ANALYSIS OF FOOD PRODUCTS FOR TOXICITY WITH SPECIAL EMPHASIS ON MYCOTOXINS USING CHROMATOGRAPHIC TECHNIQUES

By

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DECLARATION

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AFFECTIONATELY DEDICATED

TO MY

LOVING PARENTS, SISTER, BROTHERS

AND MY TEACHERS.

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ABSTRACT

Mycotoxins are the natural toxic substances in foodstuffs and feedstuffs. Contamination of those mycotoxins with foodstuffs and feedstuffs may cause various hazards to human health and his animal. However, aflatoxin is the major and critical mycotoxin compound in the world. When exporting and importing the food and feed, the free or acceptable level of aflatoxin is important. There are few types of aflatoxins; but most critical and toxic type is aflatoxin B_1. Aflatoxin B_1 causes to many diseases to human and lowers the animal production. Some of those diseases are mutagenic, carcinogenic, teratogenic, hallucinogenic, emetic and oestrogenic. By applying prevention and controlling measures of aflatoxin B_1 to foodstuffs and feedstuffs, can be controlled to the acceptable level.

This research was carried out to find the quantitative determination of aflatoxin B_1 in several major foodstuffs. There are various steps are involved to analyze aflatoxin. They are sampling, extraction, extract clean up, concentration, separation of extract components, detection, determination and confirmation of identity. Thin Layer Chromatography (TLC) visual estimation is the mostly use detection method. For this, methods has been improved by Association of Analytical Chemist (AOAC) to achieve optimal extraction of aflatoxin B_1, and called AOAC (CB) method.

R_f value and the retention time of the compound are specific to one compound. In TLC, R_f values are calculated and it was varied from plate to plate. Various factors are effected to this variation of R_f. The R_f of co-spotting and standard spotting has no significant different at 05% level. When handling High Performance Liquid Chromatography (HPLC), more reproducibility, selectivity, sensitivity and lower assay time are attributed, but HPLC needs more capital cost than TLC and TLC densitometry and high level of expertise.

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CHAPTER-1

INTRODUCTION

The term of "mycotoxin" is derived from *myco* meaning fungi and toxin meaning toxicant of biological origin. Mycotoxins are secondary metabolites of fungi that can be found as contaminants of various foods and foodstuffs (Peter R. Cheeke and Lee R. Shull 1985). Contamination can occur when the potential food is a growing crop, during storage, during processing or handling, or after the finished food product has been readied for sale or consumption. The most significant sources of contamination are in the field or in storage when plant defenses are compromised (usually as a respond to stress), fungal inoculate is present, and moisture content (water activity) and temperature are adequate to support fungal growth and mycotoxin production (Peter and Lee. 1985)

Fungi that produce mycotoxins are generally called toxigenic species. There are over 100 known species of mould s that produce mycotoxins. When animals ingest a sufficient quantity of food or feed containing mycotoxin, poisoning can occur. Mycotoxin induced disease states in animals are called "mycotoxicoses". Several mycotoxicoses in livestock and poultry have been clearly associated with the presence of feed born mycotoxins (Peter and Lee, 1985).

When food stuffs such as peanut and corn are contaminated at the preharvested stage, the mycotoxin will likely to remain associated with the raw material through subsequent stages of processing, resulting in a food product that is contaminated. Because of the obvious hazard to human health posed by the presence of mycotoxins in food, it is necessary to determine the presence of mycotoxin contamination so that such food can be derived from edible uses. These determinations must begin at the earliest stage and continue through all phases of processing (Peter R. Cheeke and Lee R. shull 1985).

Although hundreds of mycotoxins are known, relatively few are recognized as serious contaminants of food. Among those that are, the aflatoxins have received the most attention from both reseachers and regulatory agents. Aflatoxins B_1, B_2, G_1, and G_2 are (figure 1.2) produced by *Aspergillus flavus* and *Aspergillus parasiticus* and are contaminants of a variety of commodities destined for the food supply. Aflatoxin $M_1$and M_2 (figure 1.1) are metabolic products of aflatoxin $B_1$and B_2 and can be found in the milk and meat of animals that have consumed feed contaminated with aflatoxins.

Although aflatoxins are acutely toxic, the greatest concern for humans is chronic exposure to low levels because of their potent carcinogenicity. This has led regulatory agencies in various countries to impose very low tolerances for aflatoxins in food ranging from none detectable to around 20 µg /kg.

Whereas other mycotoxins are not regulated to the extent of the aflatoxins, the possible association of certain mycotoxins with certain foods and beverages dictates that monitoring and analysis is done (FAO Mycotoxins analysis of food 1995).

The many different mycotoxins have demonstrated different biological effects in laboratory animals: acute toxic, mutagenic, carcinogenic, teratogenic, hallucinogenic, emetic and estrogenic (Peter R Cheeke and Lee R. Shull 1985)

Human and animals may be exposed to mycotoxins through ingestion of toxin-contaminated food and feed, inhalation or skin contact. There are two approaches possible for the detection and determination of mycotoxins; biological and chemical. Biological methods may be useful in screening for known and unknown mycotoxins. Chemical assays are preferred because these generally are much more specific, more rapid, more reproducible, and possess lower limit of detection. Hence, chemical assays play a roe of major importance in the determination of mycotoxins (George J. Banwart. 1987)

Chromatographic techniques are the most widely used chemical assays. Chromatographic process involve the solute partitioning between two phases, a stationary phase (the chromatographic bed) and mobile phase (liquid or gas), carrying substances to be separated through the chromatographic bed. The stationary phase retards more or less the progress of substances through the bed, depending on their physicochemical properties so that a separation into components can be achieved. For mycotoxin asssays the following types of chromatographic methods are used (AOAC 12th edition 1981).

(1) Open column Chromatography
(2) Thin Layer Chromatography (TLC)
(3) High Performance Liquid Chromatography (HPLC)
(4) Gas Liquid Chromatography (GLC)

In this research the TLC analysis was emphasized, because it is very common and popular techniques to separate components, and nowadays they're still numerous applications. Initially separation was carried out in one dimension using single developing solvent. Later two-dimensional TLC was introduced to mycotoxin research (Kiermeier, 1970).

The hazards to human or animals from ingestion of mycotoxin contaminated agricultural commodities has led many countries to establish measures to control the contamination of foodstuff and animal feedstuff.

Various factors play a role in establishing limits and regulations for mycotoxin. These are:

(a) Survey data: The availability of data on occurrence indicates which commodities should be considered for legal action.

(b) Toxicological data: Without toxicological information there can be no proper assessment of whether or not the substance in question is indeed hazardous. Measures are most logically taken and the basis of specific toxicological effects.

(c) Methods of analysis –Best methods for analysis should be selected depending on the type of commodities.

(d) Mycotoxin distribution - The distribution of the mycootoxin(s) in the products may pose very difficult problems in establishing regulatory criteria.

(e) Legislation -Finally the regulations in force in other countries, with which trade - contracts exist, have to be considered and, if possible, brought into harmony with the legislation under consideration.

Mycotoxin, and particularly aflatoxins should be excluded from food as far as possible, but since the substances are present in food as natural contaminants the exposure to man cannot be completely prevented. Therefore the exposure to the population of some level of aflatoxins must be tolerated.

The adoption of regulations for aflatoxin control has been a process of continues development in some countries. (FAO Food and Nutrition paper, 1991)

Drafters of aflatoxin regulation for foodstuffs most often apply the tolerance level for aflatoxin B1 of 5µg/kg. Aflatoxin B_1 is the most important of the aflatoxins, considered from bath viewpoints of toxicology and occurrence. It is unlikely that commodities will contain aflatoxins B_2, G_1, and G_2 and not aflatoxin B_1, whereas the concentration of aflatoxin B_1 alone (Van Egmend, 1991). Aflatoxin M_1 in milk has two major tolerance levels, which are .05 and .5 µg/kg. Another recent development in community legislation is the introduction of a tolerance for aflatoxin B_1 in foodstuff ingredient at 200µg/kg. (FAO. Food and nutrition paper, Analysis of mycotoxins, 1991)

Objectives and aims

- Quantitative and qualitative determinations of aflatoxin B_1 in some foodstuffs, which are oil based products (desiccated coconut and groundnut) corn tea, and spices.
- Comparison of different extraction and cleanup methods of an aflatoxin B_1 in those products.
- Identifying the acceptable levels for aflatoxin B_1 in different countries for various commodities.

CHAPTER-2

REVIEW OF LITERATURE

2.1 GENARAL ELEMENTS OF MYCOTOXICOLOGY

The subjects of mycotoxins can be subdivided into three general areas: Mycology, mycotoxin chemistry, and toxicology.

2.1.1 MYCOLOGY

Mycology encompasses mold taxonomy as well as all the requirements, processes, factors necessary for colonization (spore germination and mould growth) and biosynthesis of mycotoxins in foods and feeds. (Peter R. Cheeke, and Lee R Shull. 1985)

There is little correlation between the pattern of toxin production by particular fungi and their phylogenetic classification (Samuels. G.J. 1994). Fungi are eukaryotes having well defined membrane bound nuclei with a number of chromosomes and as such are clearly different from bacteria. All but few fungi have a well-defined cell wall. Among fungi there are plant and animal parasites and saprophytes. They do this by growing filaments or hyphae containing hydrolytic enzymes. These hyphae form complex mycelial mat. This filamentous growth is closely associated with the production of spores. (Peter R. Cheeke.and Lee R.Shull. 1985)

Fungi are able to produce wide ranges of secondary metabolites such as pigments and toxins. Some secondary metabolites have antibiotic properties and some are toxic. (Peter R. Cheeke and Lee R. Shull, 1985)

Simpson and Batra (1984) divided toxigenic fungi into two nonphylogenetic groups.

