TRAINING MANUAL ON

"ASPERGILLUS FLAVUS SEED INFECTION AND AFLATOXIN ESTIMATION BY ELISA" AND AFLATOXIN MANAGEMENT OPTIONS IN GROUNDNUT

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1. PREFACE

Many agricultural commodities are vulnerable to attack by a group of fungi that are able to produce toxic metabolites called ***mycotoxins****. Among various mycotoxins,* ***aflatoxins*** *have assumed significance due to their deleterious effects on human and livestock health as well international trade. They are potent carcinogens and immunosuppressive agents. The simplest and least costly methods for detecting contamination with aflatoxins in groundnuts and groundnut-based foods and feeds are immunological. The main aim of this course is to acquaint the participants with these immunological methods. Although the technology may appear complicated to a novice, every effort has been made to simplify the procedures with the hope that developing countries can utilise the techniques.*

2. MYCOTOXINS - AN OVERVIEW

Mycotoxins are a group of chemically diverse secondary metabolites of fungi. They exhibit a wide array of biological effects and individual mycotoxins can be mutagenic, carcinogenic, embryo-toxic, teratogenic, oestrogenic or immunosuppressive. The aflatoxin problem came to light in the 1960's when there was a severe outbreak of a disease, referred to as "Turkey 'X' Disease" in England, in which over 100,000 turkeys and other farm animals were killed. The cause of this disease was traced to a component in peanut meal contaminated with *Aspergillus flavus*. This very singular event triggered intensive research on mycotoxins. These studies include the occurrence, chemical structure, biosynthesis, factors affecting the biosynthesis and health hazards of aflatoxins. Table 1 lists commonly occurring mycotoxins and the fungi that produce them. The UN Food and Agriculture Organization has estimated that up to 25% of the world's foods are contaminated significantly with mycotoxins.

3. AFLATOXINS

3.1. Introduction

The cultivated groundnut (*Arachis hypogaea* L.) is the most important oilseed crop in the developing countries. Groundnut seed is a valuable source of protein for human and animal nutrition. India and China alone contribute to nearly 2/3rd of the world production.

In addition to groundnut, a number of commodities are currently known to be contaminated with aflatoxins (Table 1). These include other oilseeds, cereals, legumes and spice crops. A wide range of livestock have been found to be affected by aflatoxins to a greater or lesser extent. Among cereal crops, by far the most important contamination occurs in maize. Groundnuts and maize are ingredients in the diet of many poor people and also common ingredients in livestock feeds. Health hazards from ingestion of aflatoxin contaminated food are much greater in the developing than in the developed world. Most developing countries lie in tropics, where temperatures and relative humidities often favour mold growth, and where no or only limited facilities exist for monitoring groundnut and groundnut products for aflatoxin contamination. The possible presence of aflatoxins in foods and in feeds has had a profound effect on the utilization and trade in groundnuts and its products. Developed countries which import groundnut have set aflatoxin contamination limits for food stuff ranging from zero to 10 µg kg^{-1} and this has resulted in import restrictions on aflatoxin-contaminated produce. As a result, many developing countries have been unable to export their groundnuts and groundnut products. For exporting countries to satisfy the regulations, they have to produce groundnuts with no or extremely low aflatoxin contents. This can only be achieved by following suitable management practices and by storing produce under conditions that minimize the growth of aflatoxin-producing fungi. The research, development and monitoring needed to ensure this are dependent upon having simple, specific and cost-effective methods for the detection and estimation of aflatoxins in various agricultural commodities.

Many different methods are available for the estimation of aflatoxins. They include physic-chemical and immuno-analytical methods. Chemical-chemical methods are time consuming, require expensive instrumentation, extensive clean-up of the samples using solvents; they therefore have serious limitations in cost and labour when large numbers of samples have to be processed. By contrast, immunological methods are simple, rapid, sensitive and specific. In this manual we will be concentrating on ELISA-based procedures for the estimation of aflatoxins. They will be compared with the analytical method, thin layer chromatography, to demonstrate how the aflatoxin levels determined by the two entirely different methods compare.

