values where possible.

Similar procedure was followed when using peanut butter as the sample. The difference was only the weight of the sample, which was now 20.0001g.

2.4 Limitations of Experimental Method

The major limitation uncounted in this method was the absence of the aflatoxin standard necessary to compare the results as well as to observe the impact of adding the standard to the samples upon the analysis technique(s) used in this study.

3. RESULTS AND DISCUSSIONS

3.1 Observations of Quantitation for Peanut butter and Groundnuts Samples

Only one R_F value was obtained from the measurements for the three extracts used in the peanut butter sample, as shown in the table below. However there was no observed elution in the groundnut sample extracts, that is, there was no observed movement of the spots from where they were applied to the coated surface of a chromatoplate.

Table 2: Measurements for the R_F values for the extracts

Spot	Distance moved by the spot (cm)		Distance moved by the solvent front (cm)		R_F	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
A	0.O	0.0	9.0	9.0	0.0	0.0
B_1	4.2	0.0	9.0	9.0	0.47	0.0
B_2	0.0	0.0	9.0	9.0	0.0	0.0

Where R_F = distance moved by the spot in cm / distance moved by the solvent front in cm.

The 0.47 R_F value obtained for extract B_1 does not indicate presence of aflatoxin in the peanut butter sample since it did not fluoresce under long-wave UV light (365 nm). However, the spot might be for impurities in the solvents used in extraction or for solvents used as mobile phase.

3.2 Detection Limits of Aflatoxin B_1 Using TLC Analysis Method

When Kieselgel G thin-layer chromatoplate and the conditions of fluorescent (long-wave length UV light of 365nm), the smallest weight of aflatoxin B_1 giving observable fluorescent is theoretically about0.0004µg. The obtained results give different meanings. Since no fluorescence was observed at the concentrations of the peanut butter and groundnut sample extracts used, it can be concluded that these two samples used for the detection and estimation of aflatoxins do not contain aflatoxin B1, more especially groundnut peanut sample. This is because no samples of naturally infected groundnut material appear to contain the toxic metabolite G_1 (or even B_2 or G_2) in the absence of the more highly toxic aflatoxin B1 and, further G1 is usually present in much smaller proportion.

The other possibility of the observed results could be of low levels of aflatoxin B_1 in the samples. However since no fluorescence was observed at the studied concentrations of the sampled extracts, the samples under examination , especially peanuts (naturally occurring) could have contained less than 5µg of aflatoxin B_1 per kg.

3.3 Limitations in the Analysis Method

The major problem uncounted in this experiment procedure was the absenteeism of the reference standards. The reference standard is the basis of showing how aflatoxin B1 is detected based on TLC analysis as well as its fluorescence, which results in a purple-blue fluorescent spot of R_F value 0.5 to 0.55. The reference standard would be necessary to compare the results of the sample extracts with and without the reference standards, with the results of the reference standard itself, hence observe the absence or presence of aflatoxin B_1 in the samples.

However standard aflatoxins are obtained from many sources. The standard is often a source of error in analysis. The accuracy and purity of the standard solution is the responsibility of the individual analyst. This includes storage which require low temperature and storage in dark places or containers, as well as handling since aflatoxins are highly toxic and induce cancer.

3.4 Factors Affecting the TLC of Aflatoxin Analysis

The TLC technique serves as both purification and quantification. Successful analyses of any commodity depend on the selection of appropriate methods for preparation of extracts. Also crucial to successful analysis is TLC itself. It is not sufficiently appreciated that good

quantitation requires efficient chromatography, that is, separation of the analysis from each other extractives.

Many factors can adversely affect TLC separation and quantitation. These factors, among others include the TLC plate. In this study, the plates used were commercial and coated with silica gel – Kieselgel G, which does not completely separate the four aflatoxins (aflatoxin B_1, B_2, G_1 and G_2), and differ in the retentionty for the aflatoxins. However, this is a general elution problem in partition chromatography techniques. Previous studies show that optimum separation is achieved on plates 0.25mm thick used for preparatory TLC isolation of the aflatoxins.

Another factor is the mobile phase. In choosing solvents and solvent combinations, the analyst can take advantage of the wide range of solvent selectivities for the individual aflatoxins and interfering constituents of the extracts and greatly improve the analytical results. In the technique used in this study, the suitable solvent combinations for the analysis were the chloroform-methanol mixture. However, the neutral acetonecnioroform mixture is recommended for testing the performance of the silica gels and other conditions used for TLC.

4. CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

The TLC technique was successfully used for the routine assessment of toxicity due to aflatoxin B_1 in the detection and estimation of aflatoxin B_1 in peanut butter and groundnuts (peanuts). However no fluorescence was observed at the concentrations of the extracts used. That is, no detection of aflatoxin B1 in any of the two samples was observed, hence estimation was impossible.

4.2 Recommendation

The adapted method of analysis can be used to analyze cattle feed or cattle milk (for aflatoxin M_1) in collaboration with the department of animal science.

For quick results, high performance liquid chromatography (HPLC) is the better technique to use.

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