(1) Endomycotoxic fungi and

(2) Exomycotoxic fungi

Species in the first group produce intracellular toxins that have typically acute and sometimes fatal toxic effects on animals. Certain species of the genus *Amentia* (toxic mushrooms) are endomycotoxic fungi. (Simpson and Batra, 1984)

The second group, exomycotoxic fungi, produces toxins that are extracted into the substratum. There are forty known genera of fungi that fall into this group. The most toxicology significant of these genera can be assigned to two phyla; or genetic orders: *Eurotiales*, which includes *Aspergillus*. and *Penicillium*, and *hypocreales*, which includes *Fusarium* (Peter R. Cheeke and LeeR. Shull, 1985).

Toxins produced by genera of *Eurotiales* are quite diverse and representative of several different classes of secondary metabolites including the polyketides (aflatoxins, ochratoxins, citrinin, patuline, sterigmatocystins) and the bisanhydrides (rubratoxine) more toxigenic genera are classified under Hypocreales than any other order.

Toxigenic species within the three dominant genera, *Aspergillus, Penicillium,* and *Fusarium,* occupy a wide range of habitats, sometime alone, sometimes in competition. Pitt and Udagawa (1984) distinguished these three genera in a general way as follows. *Fusarium* species attack plant crops as an active pathogen, but are seldom found in a saprophytic role. *Penicillium* species are active pathogen in a few kinds of fruit, but their dominant role in saprophytic habitats, especially a cooler climate. *Aspergillus* species are occasional pathogens in plant or animal tissues, and are dominant saprophytes in warmer regions, and under conditions of reduced water activity (Peter R.Cheeke and Lee R. Shull, 1985).

2.1.2 Mycotoxin chemistry

Mycotoxin chemistry encompasses composition and analysis of mycotoxins. Knowledge of the physicochemical properties (e.g., solubility, ionization, and molecular size and shape) established with purified mycotoxins, provide the basis for understanding their fate (absorption, distribution, metabolism, and elimination) in the body. Both quantitative and qualitative aspects of metabolism are dependent on these physicochemical properties. The availability of molecular sites for metabolism is an important determinant of whether metabolites are more or less toxic or persistent than parent mycotoxins. Similarly, the reactivity of a few mycotoxins in biological systems can be explained from their molecular structure.

For example examination of the physico-chemical and biochemical characteristics of the aflatoxin B_1 molecule reveals two important sites for toxicological activity. The first site is at the double bond in position 8,9 of the furo-furan ring. (Figure I) The aflatoxin DNA and protein interactions, which occur at this site, alter the normal biochemical functions of this macromolecule, leading to deleterious effect at the cellular level. The second reactive site is the lactone ring in the coumarin moiety. (Figure 2) The lactone ring is easily hydrolyzed; it is therefore a vulnerable site for aflatoxin degradation. (Samarajeewa.U, et. Al, 1989)

Figure: 1.1 The important sites of toxicological activity of aflatoxin B_1 structure

Lactone ring Furo-furan ring

The reactivity of all trichothecenes is attributed to the 12-13 epoxide and 9-10 double bond in the nucleus of the molecule. (Chu 1977).

The type of R group substitution accounts for the variation in toxicity of structurally related mycotoxins. The toxicity of many mycotoxins can be explained at the chemical level.

For example, the chemical basis of the cacinogenicity of aflatoxin B_1 has been elucidated (Wogan and Busby, 1980).

A related area in mycotoxin chemistry is analysis. Methods and instrumentation are now available for identification and quantification of most of the common mycotoxins in foodstuffs. Chemical testing of foods is the only definitive way determining the presence of mycotoxins.

2.1.2.1 Analysis of mycotoxins

There are numerous analytical procedures available. The methods of choice depend on several factors such as the chemical nature of the mycotoxin, the degree of quantitation required (Screening versus very accurate quantitation), the cost that can be incurred, and time that can be allotted. In general, however there are three basic steps involved in most analysis, and each step the best methodology must be developed given the constraints listed above. These three steps are extraction, cleanup, and quantitation. (Association Of Official Methods Chemist, 12th edition1984. Washington D.C.)

In this approach, analysis of aflatoxin B_1 is emphasized.

2.1.2.2. Extraction.

In the case of a solid food product, either shaking or blending the sample with an appropriate solvent usually accomplishes extraction. The choice of solvent is dependent on the chemical nature of the toxin and the matrix of the sample.

Typical extraction solvents include chloroform, ethyl acetate, methanol, acetone, acetonitrile, water and combination of one of the organic solvent and water.

Some times very non-polar solvents such as hexane are included to extract fat/oil from food containing high fat level prior to the proper extraction. The pH of the extraction solvent may need to be adjusted for certain acidic (e.g.: ochratoxin, cyclopiazonic acid) or basic (e.g.: ergot alkaloids) mycotoxins. The ratio of solvent to sample generally rangers from 2 to 5 ml/g. When extractions are performed with a high-speed blender, the blending time is usually 2-3 min, and additional particle size reduction may occur, leading to more efficient extraction.

Shaking the sample with extraction solvent for about 30 min is also commonly used extraction method. Although shaking is more time consuming than blending, automatic shakers usually have the capacity for several flasks, thus enabling simultaneous extraction of several samples.

2.1.2.3 Cleanup

In addition to extracting the mycotoxin of interest, the extraction solvent usually extracts a number of substances that can subsequently interfere with an accurate quantitation of the mycotoxin. Therefore it is usually necessary to partially purify the mycotoxin by removing interfering substances so that accurate and reliable quantitation can be achieved. In some cases then cleanup may be very simple, requiring only filtration of the extract. This is quite often true when the quantitative step is very specific, such as with an enzyme linked immunosorbent assay (ELISA). In other cases the cleanup may be the most rigorous and time consuming part of the analytical procedure, requiring a combination of several operations including centrifugation, filtration, precipitation, column chromatography, etc. The objective of cleanup step is to separate the mycotoxins from any substances that might interfere with accurate quantitation is quickly and cheaply as possible. This process may require one or a combination of several of the steps described below.

2.1.2.3.1 Centrifugation

Centrifugation is sometimes employed as the first step of cleanup when emulsions from, during extraction or when filtration is difficult. Although not commonly used, it is beneficial or necessary with some methods. It is probably most often used in analyses of dairy products. (AOAC 12th edition. Washington. 1984)

2.1.2.3.2 Filtration

Filtration is most often the first step of cleanup after extraction. It is necessary to remove solids and micro particulate mater before quantitation is attempted. Filtration may be the only cleanup step required when other extracted substances do not interfere in the quantitative step (as with ELISA). However, in most chemical analyses filtration is merle preliminary to other chemically oriented cleanup measures. In some cases filtration may be difficult and a filtering aid such as diatomaceous earth is used. (Association Of Official Analytical Chemist, 12th edition, 1984. Washington)

2.1.2.3.3 Precipitation

Many extracts contain high molecular weight pigments and proteineous substances that can be removed by precipitation and filtration through the addition of salts of lead, zinc, ammonia, iron copper, etc., as well as acids or bases (Association Of Official Analytical Chemist, 12th edition, 1984. Washington).

2.1.2.3.4 Liquid liquid partition

Liquid liquid partition is one of the oldest and still most commonly used chemical method. In many cases it is the only clean up step use in addition to filtration. It is particularly useful for acidic or basic mycotoxins (ochratoxin, cyclopizonic acid, ergot alkaloids), which, through adjustments to the pH, can be shifted from the organic phase to the aqueous phase and back to the organic phase, resulting in a high degree of purification (Association Of Official Analytical chemist, 12th edition. 1984, Washington.).

2.1.2.3.5 Column chromatography

Column chromatography is probably the most widely employed cleanup procedure. It can be highly specific and usually results in a high degree of purification. Typically, open columns are packed with silica gel (or other absorbents such as florisil, alumina, charcoal, or ion exchange resins), and many interesting substances are eluted while the mycotoxin of interest is retained. Finally, the mycotoxin is eluted, and after concentration it is ready for quantitation. Disadvantages of this classical technique are that it is time consuming and uses large volumes of solvent (Association of Official Analytical Chemists, 12th edition, 1984, Washington).

2.1.2.3.6 Solid phase extraction (SPE)

Solid phase extraction (SPE) columns or cartridges have been developed that accomplish the same purpose as the former methods but with great savings of time and solvents. Many commercial products are available. One type is a chem.- tube or Extrelut column that is filled with an inert hydrophilic matrix. The mycotoxin, in an aqueous phase, is poured into the column and allowed to sit for several minutes. It is then eluted with an organic solvent, accomplishing what liquid liquid partition does, but with much less solvent (Association of Official Analytical Chemists, 12th edition, 1984, Washington), also commercially available are disposable SPE cartridges packed with high efficiency adsorbents such as silica, alumina, florisil, ion exchange resins, and various reverse phase bonding. Because of there high efficiency, solvent consumption can be reduced greatly compared with open column chromatography.

2.1.2.3.7 Immunoaffinity columns

With the recent emergence of immunological methods for mycotoxins has come the development of highly specific immunoaffinity cleanup columns. Monoclonal or polyclonal antibodies specific to particular mycotoxin are immobilized on a solid support (agarose) and packed into small column or disposable cartridge. The filtered extract is passed through the column, and the toxin binds to the antibodies. Interfering substances are washed through the column, and the toxin is subsequently eluted with an appropriate solvent. This sample cleanup technique produces a highly purified toxin that is ready for quantitation. Because of specificity of the immunoaffinity column, extraction and cleanup of mycotoxins in lipids (e.g., milk) can take place simultaneously on the column. (George and Banwart; 1987)

2.1.2.3.8 Concentration

The final step of many cleanup protocols is the concentration of the mycotoxin in an appropriate solvent for quatitation. Usually the solvent is removed with a rotary evaporator or with gentle heating in a nitrogen atmosphere (Association of Official Analytical Chemists, 12th edition, 1984, Washington).