The organization of the contents is similar to that adopted by Mehan *et al*. (1991) for the Groundnut Aflatoxin Problem Review and Literature Database as this should facilitate reference to relevant additional data.

3.2. Risk to health of human and other animals due to ingestion of toxic groundnuts and groundnut products.

Aflatoxins are produced by strains of two fungi, *Aspergillus flavus* and *A. parasiticus*. Therefore they are referred to as "mycotoxins". This term is derived from the "mikes" (Greek word for fungus) and "torsion" (poison). Aflatoxins comprise a group of more than fifteen toxins and for developing countries they are the most important mycotoxins from the point of view of occurrence, toxicity and influence on international trade. Aflatoxins B_1, B_2, G_1 and G_2 have often been detected in groundnut, maize and other agricultural commodities. Their structures are shown in figure 1. Aflatoxins M_1 (4-hydroxy derivative of aflatoxin B_1) is of special importance because it is detected in milk from animals fed with aflatoxin B_1 contaminated feed.

The acute and chronic effects of aflatoxin ingestion have been well studied (Healthcare, 1978; Spoof, 1977). Aflatoxin B_1 is the most toxic followed by aflatoxins G_1, B_2, and G_2 in order of decreasing potency.

Table 1. Natural occurrence of selected mycotoxins

Mycotoxins[a]	Major producing fungi	Typical substrate in nature	Biological effect
Alternaria (AM) mycotoxins	*Alternaria alternata*	Cereal grains, tomato, animal feeds	M, Hm
Aflatoxin (AF) B_1 and other aflatoxins	*Aspergillus flavus, Aspergillus parasiticus*	Peanuts, corn, cottonseed, cereals, figs, most tree nuts, milk, sorghum, walnuts	H,C,M,T
Citrinin (CT)	*Penicillium citrinum*	Barley, corn, rice, walnuts	Nh,C(?),M
Cyclopiazonic acid (CPA)	*Aspergillus flavus, P. cyclopium*	Peanuts, corn, cheese	Nr,Cv
Deoxynivalenon (DON)	*Fusarium graminearum*	Wheat, corn	Nr
Cyclochlorotine (CC)	*P. islandicum*	Rice	H,C
Fumonisins (FM)	*F. moniliforme*	Corn, sorghum	H,Nr,C(?),R
Luteoskyrin (LT)	*P. islandicum, P. rugulosum*	Rice, sorghum	H,C,M
Moniliformin (MN)	*F. moniliforme*	Corn	Nr,Cv
Ochratoxin A (OTA)	*Aspergillus ochraceus, P. verrucosum*		Barley, beans, cereals, coffee, Nh,T
		feeds, maize, oats, rice, rye, wheat	
Patulin (PT)	*P. patulum, P. urticae, Aspergillus clavatus*	Apple, apple juice, beans, wheat	Nr,C(?),M
Penicillic acid (PA)	*P. puberulum, Aspergillus ochraceus*		Barley, corn Nr,C(?),M
Penitrem A (PNT)	*P. palitans*	Feedstuffs, corn	Nr
Roquefortine (RQF)	*p. roqueforti*	Cheese	Nr
Rubratoxin B (RB)	*P. rubrum, P. purpurogenum*	Corn, soybeans	H,T
Sterigmatocystin (ST)	*Aspergillus versicolor, Aspergillus nidulans*	Corn, grains, cheese	H,C,M
T-2 Toxin	*F. sporotrichioides*	Corn, feeds, hay	D,ATA,T
12-13, Epoxytrichothecenes (TCTC) other than T-2 and DON	*F. nivale*	Corn, feeds, hay, peanuts, rice	D,Nr

Zearalenone (ZE)	*F. graminearum*	Cereals, corn, feeds, rice	G,M

ATA, Alimentary toxin aleukia; C, carcinogenic; C(?), possible carcinogenic effect; Cv, cardiovascular lesions; D, dermatoxin; G, genitotoxin and estrogenic effects; H, hepatotoxic; Hr, hemorrhagic; M, mutagenic; Nh, nephrotoxin; Nr, neurotoxins; R, respiratory; T, teratogenic.