2.1.2.4 Quantitation

Many choices are available for the quantitative step. In many cases accurate quantitation is not the goal; rather, a quantitative determination of the presence or absence of a mycotoxin or determination of a broad range of contamination is all that is necessary.

Many screening methods are available to accomplish this. Most methods designed to provide accurate quantitation rely on a chromatographic separation followed by detection of mycotoxins. Thin Layer Chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC), the later often coupled with mass spectrometry (GC-MS), is the most commonly employed method for accurately quantitation.

Immunoassays, many of which are commercially available, have gained favor in recent years because they are highly specific and sensitive, do not require expensive instrumentation, and are relatively easy to use (Association of Official Analytical Chemists, 12th edition, 1984, Washington).

2.1.2.4.1 Screening methods

Minicolumn chromatography has been popular screening method for the semi quantitation of aflatoxin and other toxins. A small column is packed with layers of different absorbent, such as silica gel, florisil and alumina. The partially cleanup extract is applied to the minicolumn and eluted with an appropriate solvent. The mycotoxin is bound at the interface of two layers of absorbent and detected by fluorescence under UV light. The method is rapid, inexpensive, and, in, the case of aflatoxin, sensitive to 5-10ug/kg.
Several immunoassay - based screening kits are commercially available and have application to a wide range of mycotoxins (Taller and Hanchary, 1984). Most of these use a visual color change to indicate the presence or absence of toxin at a particular levels of contamination (e.g., 20μg/kg) some employee a range of standards so that semiquantitation can be achieved. The methods are very useful in applications where rapid results are needed and accurate quantitation is not required. (FAO, Analysis of mycotoxins, 1991)

2.1.2.4.2 Thin Layer Chromatography (TLC)

The oldest and still most often used method of quantitation of mycotoxins is TLC. Typically a thin layer of silica gel or other absorbent (allumina, reverse phase silica) is coated into a glass plate and activated by drying.

This can be done in the laboratory, but the most often commercially prepared plates are used. Micro liter quantities of cleanup extract and standard are applied in a horizontal line near one edge of the plate. This edge of the plate is placed in a tank containing an appropriate solvent system, which migrates through the sorbent layer, separating the components of the sample. Mycotoxins are usually visualized as fluorescent spots under UV light, or plates can be sprayed with or exposed to various reagents to effect a chemical change in the mycotoxin that makes it visible.

Quantitation is achieved by comparison of the intensity of the fluorescence or color of the sample spot with those of a series of standards. This can be done visually or with the aid of a densitometry. (Taller and Hathary, 1984)

2.1.2.4.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is similar in principle to TLC in that a solvent (mobile phase) is passed through a sorbent (stationary phase) to effect the separation of the mycotoxin(s) from other components. It differs in that the high efficiency sobent is packed in to a column and the solvent is pushed through with a high-pressure pump. In normal phase HPLC, columns are packed with silica gel and mobile phases are composed primarily of low polarity solvents. In reverse phase HPLC, columns are packed with silica to which has been bound a particular functionality (e.g., C_8, C_{18}, phenyl, cyano), which produces a relatively non -polar stationary phase. Mobile phases are quite polar, normally consisting of combination water, methanol, acetonitrile, etc. After mycotoxins elute from the column, they immediately pass in to a detector, which usually measure the fluorescence (fluorescence detection) or absorbance (UV detection) of the mycotoxin. This can be compared with similar measurement of known concentrations of standards to produce accurate quantitation (Taller and Hathary, 1984)

2.1.2.4.4 Chromatography (GC) and Gas Chromatography- Mass Spectrometry (GC-MS)

Although used to minor extend for quantitation of variety of mycotoxins, gas chromatography (GC) has become the method of choice for the trichothecenes. This group of toxins lacks an UV chromophore, making them unsuitable for HPLC analyses requiring UV or fluorescence detection. However, volatile derivatives of trichothecenes can be prepared, and GC analysis with flame ionization or electron capture detector detection provides a highly sensitive quantitation tool for this group of toxins.

The most popular columns for GC analysis today are fused silica capillary columns coated with a thin polymeric film. The length of these columns (15-30m) gives them tremendous resolving power.

When a gas chromatograph is coupled with a mass spectrometer (GC-MS), the most reliable identification of GC peaks can be achieved. This method can be used to separate and identify complex mixtures of trichothecenes with high sensitivity and selectively. (Taller and Hathary, 1984)

2.1.2.4.5 Immunoassays

The production of antibodies to specific mycotoxins in recent years has led to development of many immunoassays, which have the advantages of selectivity and sensitivity. Immunoassays for mycotoxins are two basic types: radio immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Both are based on the competition between unlabeled toxin in the sample and labeled toxin in the assay system for antibody binding sites.

2.1.3 Toxicology

Mycotoxin can cause a broad spectrum of biological effects, including both acute and chronic toxicoses. Evidence of mycotoxicoses was apparent long before their identities were known for example, in 1908, a disease characterized by photosensitization in ruminant in New Zeland was described (Peter R. Cheeke and Lee R. Shull. 1985).

Alimentary toxic aleukia (ATA); or septic ongina, has occurred in Russia at infrequent intervals. Symptoms of this illness include a burning sensation in the mouth; the tongue feels stiff, with diarrhea, nausea, vomiting and perspiration (George J. Benwart., 1987). More than 100,000 turkey pouts died within a few months in England during 1960.Investigation revealed that ducklings, young peasants and partridges also had a high motility.

About this same time a trout hatchery in California experienced severe losses due to hepatomas. Reports from Africa also revealed heavy losses ducklings when the information of these incidents was accumulated and examined, all of these isolated losses were due to the presence of aflatoxin. N general, toxic effects are initiated at the sub cellular level and may or may not be accompanied by discernible pathological changes. (George J. benwart.1987).

Table: 2.1 some mycotoxins that might cause human illness.

Mycotoxins	Some producing organisms
Aflatoxin	*Aspergillus flavus, Aspergillus parasiticus*
Citreoviridin	*Penicillium citrinum, Penicillium viridicatum*
Cyclopiazonic acid	*Penicillium cyclopium*
Ergotoxins (alkaloids)	*Claviceps purpurea*
Luteuskyrin	*Penicillium islandicum*
Ochratoxins	*Aspergillus ochraceus, Penicillium viridicatum*
Patulin	*Penicillium expansum, Penicillium patulum, Penicillium urticae,Aspergillus cavatus, Byssochlamys nivea.*
Pennicilic acid	*Penicilliummartensil, Penicillium viridicatum Penicillium cyclopium, Pnicillium* um, Penicillium patitans
Roquefortine	*Penicillium roqueforti*
Rubratoxin	*Penicillium rugulosum*
Stachybotrytoxin	*Stachybotrys atra*
Sterigmatocystin	*Aspergillus versicolor, A. nidulans*
Tenuzonic acid	*Alternaria tenuissima, Alternaria alterata*
Trichothecenes	*Fusarium graminarium, giberella zeae*
Fusarenon-x	*Fusarium nivale*
Nusolaniol	*Fusarium solani*
Nivalenol	*Fusarium nivale, Fusarium roseum*
Fusarium- T-2	*Fusarium tricinctum, Fusarium solani, Fusarium roseum*
Trichothecenes	*Trichothecium roseum.*
Zearalenone	*Fusarium graminarium, Fusarium tricinctum, F.culmorum*

(Source: George J. Banwart, Basic food microbiology. 1987)

2.1.3.1 Biological effects

The range of biological effect is as varied as the chemical structures of the different mycotoxins and, as would be expected, their individual toxicology is greatly dependent on dose and frequency and duration of exposure (Peter R.Cheeke and Lee R Shull. 1985).
Aflatoxin can cause a response in microorganism, cell culture, plants and animals. The effects of aflatoxins can be toxigenic, mutagenic, teratogenic or carcinogenic.

Several species of *Bacillus* and *Flavobacterium* are inhibited by aflatoxins.

Other microorganism including Actinomycetes, and various yeast and moulds are altered by the presence of aflatoxin in the substrate. Aflatoxins can interfere with plants by inhibiting the germination of seeds and / or chlorophyll synthesis.

In cell cultures, aflatoxin causes a decrease cell numbers, protein level, RNA, and DNA. There is an inhibition of mitosis, the formation of giant cells and accumulation of cellular debris. (Fujimoto and OHBA, 1975). (Mutagenic effect of aflatoxin were described by Ong 1975.)

Species of animals vary in their resistant. For farm poultry, ducklings are the most susceptible, followed by turkey poults, pheasant chicks, goslings, chickens and quail. For farm animals, the order from high susceptibility to resistance is young pigs, pregnant sows, and calves, fattening pigs, mature cattle and sheep. Ducklings are biological system for assaying of aflatoxins. The acute lethality of aflatoxin is very evident in rainbow trout (LD 50 about 0.5mg/kg).

Tables 2.2 indicate the wide diversity of toxic effects caused by mycotoxins.

Table 2.2 Classification of mycotoxins based on biological effects

Biological effect	Types of mycotoxins
Hepatotoxins	Aflatoxin, sporidesmin, rubratoxin B, Sterigmatocystin, Tricothecenes, Ochratoxin A, phomopsin
Nephrotoxins	Ochratoxin A, Citrinin, Aflatoxin, Oxalate
Neurotoxins	Tricothecenes (e.g., vomitoxin, satratoxin), Slaframine, penitram A, (And other tremorgens) Ochratoxin A, Ergot alkaloids, Lupinosis (Ammonia accumulation).
Genitoxins	Zearalenone, Ergot alkaloids, Aflatoxins
Dermitoxins	12-13- Epoxy trichothecens (e.g., T-2 toxin, nivalenol)
Photosensitizing agents	Sporedesmin (facial eczema)
Carcinogens	Aflatoxin, Sterigmatocystin, luteoskyrin, patulin, pennicilic acid, T-2 Toxin (possibly), Ochratoxin A, (possibly) citrinin, (possibly)
Teratogens	Aflatoxin, (possibly), Ochratoxin A
Immunosuppressants	Aflatoxin, Trichothecenes (T-2 toxin), Ochratoxin (possibly)
Hematologic agents	Dicoumarol, aflatoxin, trichothecenes

(Source: Peter R. Cheeke, Lee R. Shull, Natural toxins in feed, 1985)

2.1.3.2 Effect on metabolic process

These include interference with carbohydrate and lipid metabolism and microbial respiration. However the contribution of alteration in these essential processes to the overall toxicity has been difficult to access.