[a]The optimal temperatures for the production of mycotoxins are generally between 24 and 28°C, except for T-2 toxin, which is produced at 15°C.

Source F.S. Chu, 1995

Human beings: The susceptibility of animals to aflatoxins varies from species to species. Primary liver cancer is one of the most prevalent human cancers in the developing countries. Epidemiological studies support the association between the incidence of hepatocellular carcinoma and consumption of foods contaminated with aflatoxin. It is currently known that there are synergistic effects between aflatoxin and hepatitis B virus (which causes jaundice) infection causing primary liver cancer.

Livestock: Outbreak of aflatoxicosis was first reported in turkeys in the UK in 1960. Affected birds lost appetite, became lethargic and died within 7 days from the onset of symptoms. A similar disease was also reported in chickens. More recently in 1994 around Hyderabad city over 200,000 chickens died due to inclusion of aflatoxin-contaminated maize and groundnut meal in their feed. Several reports have appeared of outbreaks of aflatoxicosis in cattle. Initial symptoms appear as lesions, ultimately leading to diffuse cirrhoses of liver. Sheep do not appear to be susceptible to aflatoxins. Dogs and pigs are susceptible. Mice are resistant, to a certain extent, to aflatoxins.

It is important to realise that acute toxicity due to aflatoxins for any given species of animal is influenced by such factors as age, size, breed, condition of animal and composition of diet. Young animals tend to be more sensitive than mature animals.

The tolerance level for aflatoxin B_1 is 5 $\mu g\ kg^{-1}$ of material. It is unlikely that contaminated commodities will contain only aflatoxin B_1 and often other aflatoxins may also be present, but the sum of aflatoxins B_2, G_1 and G_2 present is generally less than the concentration of aflatoxin B_1 alone.

3.3. *Aspergillus flavus* infection and aflatoxin contamination of groundnuts

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. Therefore the pod is associated with soil microflora over an extended period of time and this facilitates its invasion by various soil-inhabiting organisms. Research institutions in a number of groundnut producing countries placed a high priority on determining the stage or stages in crop production at which groundnuts are most likely to be invaded by *A. flavus* and become contaminated with aflatoxins. Now it is established that *A. flavus* infection could occur at preharvest, during harvest and postharvest stages.

Pre-harvest: Many investigations have demonstrated that shells of immature and mature pods were commonly infected by *A. flavus* but that very little seed infection occurred at this stage. Late harvested (over-mature pods) were more likely to have contaminated seeds. The stage at which the crop is harvested can therefore influence aflatoxin contamination.

Drought stress (mid or late in the season) increased levels of *A. flavus* infection resulting in high amounts of aflatoxins. This was attributed to lowering of seed moisture content, over-maturity and decreased plant vigour.

Pods and shells that were damaged were more likely to contain toxic seeds than were pods with undamaged shells. A number of soil inhabiting pests including pod

borers, millipedes, mites, white grubs, termites and nematodes have been implicated in *A. flavus* infection of groundnuts before harvest. Mechanical damage to pods during field operations and at the time of harvest can lead to invasion of seeds by *A. flavus*.

During postharvest drying: When groundnut pods infested with *A. flavus* are harvested and kept for drying, they are subjected to rapidly changing environmental conditions which cause shifts in the dominant and subdominant fungal species on and within the pods. When high moisture groundnuts are lifted and dried in windrows there may be considerable invasion of seeds by *A. flavus* and other fungi already established in the shell. This is further enhanced if drying is slow and seeds have a moisture content ranging from 12% to 30% for extended periods.

During storage: The main factors which can influence the growth of *A. flavus* are moisture (relative humidity), temperature, time and gaseous composition of the atmosphere.

A. flavus infection and aflatoxin concentration are likely to increase in groundnuts during storage until the moisture content drops below 9%. Natural accumulation of CO_2 and decreased levels of oxygen in closed storage can reduce development of the fungus. Aeration is necessary to reduce aflatoxin contamination during storage. High relative humidity and temperatures, rain water leakage, condensation and insect infestation are all important factors that contribute to *A. flavus* growth and aflatoxin contamination of groundnuts in storage.

***A. flavus* isolates and aflatoxin production**: Various isolates of the fungus collected from different geographical regions were evaluated for aflatoxin production on natural and artificial media. The majority of the isolates produced aflatoxins, usually aflatoxins B_1 and B_2. *A. parasiticus* can produce all the four aflatoxins, B_1, B_2, G_1 and G_2. Some of the *A. flavus* strains do not produce aflatoxins.

3.4. Occurrence of aflatoxins in groundnuts and groundnut products

Important problems encountered when trying to obtain an accurate record of extent and degree of contamination of groundnut and groundnut products with aflatoxins are the variation in efficiency of sampling and the sensitivity and applicability of the analytical procedures. Since this training course is for participants from Asia, only the information available from Asian countries is given.

India: Many commercial groundnut cake and seed samples tested contained aflatoxin, levels even reaching as high as 8000 $\mu g\ kg^{-1}$. Aflatoxins were also detected in unrefined groundnut oil. Samples of refined groundnut oils or hydrogenated oils did not contain any aflatoxins.

Indonesia: Around 70% of the marketable groundnuts tested were found to be contaminated with aflatoxin at levels from 40 to 4100 $\mu g\ kg^{-1}$. All groundnuts and groundnut products sampled from markets, stores, and food manufacturers contained aflatoxins.

The People's Republic of China: The samples from southern China contained the maximum amount of aflatoxins (42% of samples of seeds and 68% of oil samples being toxic). Aflatoxin contamination was negligible in groundnut samples from northern China. Warm and humid weather in southern China may be contributing to the high levels of contamination of groundnuts in that area.

Nepal: Only limited surveys have been conducted to determine aflatoxin levels in groundnuts. Many samples were found to be contaminated. Peanut butter samples and maize and groundnut cake used as feed ingredients, were also mostly contaminated.

Pakistan: Many samples of roasted groundnuts from local markets were found to be contaminated. Aflatoxin levels of up to 300 $\mu g\ kg^{-1}$ were reported.

Philippines: Over 90% of the produce is used as human food, in the form of snack items and peanut butter. All processed groundnut samples contained levels of aflatoxin ranging from a trace to more than 1000 $\mu g\ kg^{-1}$. Although aflatoxin levels at the time of harvest were low, the levels increased at least ten-fold in storage during the main cropping season.

Thailand: Groundnuts are utilized in various types of confectionery products and for cooking oil. Oil cake is used for animal feed. Nearly 50% of the samples collected from towns and villages showed aflatoxin contamination, levels reaching as high as 1530 $\mu g\ kg^{-1}$. Contamination was most frequent in samples collected in the rainy season, when the average concentration of total aflatoxin in all groundnut samples was nearly twice that observed in samples harvested during the dry season.

CONCLUSIONS

It is apparent that aflatoxin contamination is influenced by weather conditions such as rainfall and temperature. Surveillance and monitoring of farm as well as confectionery and feed products are yet to be conducted in a systematic way from many countries in Asia. Nevertheless, it is fortunate that several important groundnut producing countries have recognized the problem and are supporting research and monitoring/control activities aimed at mitigating the problem.

3.5. Limits and regulations

The hazards to human and livestock from ingestion of aflatoxin-contaminated groundnuts and groundnut products are well known. As a result many countries have established permissible limits for aflatoxins on products involving groundnut, and have established measures to control the contamination of food products and livestock feeds.

Factors which played a role in establishing limits and regulation are listed below:

1) Survey data:

Data on aflatoxin levels in various products indicate commodities to be targeted for legal action. These data are expected to allow an estimate of the effects of enforcement on the availability of food, including animal products and of feed. In

developing countries where food supply is limited, drastic measures to regulate permissible aflatoxin levels may lead to lack of food and to higher prices.

2) Toxicological data:
This information is vital to determine permissible aflatoxin levels for each commodity. In the case of aflatoxin this is relatively difficult to assess because of carcinogenic effects. The current assumption is that it is difficult for aflatoxins to determine the threshold levels below which effects cannot occur, and that, therefore, any small amounts can lead to a proportionally small probability of inducing some ill effects. Therefore zero tolerance would be most appropriate. Nonetheless aflatoxins are natural contaminants and therefore cannot be eliminated unless all vulnerable foods or feeds are outlawed. This makes regulatory judgements particularly difficult.