2.1.3.3. Acute intoxication

The principal target organ in all species is the liver. Numerous liver functions are affected, and the cumulative impacted can be fatal to animals. After administration of a large single dose of AFB1, hepatocytes undergo progressive changes, which include infiltration with lipids, eventually ending in necrosis (cell death). These toxic effects are believed to be result of wild spread and nonspecific interaction between AFB_1 or its activated metabolites and various cell proteins. Interaction with key enzymes can disrupt basic metabolic process in the cell, such as carbohydrate or lipid metabolism, and protein synthesis. A s the liver loses its functionality, other effect appear such as derangement of blood clotting mechanisms (cougulopathy), icterus (jaundice), and reduction of essential serum proteins which are synthesized in the liver. (George and Banwart, 1987)

2.1.3.4 chronic intoxication

Chronic poisoning, or aflatoxicoses, can result when low levels of toxin are ingested over a prolonged period. The toxic effects are not nearly as specific or clinically evident as in acute intoxication. In general, affected livestock exhibit decreased growth rate, lowered productivity (milk or eggs), and immunosuppression. (George and Banwart, 1987)

Reduced growth rate is considered the most common effect associated with chronic aflatoxicosis in farm animals. Liver damage is also prevalent in chronic aflatoxicosis in all species. At necropsy, the liver is usually pale to yellow, and the gall bladder may be enlarged. (George and Banwart, 1987)

2.2 Aflatoxins associated with concentrate foods

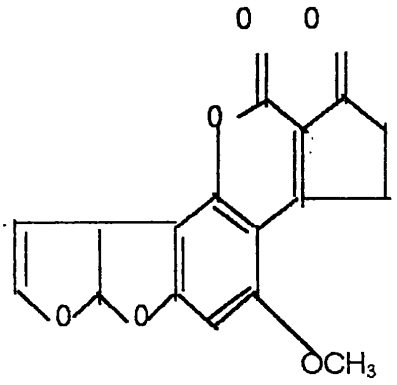
Aflatoxins are a family of family of bisfuranocoumarin compounds produced primarily by toxigenic strains of *Aspergillus parasiticus* only about one half of the known strains of *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxins.

Other fungi such as *Penicillium* spp.,*Rhizopus* spp., *Mucor* spp., and *Streptomysces* spp., are capable of producing aflatoxins. The name "aflatoxin" derives from *Aspergillus* (a), flavous (fla), and toxin. *Aspergillus flvous* and *Aspergillus parasiticus* produce for major toxins; B_1, B_2, G_1 and G_2.These were named according to their fluorescence properties under long wave ultra violet light on thin layer chromatographic (TLC) plates; B_1 and B_2 fluorescence blue, whereas G_1 and G_2 fluorescence green.

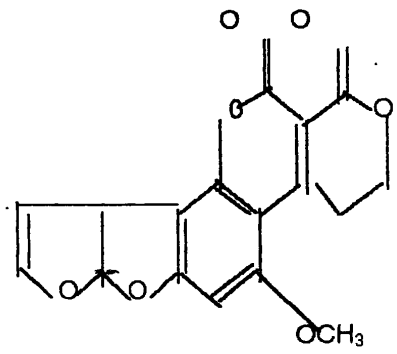
Fourteen other aflatoxins are known but most of these are metabolites formed endogenously in animal administrated one or more of the four major afltoxines.

Metabolites of toxicological significant include: aflatoxin B_{12}, 3-oxide (AFB1 2,3, -oxide) aflatoxin M_1 (AFM_1); aflatoxin, and aflatoxin B_{2a} (AFB_{2a})

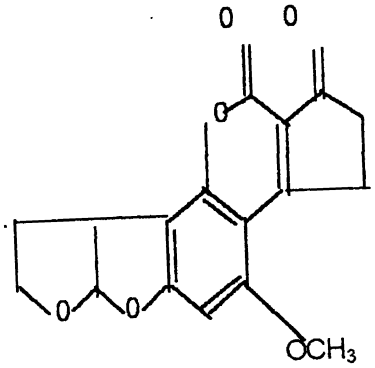
Figure 1.2 structures of aflatoxins



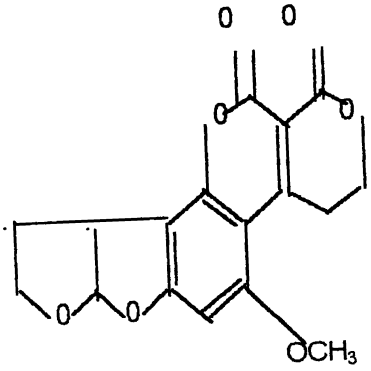
Aflatoxin B_1



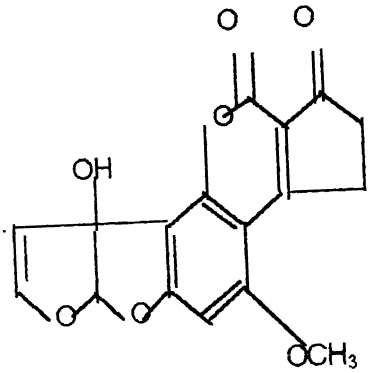
Aflatoxin G_1



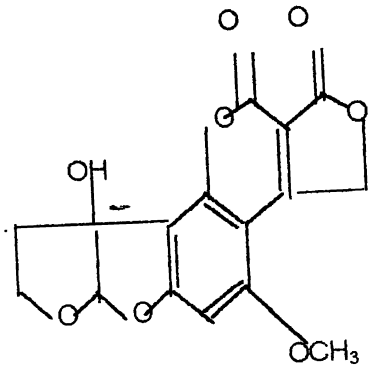
Aflatoxin B_2



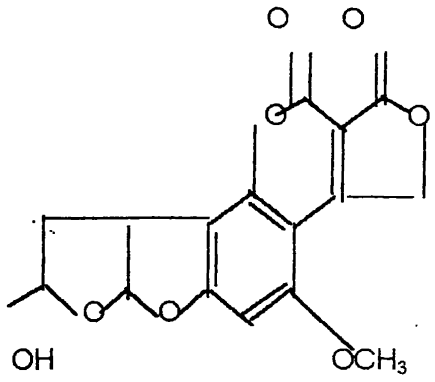
Aflatoxin G_2



Aflatoxin M_1



Aflatoxin M_2



Aflatoxin B_{2a}

2.3 Occurrence of aflatoxins

Aflatoxin producing strains of *Aspergillus* are distributed worldwide in soil and air. When environmental conditions are favorable and a substrate (feed or seed) is accessible as a nutrient souse, colonization and mould growth can easily occur. The resulting profile of aflatoxins and their individual concentrations will vary greatly according to the existing environmental conditions (temperature, moisture, and aeration), the substrate, and the type of mould involved. For example, *Aspergillus flavus* growing on corn produces primarily B_1, B_2 where as *Aspergillus paraciticus* on corn products all four major aflatoxins: B_1, B_2 ,G_1, G_2.On soybeans, only negligible concentrations of B_1 are produced by both species. *Aspergillus flavus* is primarily a seed colonized mould and is usually referred to as a storage mold.
It is capable of colonizing most of the important gram crops including corn, small grains, peanuts, cottonseed, cassava, copra, and most nut crops, if moisture and temperature condition are favorable. The moisture content of mould growth and aflatoxin formation require a moisture content of grater than 14%, a temperature at least 25°C, and some degree of aeration (O2). When these requirements are met, mould infestation followed by aflatoxin formation in target crops is likely to occur.

This major foodstuff with high potential for invasion by *Aspergillus* species during growth, harvest, transportation, or storage of corn, cottonseed, and peanuts. Colonization of small grains and soybeans generally occurs in storage. Storage condition for soybeans that promote aflatoxin formation, aside from optimal moisture and temperature conditions, are lack of aëration system or their improper use (temperature differentials can cause moisture migration), kernel damage and spore dissemination caused by storage insects, presence of fines (dust, weed seeds, and broken kernels), and poor sanitary practices in foods.
Ensuring the maintenance of the correct temperature and dryness during storage and distribution could control occurrence of aflatoxin in food and animal feed.

Unfortunately the contamination of commodities such as groundnuts and maize with aflatoxin is far more complex and may involve infection with these two species of *Aspergillus* and productive of aflatoxin in the field. This is specially associated with drought stress and insect damage. However even healthy plant the stigma of the developing flowers.

2.4 Detoxification of Aflatoxins in Foods and Feeds

The sensitivity of aflatoxins to physical or chemical treatments is affected by many factors including moisture content, location of the toxin in the foods, and forms of the foods, and interactions of the toxins with food components (U. Samarajeewa, U. et al. 1991).

Thus, it is important to understand these factors before a specific detoxification method can be recommended. In addition, the use of any applicable treatment conditions Should not a use undesirable altercations to the nutritional and organoleptic qualities of the foods. The combined uses of physical and chemical treatments procedures appear to provide use a better prospect than the use of only a single treatment procedure. (U. Samarajeewa, 1990).