3) Methods of analysis:
These are extremely important for determining the aflatoxin levels in various commodities. Simple and cost-effective methods are absolutely essential especially for developing countries. In the absence of reliable methods of analysis, it would be difficult to establish relevant tolerance limits. It is worth mentioning that tolerance limits cannot be lower than the actual limits of detection of the method employed for analysis.

4) Aflatoxin distribution:
Distribution of aflatoxin contamination in groundnut is particularly important as it has been shown to be non-homogenous. Therefore, it is vital to establish reliable sampling procedures. Risks to both consumer and producer should be considered prior to establishing sampling methods and analysis criteria for groundnuts and groundnut products.

5) Legislation:
Regulations especially imposed by developed countries are a handicap for developing countries wishing to export their groundnuts. Aflatoxins are natural contaminants of groundnut and therefore exposure of humans to some levels of aflatoxin 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. When local foods are also export items, selective exportation of the most wholesome food may lead to concentration for local consumption of the more contaminated food, thereby increasing the risk of toxic effects in the indigenous population (see Table 2).

Aflatoxin limits in animal feeds. A few countries e.g., Cuba, Dominica Republic, Malaysia and Portugal have a zero tolerance limit. Many European countries have set a reasonable limit of 3 µg kg^{-1} of aflatoxins (Table2). It is not clear whether tolerance for total aflatoxins contributes to better protection of public health than a tolerance for aflatoxin B_1 alone. Aflatoxin B_1 is the most common toxic and carcinogenic aflatoxin.

Currently some 35 countries regulate aflatoxins in animal feedstuffs. The European Economic Community (EEC) (new European Union or EU) has given directives on the maximum permissible levels of aflatoxin in groundnuts and groundnut cake imported into EU. The first directive 74/63 became effective on 11 February 1974 (EEC's official

Journal No. L-38, page 31). The maximum permissible levels ranged from 20 µg kg^{-1} (in whole feeding stuffs for pigs and poultry except piglets and chicks) to 50 µg kg^{-1} (in straight feeding stuffs and whole feeding stuffs for cattle, sheep and goats except dairy cattle, calves, lambs). The EEC limits on aflatoxins in animal feeds were set after the monitoring program of milk in the U.K. showed that many milk samples contained aflatoxin M_1. Groundnuts and cottonseed (raw materials used in dairy feeds) were regarded as the sources of aflatoxin contamination. Consequently, in 1982, the U.K. Government introduced a ban on imports of groundnut and cottonseed. However this was soon changed to a ban on imports of groundnuts/meal when contaminated with aflatoxin exceeding 50 µg kg^{-1}.

The 1974 directive was tightened in 1984 when the tolerance for aflatoxin B in complementary foodstuffs for dairy cattle was reduced from 20 to 10 µg kg^{-1}. This was in accordance with the trend in Western European countries to establish tolerances for aflatoxin M_1 in milk at a level of 0.05 µg L^{-1}. Current permissible levels of total aflatoxin and aflatoxin B1 are given in the table 3. It is possible that EU regulations for aflatoxins in foods and animal feedstuffs will be more stringent in the near future.

Concluding Remarks

Differences in maximum permissible levels of aflatoxin(s) in groundnuts and groundnut products vary widely among countries that have regulations on aflatoxins. It is imperative to follow reasonably uniform regulations and limits on aflatoxins in groundnuts and groundnut products in order to prevent any unnecessary damage to the exporting countries/agencies and to freedom of international trade in oilseeds. Efforts towards establishing uniformity of aflatoxin regulations should be supported by knowledge about the rationale for the decisions that resulted in the enforcement of current regulations in various countries. International organizations including FAO, WHO, UPAC, and EU are currently attempting to develop uniform aflatoxin regulations for various foods and feeds. The permissible levels of aflatoxin(s) in foods and feeds must be based on the current knowledge on the toxicity of the toxins to humans and animals and on the methods available for their detection.