Examination of the physico - chemical and biochemical characteristic of the AFB_1 molecule reveals two important sites for toxicological activity (Buleman, L.B H.M. Bamhart, and T.E.Hartung.1973). The first site is at the double bond in position 8,9 of the furo- furan ring. The aflatoxin DNA and protein interactions, which occur at this site, alter the normal biochemical functions of these macromolecules, leading to deleterious effects at the cellular level. The second reactive site is the lactone ring in the coumarin moiety. The lactone ring is easily hydrolyzed; it is therefore a vulnerable site for aflatoxin degradation. Degradation treatments of aflatoxin B_1 should therefore be aimed either at removing the double bond of the terminal Furan ring or in opening the lactone ring.

2.4.1 Physical methods

. The appropriate Aflatoxins, after absorbing energy to reach an excited state, may undergo transformations to produce non-toxic molecules or molecules of reduced toxicity. Energy source of and treatment conditions that can be used to effectively cause aflatoxin breakdown may provide an acceptable means for detoxification of contaminated foods. The energy used for such treatment can be provided in the form of heat, gamma radiation, ultraviolet (UV), or visible light.

2.4.1.1 Heat

Sold aflatoxin B_1 is stable to dry heat up to its melting points of 260 ^{0}C, the thermal decomposition temperature is 269 ^{0}c (Chu, F.S.C.C Chang, S.H. Ashoor, and N. PenticeE. 1975).

However temperature as high as 300 ^{0}C have been observed to be necessary to degrade aflatoxin B_1 in certain foods. The sensitivity of aflatoxins to heat is governed by environmental conditions. On one hand, the presence of moisture in foods may enhance degradation by hydrolyzing the lactone at a critical moisture concentration and temperature (Bulliman L.B. 1973). On other hand, their "binding" or association with proteins and other food constituents may protect aflatoxin in foods in part.

2.4.1.2 Irradiation

Ionizing radiation is currently recommended and used to eliminate pathogenic microorganisms in food (111). Gamma radiation penetrates effectively through both liquid and solid media. However, aflatoxin are resistant to direct treatment by gamma radiation; indirect effects such as radiolysis of water or other simple food constituents may initiate free radical reactance leading to degradation of aflatoxin (55).

2.4.1.3 Ultraviolet and visible light

Aflatoxin B_1 absorbs ultraviolet (UV) radiation at 222, 265, and 362 nm, with the greatest absorption occurring at 362 nm.

Irradiation at 362 nm activates aflatoxin B_1 and increases it susceptibility to degradation. The accumulated evidence from ultraviolet and solar radiation studies indicates that the double bond in the terminal furan ring of aflatoxin B_1 is the likely site of photo degradation.

2.4.2 Chemical methods

Chemical degradation of aflatoxins is currently viewed as a more practical approach for its decontamination in foods. Many chemicals have been screened for their ability to detoxify pure aflatoxin B_1 (170).

CHAPTER-3

Materials and methods

3.1 Samples analyzed in the study

The investigation carried out covered following commodities; desiccated coconut, black tea and green tea, groundnuts, corn, cinnamon quills, nut-meg, clove, cardamom pods, coriander, dried chili, and wine spices.

The samples of groundnuts and corn were collected from Colombo market. Desiccated coconut, tea and other spices samples were obtained from lots, prepared for export, reached by S.G.S Lanka (Pvt.) Ltd. Laboratory services, for various chemical testing.

3.2 Preparation of samples

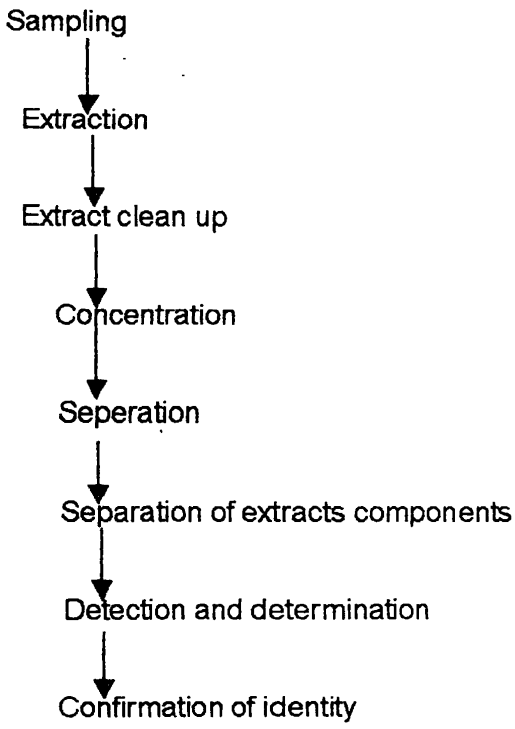
A properly drown sample, in the range of 2-40 Kg, is too large for analysis. Therefore, it must be sub sampled to obtain an analytical sample in the range of 25-200 g (most often 50g). Quartering techniques sub sampled groundnut, corn, tea and some spices. Because mycotoxin contamination is still quite heterogeneous at this point, another error introduced. However, this error can be greatly minimized by thoroughly grinding and blending the sample.

Batch type size reduction equipment, (Hobert vertical cutter/ mixer (VCM) Waring blender; food cutter) reduce particle size and mix in one operation. With other types of size reduction equipment and when product is received in finely ground state, mixing is needed. Free flowing dry materials can be mixed in double cone or twin shell blender such as twin shell blender (Cereal food world 29,771, (1984))

3.3 Drawing of analytical sample

Analytical samples were prepared by sub-dividing the lot samples. When homogenized samples were not able to obtain by sub-dividing, composite number of randomly selected samples were taken. (Cereal Food World 29,771, 1984 JAOC 62,1182 (1979)).

Figure 3.1 Basic steps of all chemical analytical methods for the detection and determination of mycotoxin



3.4 Extraction techniques

Three different extraction methods are used and the results were compared.

3.4.1 (a) Apparatus

(1) High speeds blender - Explosion-proof with 1L (qt) jar. Drill32 min (1/8") hole. Ca 1 cm from center of lid permits escape of vapors.
(2) Wrist action shaker -Burrell
(3) Stir motor, 1/30 hp, and 1403-1600 rpm with stainless steel shaft and propeller blade.
(4) Funnel- 150 min with fluted S & S 588 or equiv., paper no fit: or burchner . 32-cm diam., with Whatman No. 42 or equiv: to fit.
(5) Glass stoppered conical flask 100 ml, 500ml
(6) Measuring cylinder.
(7) Analytical microbalance.

3.4.1 (b) Reagents

(1) Diatomaceous earth- Hyflo super- cell.
(2) Sodium sulfate- anhydrous granular ACS grade.
(3) Solvents ACS grade in glass: chloroform (CHCl3), acetone, Petroleum ether (anhydrous. < 0.01%alcohol)

3.4.2. Modified Association Of Official Analytical Chemists "Contaminated Branch" Methods (AOAC CB)(Basil A Roberts JAOAC)

A grand homogenized sample (10g) was measured accurately and transferred to 100 ml glass stoppered conical flask. To this was added chloroform (50 ml) and water (5ml) with diatomaceous earth. The mixture was well shakers for 30 minutes using wrist action shaker or magnetic stirrer, filtered through a 12 cm No. 42 Whatman type filter paper and sufficient filtrate (5ml) was collected to allow pippeting of a 5 ml aliquot. Anhydrous sodium sulfate (2g) was added into the mixture, which was then re-filtered after 15 min.

3.4.3 Association of Official Analytical Chemist "Contaminated Branch" method

Sample of groundnuts, desiccated coconut, spices, were weight (50g) into conical flask (500ml). Diatomaceous earth (25g), chloroform (250ml) and water (25ml) were added and the flask stoppered. The mixture was shaken for 30 min. in wrist action shaker and filtered through fluted filter paper (Whatman No. 42) or transferred to a Burchner funnel precoated with layer of diatomaceous earth (5mm). The filtrate (50-ml) was collected in a flask (100-ml). Anhydrous sodium sulfate (5g) was added to filtrate which was then well shaken. The flask was stoppered and retained for the AOAC silica column cleanup step.

3.4.4 Aqueous acetone method: That is the method described by the tropical products Institute G- 70 report (EEC method)

Groundnut or desiccated coconut (20g) samples were crushed in a motor and defatted in a soxhlet apparatus using petroleum ether (b.p 40 ^{0}c-60 ^{0}c) for 3 hours. The defatted material was dried for a ^{1} h. at 105 ^{0}c in forced draft oven. Acetone: water (70:30)(100ml) was added to the sample of defatted groundnut or desiccated coconut (20g) in conical flask (250ml).
The flasks were well stoppered and shaken for 30 min. in a wrist action shaker. The mixture was then filtered through a filter paper into a measuring cylinder. The clear extract was retained for the lead acetate cleanup state.

3.5 Clean-up procedure

3.5.1 (a) Apparatus

(1) A glass column - Uniform length (20 cm) and internal diameter 1 cm
(2) Seppak silica cartridge
(3) Rotary evaporator- with continuous feed
(4) Vials 1,2,and 4 dram, screw cap (kimbile glass No. 60910-l, but Al foil- or Teflon linked)
(5) Funnel - 150-mm. diameter with fluted No. 42 Whatman filter paper (32cm diameter).
(6) A glass hypodermic syringe
(6) A round bottom flask (500ml)
(7) Separator funnel- 250 ml capacity

3.5.1 (b) Reagents

(1) Silica gel 60 Brinkman #7734, 70-230 mesh. Dry 2hs.at 110 ^{0}C
(2) Sodium sulfate - Anhydrous granular ACS grade
(3) Solvents - ACS grade in glass: acetone, $CHCl_3$, n- hexane, diethyl ether (Anhydrous -, <0.01%alcohol)

3.5.2 Silica gel minicolumn clean up

A glass column length (20cm) and internal diameter (1cm), was used as a minicolumn. This was about one length the size of the usual AOAC CB column. Silica gel (2g) prepared according to AOAC standard procedures and anhydrous sodium sulfate (3g) was packed into the column. The relevant extract was introduced in to the top of the column using a small amount of chloroform. The column was then washed successively three times with portions (30 ml) of (1) n- hexane (11) diethyl ether (111) chloroform methanol (97:3). The last fraction was collected and evaporated in a rotor vapor (below 50c).The dried extract was used for TLC and HPLC analysis.

3.5.3 Seppak silica cartridge cleanup procedure

To a glass hyperdermic syringe (50 ml), a seppak cartridge was attached. Diethyl ether (5ml) and n- hexane (5ml) were introduced into the hypodermic syringe. This eluted any pigments present and fat in the sample.

Finally the aflatoxins were eluted using chloroform: methanol (97:3)(20ml) into a round bottom flask (50 ml). The extract was evaporated using a rotavapour and retained for TLC and HPLC analysis.

3.5.4. Lead Acetate clean up (Jones B.D., 1972)

The filtrate from acetone: water extraction (75ml), distilled water (30 ml) and of 20% lead acetate solution (10 ml) were added into a beaker (500ml). The total volume of the content in the beaker was reduced to approximately 75ml over a steam bath; the contents were cooled and transferred into centrifuged tubes for centrifuging for 5 min. at 20000rpm in a Labofuge Heraeus model 1 centrifuge.

The supernatant was decanted into a separating funnel. The precipitate was dispersed in the centrifuge tube with acetone: water (80:20) [75ml] and stirred well and re- centrifuged for a further 5min. The supernatant liquid was decanted into aspirating funnel. Aflatoxin was extracted by shaking with three portions (25ml) of chloroform. The separated chloroform layer was run through a bed of anhydrous sodium sulfate (2mm). The chloroform extract was collected into a round bottom flask (100-ml) and evaporated to dryness using a rotavapour.

3.6 Preparation of aflatoxin B_1 standards

3.6.1 Aflatoxin standard solution

Individual crystalline aflatoxins, B_1, B_2, G_1, and G_2 purchased from Sigma Chemical Company, St louuis, MO 63178, USA, were dissolved separately in chloroform to result in a stock solution of about 8uml-of each aflatoxin. Their respective concentrations were accurately determined by UV absorbency.

Appropriate aliquots were taken to give specific concentrations of the individual or a mixture of four aflatoxins. Aliquots from the stock standard solutions were evaporated in small vials (5 or 2 ml) under steam or nitrogen and derivatized with trifluoroacetic acid in the case of HPLC analysis.

Add 5ml of benzene: acetonitrile (1:9) mixture to container of dries aflatoxin and mix well. Take 200 μg of that and make up with chloroform in a 50-ml volumetric flask. To 50 ml chloroform, add 200ul of benzene acetonitrile (1:9) mixture make it up to 50 ml, and shake keep this as blank.

3.6.2 Determination of the concentration of the standard

Measure absorbance at 360 nm and calculate the concentration of the standard using the following equation.

$$\text{Concentration} = \frac{\text{absorbance* molecular mean *CF *1000}}{E}$$

CF = Correction factor obtained for the UV spectrophotometer. (=10)

E = the absoptivity of the particular aflatoxin being examined in the particular solvent used.

The absoptivities of the aflatoxins in most widely used solvents and their molecular weight are given in the following table 3.1.

Table 3.1 Absoptivity of aflatoxins in various solvents.

Aflatoxin	Molecular mass	Wave length (nm)	Absorptivity E in mol/cm
B1	312	350	19800
B2	312	350	20900
G1	328	350	17100
G1	330	350	18200

(Source: FAO Analysis of mycotoxins, food and nutrition paper. 1991)

3.7 TLC Analysis

Chloroform (88ml), acetone (11.5 ml) and water (.5ml) mixture is used as the mobile phase. Spot the standard TLC plate in different concentration along with the sample on the same TLC plate.

Develop the TLC plate in a chamber if aflatoxins are present in sample spots compare with fluorescence intensity of quantitative standard using UV light. Select spots, which most nearly match in intensity. Calculate approximate concentration on aflatoxins present in the sample.

TLC plate was then developed in unlined, unequilibrated chloroform: acetone: water (88.5:11:0.5) solvent system. The developed plates were examined in the dark using and ultraviolet lamp (365-nm wavelength). The intensity of each standard spot was matched with sample spot.

For spice samples two-dimensional TLC was performed. Plates were first developed with chloroform: acetone (9:1) and then run in the second direction with toluene: ethyl acetate: 90%formic acid (5:4:1).

3.10 Visual estimation

The fluorescence intensities of the aflatoxin B_1 spot in the sample with which the aflatoxin B_1 standard spot was compared in order to determine which of the sample spots matches to that of the standard. If the sample spot intensity is found to lie between that of the standard spot, the actual intensity could be roughly estimated. If the spots of smallest volume of sample are too intense to match the standard, the sample was diluted and chromatography repeated. The concentration of aflatoxin B_1 in the sample, in µg/kg was calculates from the following formula.

3.10.1 Calculation

$$\text{Aflatoxin } B_1 \text{ content } (\mu g/kg) = \frac{S \times Y \times V}{W \times Z}$$

S =µl of aflatoxin B_1standard equal to that of material being evaluated on the plate (Volume of standard spotted.)

Y = Concentration of aflatoxin B_1 standard in µg/ml

W =Weight in grams of sample contain in final extract, (Final mass of the sample.)

Z =µL of sample extract spotted to give fluorescence intensity equal to S, the B_1 standard.

V = µl of solvent required to dilute final extract (200 µl).

3.11. Determination of concentration of aflatoxin B_1 standard solution

3.11.1. (a) Apparatus

- Analytical microbalance, sensitivity of .001 mg.
- Spectrophotometer, capable of measurements 200-400 nm, with 1 cm quartz- face cell.

3.11.2 Calibration of spectrophotometer

Prepare three solutions of $K_2Cr_2O_7$ in H_2SO_4 .

1. K2Cr207, 0.25 mmol/L in H2SO4, 9mmol/l (Dissolve 78 mg $K_2Cr_2O_7$ in 1.0l H_2SO_4, 9 mmol/l water.)
2. $K_2Cr_2O_7$, 0.125 mmol /l in H_2SO_4 9mmol/l (Dilute 25 ml of (1) to 50 ml with H_2SO_4, 9mmol/l water).
3. $K_2Cr_2O_7$, .0625 mmol/l in H_2SO_4, 9mmol/l (Dilute 25 ml of (2) to 50 ml with H_2SO_4, 9 mmol/l water).

Determine the absorbance (A) of solution (1), (2), (3) at maximum absorption near 350 nm, against H_2SO_4 9 mmol/l as solvent blank.

Calculate the molar absorption co- efficient (Σ) at each concentration.

$$\Sigma = A/C^* L$$

Where,

C = Concentration in mmol/l

L = Path length in meters

A = Absorption

If the three values vary by more than the guaranteed accuracy of the A scale, check either technique or instrument. Average the three Σ values to obtain Σ. Determine the correction factor (CF) for the instrument and cells by substituting in equation:

$$CF = 316/\Sigma$$

Where, 316 = The values for Σ of $K_2Cr_2O_7$ solutions. (Σ ln m^2 / mol)

If CF is, 0.95 o .1.05, check either instrument or technique to determine the cause.

Record a UV spectrum of the aflatoxin solution obtained above from 330 to 370 nm against the solvent used for solution in the reference cell. Determine the concentration of the aflatoxin standard solution by measuring the absorbance (A) at the wavelength of maximum absorption close to 350 nm and using following equation.

$$\mu g \text{ aflatoxin/ml} = \frac{A \times MW \times CF \times 1000}{E}$$

A = Absorbance

CF = Correction factor

E = Absoptivity

3.8 High Performance Liquid Chromatography (HPLC) estimation

3.8.1 Equipment

The equipment consisted of Waters 501 solvent delivery system attached to a Simadzu fluorescent detector 535 with a neon lamp, Rhedyne injector with 20ul loop, a 25cm long stainless steel u bond pack C_{18} column and Waters guardpack column assembled and connected with stainless tubing.

3.8.2 Mobile phase

High purity water was obtained by the Millipore water refilter through Millipore filtration equipment. Measured amounts of solvents were mixed in following ratios.

Table: 3.2. Mixed ratios of solvent for mobile phase of HPLC

Water	280
Acetonitrile	80
Methonol	20
Tetrahydrofuran	12

(Source: FAO Analysis of mycotoxins, food and nutrition paper.1991)

The solvent mixture was held in an ultrasonic bath for 3 min. for the complete mixing of each component.

3.9 Thin layer chromatography and visual estimation

The extracted aflatoxins from AOAC (CB), aqueous acetone or scale down Roberts extraction were dissolved in chloroform ((1-3ml) and transferred into 5 ml or 2ml vials. Chloroform was evaporated in a steam or nitrogen and the residue dissolved in a measured amount of chloroform (200ul). The vials were well stoppered to prevent evaporation.

These extract were spotted on TLC plate prepared according to the AOAC standard procedure or precoated Merck TLC plate (silica-gel G, 250µ thickness without fluorescence indicator). From the aflatoxin standard mixture 1,2,4,8 and 10 µl aliquots were spotted along with 1, 2, 3, 4, and 5µl sample extracts on the same TLC plate. Each amounted to approximately 0.4-12 ng.

Table 3.3 Molar absorption co-efficient (Σ) of aflatoxin in methanol and 95% Confidence Limits expected from single determination of molar absorption of co efficient.