Table 2. New International Regulation on Aflatoxin Contamination in Groundnut (July 1988)

Country	Aflatoxin total (ppb)
Australia	16
Canada	15
Denmark	10
France	1

Italy	50
USA	20
Japan	10

Decontamination of products by chemical treatments as well as any blending of contaminated products with good quality products in order to reach admitted level for human consumption is forbidden. (Source: Mycotoxicology News Letter, Aug 1998, Vol. IV, No. 2.)

Table 3. Proposed maximum permitted levels of aflatoxins in groundnuts and spices

	Aflatoxins: maximum admissible levels (μg/kg)	
Products	B_1	Total
Groundnut and groundnut-based products		3 5
Spices	5(1)	10(1)
- *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika)		
- *Piper* spp. (fruits thereof, including white and black pepper)		
- *Myristica fragrans* (nutmeg)		
- *Zingiber officinale* (ginger)		
- *Curcuma longa* (turmeric)		

(1) The maximum limits shall be reconsidered and, if necessary, reduced before 1 January 2003 according to the progress of scientific and technological knowledge.

3.6. AFLATOXIN ANALYSIS IN GROUNDNUTS AND GROUNDNUT PRODUCTS

3.6.1. Sampling

Sampling is an important step in testing groundnuts for aflatoxin contamination. If aflatoxins were homogeneously distributed throughout the samples intended for analysis, then sampling should not have posed any problem. Unfortunately the highly skewed nature of the distribution of aflatoxin complicates the sampling procedure. Toxicity often resides in only a few contaminated kernels. Additionally there is extreme variation in the level of aflatoxin among contaminated kernels.

The total error made in a test procedure consists of three parts, namely the sampling error, the sub sampling error (after grinding the original sample, a relatively small portion taken for analysis) and the analytical error. In figure 2 possible errors arising from the three components to the total error are represented. The major error component is the sampling error, whereas the sub sampling and analysis errors (intra-laboratory) vary only slightly across all concentrations.

It is possible to draw a curve indicating the relationship of the probability of acceptance of a lot versus the aflatoxin concentration in the lot, given a certain tolerance. An example is given in figure 3. It is apparent from the figure that the probability of accepting a large lot approaches 1, when the concentration of aflatoxin approaches zero; as the concentration increases, naturally the probability of accepting the lot approaches zero. It is also apparent that there are two risks, the producers' risk and the consumers risk. The consumers risk is an assessment of the likelihood of accepting a batch of groundnuts whose aflatoxin content exceeds the maximum level

imposed for the toxin and the producers risk is an assessment of likelihood of rejection of a batch that contains less toxin than the statutory maximum level. Increasing the sample size will reduce both the consumers risk and the producers risk (Fig. 4).

The ideal operating characteristic (oc) curve is obtained when the entire sample is ground and analysed. Obviously this is not possible because nothing would be left to sell or to buy the produce in its original form. The choice of the sample size depends on the risks that can be accepted and the costs one is willing to bear. Another possibility of influencing the producers' risk and the consumers risk is changing the level of decision (if test sample contains toxin levels that exceeds the limits imposed, then the lot will be rejected).

Lowering the levels of decision reduces the consumers risk. However it leads to increase the producers risk. In order to limit these risks sampling procedures have been developed in which two (or more) decision levels are used, an acceptance level and a rejection level. In such cases a lot is accepted if the outcome of the analysis of the test portion is lower than the acceptance level, rejected if it is higher than the rejection level, and reanalysed when the outcome is in between the two levels (Fig. 5).

An example of such a sampling plan is that adopted by the Peanut Administration Committee (PAC) in the USA for sampling large lots of peanuts, before they are shipped to the manufacturer. This sampling procedure involves multiple sampling and assay from representative units of 22 kg of the lot and a tolerance for the sum of the aflatoxins B_1, B_2, G_1, and G_2 of 25 µg kg^{-1}.

Although it would be a costly exercise, very large samples of many kilograms of groundnuts must be taken to obtain a low risk of wrong decision for both the consumer and the producer. These large samples are required to be divided into sub samples to permit adequate analysis. Devices such as the Dickens-Salterwhite sub sampling mill are available, for preparing the sample (see section on "Sample Preparation"). This is followed by the analysis of the whole sub sample and the size of the sub sample is further reduced until a test portion, in size generally ranging from 20-100 gm, is obtained. The compromise between solvent economy and a representative sample appears to be 50 gm.