Aflatoxin	λnm	Σ(m^2/mol) in MeoH	**95%** Confidence limit (+)
B_1	223	2210	160
	265	1240	80
	360	2180	110
B_2	222	1860	100
	265	1210	60
	362	2400	50
G_1	216	2740	250
	242	960	30
	265	960	120
	362	1740	70
G_2	214	2350	230
	244	1050	30
	265	900	110
	362	1350	86

(Source: FAO, Food and nutrition paper, Analysis of Mycotoxins, 1991)

3.12 Densitometric evaluation of the developed thin layer chromatographic plate

Developed plates were placed in the densitometric scanner (CAMAG model 76502) with silicagel layer on the upper side. Each unknown and standard spots on the same plate were scanned along the solvent path. Following instrument parameter were used throughout the analysis.

Lamp	mercury
Wavelength	365 nm for existing the aflatoxin
Out of filter	400 nm
Scanning speed	2 mm /sec
Sensitivity	10 units

The output signal of the densitometer was connected to a W+W MODEL 1100 strip chart recorder and therefore the chromatogram was recorded on the chart paper with each spot being recorded as a peak.

Recorded parameters were as follows;

Sensitivity input	100 mv
Chart speed	0.5 cm/min.

Each was scanned as soon as possible after varying the parth and precautions were taken to radiation of any particular aflatoxin spot. The area corresponding to these each peak was calculated (1) by measuring height .5 bases or (2) by an integrator printout of the pulse unit corresponding to peak areas.

3. 12 .1. Fluorodensitometric analysis

Usually 20cm X 20 cm thin layer chromatographic plates are used for densitometric assay. Standard aflatoxin mixture was run on the same plate with the sample (in duplicate) and plate was scanned along the pathway of each spots beginning from origin of the spot The beam UV lamp was placed on the TLC plate until maximum deflection of photo multiplier reading is obtained. Record areas of each peak corresponding to standards and samples were measured manually and the average peak areas were taken for the calculation.

3.12.2 Calculation

Area corresponding to aflatoxin B_1 standard A_1

Area of sample spot correspondents to same standard A_2

Amount of aflatoxin B_1 in the standard spot µg (ng)

Volume of sample extract spotted on to the TLC v µl

Total volume of sample extract w µl.

Amount of sample representing the final extract m g.

Aflatoxin B_1 in the sample (µg/kg) = $\frac{\mu g \times A_2 \times w}{A_1 \times V \times m}$

3.13 HPLC with fluorescence detection

In HPLC analysis, the detector was always connected with the integrator (Shimadzu CR – 3A). Once the aflatoxin standard mixture was separated by the correct solvent system it was found possible to feed actual amount of each standard din to the CR- 3A integrator.

Thereafter computation of the values representing the corresponding aflatoxin standards in the sample is automatic. After a known amount (20 ul) of sample extract (prepared according to HPLC dramatization method) was injected into the system it was possible to calculate the absolute amount of aflatoxin present in the sample. Care was taken to ensure that standard curves in the concentration ranges measured were linear.

3.14 Confirmation of aflatoxins

3.14.1 (a) Apparatus

1. TLC plate (precoated)
2. Micro caps (1ul, 2ul, 5ul, 10ul, 25ul)
3. Long wave UV light source
4. Sprayer

3.14.1 (b) Reagents

ACS grade chemicals of Acetone, chloroform, water, isopropanol-water, ether, methanol

3.15 TLC with different solvent systems

Occasionally other blue fluorescence spots appear which can easily be mistaken for aflatoxin B_1. Therefore, chemical confirmation of the identity of toxin in all samples is essential. Other compounds have been found giving similar physical properties to aflatoxins hence it is possible to be missed by spots on the TLC plates having the same R_F values as aflatoxin. In this case the TLC solvent system can be varied and if the R_F value obtained for sample and standard continues to be the same, the probability of the presence of aflatoxins increases.

Following solvent system were used for the TLC confirmation (Stoloff and Scott, 1972)

(1) Acetone; Chloroform; water 11.5:88.0: 0,50

(2) Acetone: chloroform: isopropanol-water 12:88:1.5:0.5

(3) Ether: methanol: water 96:3:1

3.15.1 TLC with spry reagents

Significant change of color of the fluorescence can be obtained with dia. Sulphuric acid or hydrochloric acid (Stoloff and Scott 1984).
When these acids are sprayed on to TLC plates, the aflatoxin spots which are blue or green under UV light turn to yellow. This indicates the presence of aflatoxins. In this study 25% sulphuric acid was used as the spray reagent.

3.15.2 Trifluoroacetic acid (TFA) derivatition (Nesheim, S. and Brumley. J.AOAC, 1991)

Aflatoxin B_1 or G_1 could be converted into hemiacetals by treatment with TFA. Hydroxilated compounds of aflatoxin B_1 and G_1 are nearly 10 times more intense than their corresponding mother compounds; hence detection of such hydroxylated compounds can be very sensitive and also show definite changes in TLC and HPLC systems. By dramatization of standard aflatoxin and the sample extract at the same time it is possible to confirm the presence or the absence of aflatoxin in any suspected sample if followed by TLC or HPLC.

3.16 R_F value

RF value defined as the ratio of the distance traveled by the compound at its point of maximum concentration to the distance traveled by the solvent. Both the distance are measured from the point of application of the sample. RF value has no unit.

3.17 Determination of chromatographic purity

On to a Thin Layer chromatographic plate (TLC), apply spots of 5µl of the standard solutions an imaginary line, at distance of ca 2 cm from the surface of the developing solvent. Develop the plate in one of the developing solvents indicated in table 11. Repeat the procedure with a second plate, developed in a different solvent system indicated in table 11.

Table 3.4. Developing solvents for TLC aflatoxins.

Chloroform –Acetone (90-10) saturated tank
Diethyl ether- methanol- Water (96:3:1) saturated tank
Diethyl ether- methanol- Water (94:4.5:1.5) saturated tank
Chloroform- methanol (94:6) saturated tank
Chloroform – ethanol (97:3) saturated tank
Benzene – methanol- acetic acid (90:5:5) unsaturated tank
Dichloromethane- trichloroethone- n- amyl alcohol formic acid (80:15:9:1) unsaturated tank (Order of RF changed to B_1,G_1, B_2, G_2).
Chloroform – trichloroethane- n- amylalcohol- formic acid (80:15:4:1) saturated tank.
Chloroform: Acetone: water (88:12:1.5) unsaturated tank.
Chloroform- acetone- isopropanol- water (88:12:1.5:1) unsaturated tank
Chloroform: isopropanol (99:1), unsaturated tank
Toluene- ethyl acetate- formic acid (6:3:1) unsaturated tank

(Source : FAO, Food and nutrition paper , analysis of mycotoxins, 1991)

CHAPTER-4

RESULTS AND DISCUSSION

4.1 Comparison of extraction and cleanup procedures.

Commodity: groundnut, estimated by the visual estimation of fluorescence.

The major aim of the first part of the study is to determine the best extraction, and cleanup techniques for the oil base product.

The groundnut sample had to be extracted separately by all three extraction and cleanup techniques.

Table: 4.1. The concentration of aflatoxin B_1 in groundnut samples. (Use same size of TLC plate, adsorbent, and same mobile phase. (µg/kg))

Sample no	AOAC minicolumn	AOAC (Roberts)	Aqueous acetone
01	12.5	56.25	37.5
02	37.5	75.0	52.5
03	12.5	56.25	37.5
04	37.5	93.75	37.5
05	37.5	75.0	62.5
Mean	27.5	71.25	45.5
Standard deviation	12.24	14.03	10.29

The best extraction method for oil based product is modified AOAC (CB) method (According to mean values of detection). Groundnut and desiccated coconut have more fat content than other commodities. Therefore in AOAC (CB) mini column method used more volume of fat eluting solvents (n-hexane, diethylether). These solvents are less polar, and elute less polar compounds.

Aflatoxin B_1 will be detoxified by UV and visible light fluorescence in experimental environment. In AOAC (CB) and aqueous acetone methods get larger time to exposing visible light and it may also effect the less detection those methods. The modified AOAC method is used less time, less expensive than other methods. Therefore, it is more efficient, effectiveness, and economical than other two methods.

4.2 Study on aflatoxinin B_1 in spices

Export prepared samples of cinnamon bark, clove, red chillies, nutmeg, coriander, cardamom pods and wine spices were analyzed for aflatoxin B_1. The AOAC (CB) scale down procedure adopted by Roberts was carried out using seppak cartridge cleanup. Aflatoxin B_1 was estimated by thin layer chromatographic separation.

Firstly recovery experiments were carried out using low levels of spiking with aflatoxins. Results (table 4.3) showed that recoveries of aflatoxin B_1 . 100% were observed indicating cross contamination with non-aflatoxin fluorescence compound.

Table 4.2 Recovery of spiked aflatoxin B_1 from spices.

Spices	% Recovery of aflatoxin B_1
Cinnamon bark	92
Clove	112
Red chilies	138
Nut-meg	120
Coriander	88
Cardamom pods	98
Wine spices	143

Recovery of aflatoxin B_1 from spiked samples of spices, were spiked with the level of 200µl 1.875 µg/ml Aflatoxin B_1 standard Aflatoxin were extracted by the Modified AOAC CB procedure with seppak cleanup.

Aflatoxin contamination was measured in selected spices. Results show that contamination indicated was very low. (Table 4.3.)

Table 4.3. Aflatoxin B_1 contamination in spices using TLC visual estimation.

Spices	Aflatoxin B_1 (μg/kg)
Cinnamon bark	Not detected
Clove	Not detected
Red chilies	18.75
Nut-meg	Not detected
Coriander	Not detected
Cardamom pods	Not detected
Wine spices	Not detected

4.3. Recoveries, co-efficient of variation of techniques

The retention time and other data for aflatoxin standards for HPLC are shown in table 4.4. Figure 4.1 shows the HPLC profile of the aflatoxin B_1 standard.