3.6.2. Sample preparation

Proper grinding and subdivision of the sample is obviously essential before performing analysis for aflatoxins. Ideally, a sub sampling mill should simultaneously grind and subdivide the sample, and that developed by Dickens and Salterwhite is suitable for this purpose. Alternatively, the sample may be ground and subdivided in separate operations. Rotary sample dividers such as "spinning riffles" and the "cascade sampler" are capable of producing several representative sub samples which can be useful in aflatoxin analysis. The size of the sub sample may vary, but for most cases it ranges from 20 to 100 gm. A sub sample size of 50 gm is used in most methods and appears to be the best for obtaining both solvent economy and representative sample.

3.6.3. METHODS FOR ANALYSIS OF AFLATOXINS

Three types of assays have been developed for the detection of aflatoxins. These include biological, chemical and immunochemical. Biological assays were used when chemical and immunochemical methods were not available for routine analysis. Biological assays are qualitative or at best semi quantitative, and often are non-specific. They are time consuming.

Chemical and immunological assays are suitable for routine analysis. They are invariably quick, and are cheaper and more specific than biological assays. Additionally these tests are reproducible and sensitive. Chemical tests available for aflatoxin analysis are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), high performance liquid chromatography (HPLC) and fluorometric methods. HPLC is more sensitive than TLC and provides results with a high degree of accuracy. In order to achieve rapid results minicolumns were developed. Immunological methods were developed in late 1970's and since then many improvements have been incorporated. Immunochemical methods are more desirable than chemical methods for rapid and relatively inexpensive assay of aflatoxins.

4. IMMUNOLOGICAL METHODS FOR AFLATOXINS

The two major requirements for the application of immunochemical methods are high quality antibodies and methodologies to use the antibodies for the estimation of aflatoxins.

4.1. Principles of antibody production

An antigen is a molecule which can elicit production of antibodies when introduced into warm blooded animals. Proteins, peptides, carbohydrates, nucleic acids, lipids, and many other naturally occurring or synthetic compounds can act as antigens, especially those having a molecular weight of 10,000 daltons or higher with a definite molecular structure and which are not normal constituents of the animal being immunized. Antibodies are glycoproteins which are produced as a result of immune response following introduction of antigens. Blood serum containing antibodies is referred to as antiserum.

When antigens are introduced, into an animal, a series of interactions between macrophages, T lymphocytes, and B lymphocytes lead to antibody production. The first exposure of animals to antigens leads to a relatively weak reaction, referred to as the primary response. A series of specialized events occur during the primary response. These events prepare the animal to respond with quick and intense production of antibodies (secondary response) when the antigen is reintroduced. Both the primary and secondary responses occur in plasma cells. When antigens are first introduced, antigen presenting cells (APCs), (Langerhans cells in the skin, dendritic cells in the spleen and lymph nodes and monocytes in the blood), T cells and B cells act in concert to stimulate the production of antibodies. Many techniques for the preparation and introduction of antigens, such as selection of appropriate injection site (intramuscular, subcutaneous, intravenous, intraperitoneal etc.), mixing of antigen with adjuvants etc. influence the uptake of antigen by the APCS. Adjuvants act by protecting the antigen from being rapidly degraded in the blood stream, and they also contain substances that stimulate the secretion of host factors that facilitate the

macrophage movement to the site of antigen deposition and increase the local rate of phagocytosis.

After an antigen is engulfed by APCS, it is partially degraded, appears on the cell surface of APC and binds to it with a cell-surface class II glycoprotein. In the next step, antigen-glycoprotein complex on the APC binds to T-cell receptors. This leads to T-cell proliferation and differentiation. While T-cells are proliferating, antigens are also processed by virgin B-cell lymphocytes in a similar manner as by APC's. However, the uptake of antigen by B-cells is specific, unlike that by APC'S. As in the case of APC'S, the antigen forms a complex with a surface antibody (Class II protein) on the B-cell surface. This complex also stimulates the same helper T-cells which now bind to B-cells. This leads to division of B-cells and the production of the antibodies. Therefore the contact between B cells and helper T-cells is a major event in the regulation of production of antibodies.