Table 4.4. The retention time and other data for aflatoxin B_1

Detector B
(Ex:364nm,
Em:455nm)

Pk #	Retention Time	Area	Area Percent	Name
1	1.850	45349	3.36	
2	2.558	17612	1.30	
3	2.708	9063	0.67	
4	2.917	5033	0.37	
5	3.050	7849	0.58	
6	4.583	281955	20.89	
7	5.575	244613	18.12	
8	6.000	146401	10.85	
9	6.992	536921	39.78	
10	7.425	31693	2.35	
11	7.867	23373	1.73	
Totals		1349862	100.00	

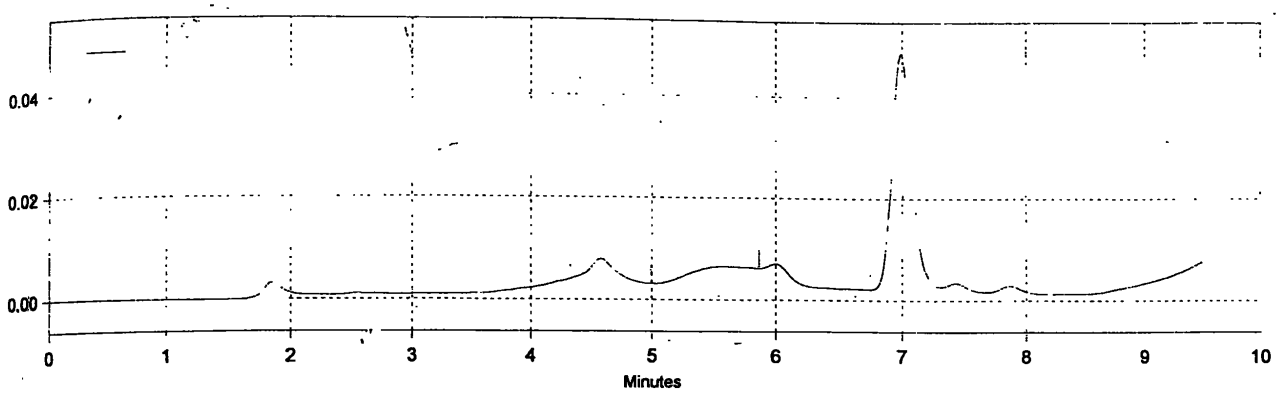


Figure 4.1 HPLC profile of the aflatoxin B_1 standard

4.4. General discussion

Statistical sampling is an essential pre-requisite for analytical study. In the case of aflatoxin this poses special problems as the contamination is due to the growth of a mould and is therefore in variably heterogeneous. This is further complicated in the case of commodities like maize and groundnut (Pettit: *et . al.*, 1971) where the fungal contamination can begin at the stage of flowering. This can lead to isolated contamination for example, in the case of groundnut a single contaminated kernel can have an aflatoxin levels high as 35,800 of ppb (Pettil R: et.al.1971) and this would be found among uncontaminated kernels. Another problem in sampling posed by commodities where the particle (Frequently grain or kernels) size is large (e. g., groundnut). This results in extreamly large bulk samples needing to be taken in order to produce meaningful results.

In the case of materials containing high fat content, however acetone: water method seems to be less satisfactory. The reason being the necessity of a prior de- fatting step, according to TP1 G70 report (Jones, B.D 1972) which presumably remove aflatoxins in the sample.
High fat is also a problem when dealing with the AOAC (CB) and modified AOAC method as this factor causes the need to use large amounts of solvents.

The chief advantages of the AOAC (CB) method is that it not only gives efficient extraction but also uses a large sample size However, large sample size is has drawbacks as it causes the use of larger volumes of solvent and additional analysis time. The modified AOAC methods therefore sacrifice the advantage of working with a large sample while aiming at a cheaper faster assay.

Regarding cleanup techniques they all appear to work satisfactorily. The lead acetate method was linked to the acetone: water extract and other two are interchangeable with extraction procedure. The seppak cleanup proved not only more efficient but also less time consuming and less costly. Result showed that the AOAC and Roberts methods to yield results of very similar nature.

However, the ultimate choice of a method will also depend on the commodity (aflatoxin containing substrate and it is likely that further commodity specific refinements to techniques can be made). A quantitative evaluation of extraction and cleanup techniques is given in Table 4.5.

Table 4.5. - Comparison of Extraction/ Cleanup techniques

Attribute	Pons-Lead Acetate	AOAC (CB) mini column	Modified AOAC (CB) Roberts Seppak
1) Reproducibility	Variable	Good	Good
2) Recovery	Variable	Good	Good
3) Assay time	45 min.	2h.	5 min.
4) Sample throughput (per week)	20	15	50
5) Running cost (Consumable)	Rs.75/-	Rs.110/-	Rs.200/-
6) Expertise	Middle level	Middle level	Middle level

The TLC visual method is has a very high co-efficient of variation and therefore accuracy will also be in doubt. Further, the TLC method cannot be used below 5 ppb due to the low sensitivity of the technique. Further, uses of TLC plates for analysis of aflatoxins are faced with the problem of fading of spots due to degradation by laboratory environmental factors (acid fumes, etc.) and by light (visible and UV). These probably are some of the factors that cause a high co- efficient of variation in the results obtained by TLC based techniques, the other being the degree of subjectivity imposed by the estimation being carried out visually.

Still there are factors in the HPLC techniques, which can detract from its use: (1) Solvent of high purity is needed, and (2) a good cleanup techniques must be utilized. These add to both cost and expertise.

Furthermore HPLC equipment (with a fluorescent detector) cost approximately (Rs./-500,000) and such capital cost will be a deterrent to its common use. Furthermore, it must be remembered that an instrumental techniques is only as good as the analyst and for good results with the backup maintenance, repair and operational facility and guidance. For this reasons this ultimate tool may not be practical preposition in many instances especially in remote areas of the third world.

A small amount of (5-20 ppb) of aflatoxin was expelled in the oil and it was found that this could be detoxified by sunlight (Samarajeewa, U; et. al. 1985) but only after removing a quenture (brown pigment) by purifying the oil. Aflatoxin content could also be lowered by ultra – filtration (Basappa, S.C.and Murthy, S. Ind. Tech. 1977) but this was not tested.
Export commodities, spices, tea, cocoa, were the next to come under examination. The rational for this study was that global trade is becoming more demanding on aflatoxin contamination and therefore there is a need to identity methods of detecting aflatoxins unambiguously at level of ~5 ppb.

CHAPTER - 5

CONCLUTION AND RECOMMENDATION

5.1 CONCLUTION

The efficient method of extraction and clean up of an oil based product are the Modified AOAC (CB) method and seppak silica cartridge clean-up methods. But oily compound of commodities may interfere the extraction and clean-up procedure of modified AOAC (CB) method. Further, de-fatting step may introduce some false results due to eluting of aflartoxins with solvent used for eluting fat compounds.

The R_F values of aflatoxin B_1 vary. The factors affecting for the variation of R_F of aflatoxin B_1 are, type of mobile phase, concentration of mobile phase, type of absorbance used for TLC plate, the size and thickness of the TLC plate, state of saturation of chamber of TLC. The overall mean of R_f value of aflatoxin B_1 is 6.69 (See appendix-2).

There are no significant difference between standard R_F values and co-spotting (spotting of both aflatoxin B_1 standard and final sample extraction) R_F values at 5% level. The retention time of aflatoxin B_1 is ranged from 7.49 – 6.49 min.
Visual estimation Method is not much accurate for quantitative analysis. It makes approximate analysis.

The some recovery practical shows the over 100% recoveries. Therefore those commodities must contaminate with non-aflatoxin B_1 compound or inherent compounds. That false fluorescence will mislead with aflatoxin B_1 fluorescence. It must be confirmed with suitable confirmatory chemical test.

5.2 RECOMENDATION

Comparison of detection techniques In these studies three detection techniques namely TLC (visual fluorescence), TLC densitometric (fluorescence), HPLC (fluorescence) can be used. The extraction and clean-up techniques should be the same in each case.

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APPENDIX-1

Final mass of the sample,

For modified AOAC (CB) Seppak cartridge methods $= \frac{10g \times 5g}{50\ ml}$

$= 1g$

For AOAC (CB) mini column methods $= \frac{50\ g \times 50\ ml}{250\ ml}$

$= 10g$

For aqueous acetone and lead acetate clean up $= \frac{20g \times 75\ ml}{100\ ml}$

$=15g$

Final made up volume of sample to be spotted on TLC $= 200\mu l$

Spectrophotometer reading for absorbance $= 0.119$ (A)

Molecular mass of aflatoxin B_1

$= 312$

Correction factor

$= 1.0$

Absobtivity of aflatoxin B_1 $=19,800$

Concentration of aflatoxin B_1 $= \frac{0.119 \times 312 \times 1.0 \times 1000}{19,800}$

$= 1.875\ \mu g/ml$

Volume of sample spotted

$= 25\ \mu l\ (v_1)$

Voume of spotted aflatoxin B_1 standard $= 10\ \mu l\ (v_2)$

Concentration of aflatoxin B1in sample $= \frac{C_1 \times V_2 \times 200\mu L}{V_1 \times 1g}$

$= 150\ \mu g/g$ (150ppb)

Mean retention time of aflatoxin B_1 $=7.79$ min.

Standard deviation of retention time of AF B_1 $= 0.058$

Co-efficient of variation of retention time of AF B_1 $= 0.008$

APPENDIX-2

SAS 4:31 Thursday, May 6, 1993 1

TTEST PROCEDURE

Variable: DISTANCE

GROUP	N	Mean	Std Dev	Std Error	Minimum	Maximum
C	17	0.64611765	0.09748839	0.02364441	0.55200000	0.85700000
S	18	0.69283333	0.12003394	0.02829227	0.56200000	0.94600000

Variances	T	DF	Prob>|T|
Unequal	-1.2670	32.3	0.2142
Equal	-1.2594	33.0	0.2167

For H0: Variances are equal, F' = 1.52 DF = (17,16) Prob>F' = 0.4109

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