In order for a compound to be good antigen, it should possess one or more epitopes (an antigenic determinant of defined structure), which can bind to the surface antibody on virgin B cells. After the antigen is dissociated, each epitope should be able to bind simultaneously to both the Class II protein and T- cell receptor. Any epitope that is exposed is expected to stimulate strong response to antibody production.

4.2. Structure of immunogammaglobulins and function

Antibodies are glycoproteins present in the serum and tissue fluids of mammals. They are referred to as immunoglobulins because of their role in adaptive immunity. Although all antibodies are immunoglobulins, it is important to realise that not all the immunoglobulins produced by a mammal have antibody activity. There are five classes of antibodies, IgG, IgM, IgA, IgE, and IgO, separated on the basis of the number of Y-like units and the type of heavy-chain polypeptide they contain. There are also significant differences within each class of gammaglobulins.

The basic polypeptide structure of the immunoglobulin molecule is shown in the figure 6. It contains a unit of two identical light polypeptide chains and two identical heavy polypeptide chains linked together by disulfide linkage. The class and subclasses of an immunoglobulin molecule are determined by the type of heavy chain. The most common immunoglobulin is IgG and therefore the description given is for IgG.

IgG molecule contains one structural "Y" unit (Fig. 6). The two arms of Y are made of two identical light chains of molecular weight 23,000 daltons and two identical heavy chains of molecular weight 53,000 daltons. Each light chain is linked to the heavy chain by non-covalent bonds and by one covalent disulfide bridge. Each light-heavy chain pair is linked to another IgG by disulfide bridges between the heavy chains. Carboxytermini of the two heavy chains fold together and form the "FC" domain. The region between the Fab and Fc fragments is called the "hinge". Digestion of IgG with pepsin yields two Fab fragments attached to each other by disulfide bonds and a Fc fragment.

In both heavy and light chains, at the N-terminal portion, the amino acid sequence vary greatly from IgG to IgG. In contrast, in the Fc portion (C-terminal portion of both heavy and light chains) the sequences are identical. Hence the Fab domain contains "Complementary Determining Regions (CDRs)", or hypervariable regions. The six CDR's (three on either side of Fab) comprise the antigen combining site or "paratope" region of IgG. The antigen binds to IgG at this paratope region. The paratope is about 110 amino acid residues in length (both for light and heavy chain). The constant region of the light chain is also about 110 amino acids but the constant region of the heavy chain is about 330 amino acid residues in length.

The antigen combining site (paratope region in IgG) is a crevice between the variable regions of the light and heavy-chain pair. The size and shape of crevice can vary because of differences in the variable light and variable heavy regions, as well as differences in the amino acid sequence variation. Therefore specificity between antigen and antibody results from the molecular complementarity between determinant groups on the antigen (called "Epitope") and the paratope region of the IgG. A single antibody molecule has the ability to combine with a range of different antigens. Stable antigen-antibody complexes can result when there is a sufficient number of short-range interactions between both, regardless of the total fit. This interaction can be as a result of hydrogen bonds, salt bridges, electrostatic charges, hydrophobic bonds, van der Waals' forces and so on. Therefore it is important to realize that the interaction between antigen and antibody is not covalent and therefore is reversible. Various factors such as pH, temperature, detergents, and solvent conditions can influence these interactions.

4.3. Polyclonal antibodies

These are obtained from serum of an animal following injection with an antigen which contains many antigenic sites. Therefore the antibodies produced react with more than one epitope.

4.4. Monoclonal antibodies

They are produced by a single antibody-producing B lymphocyte, immortalized either by mutation or fusion with a myeloma cell line. They react with a single epitope.

4.5. Production of polyclonal antibodies

Aflatoxins have a molecular weight too low to directly evoke antibodies, when injected into animals. Therefore aflatoxins (referred to as a "hapten") must be conjugated to a protein "carrier" before immunization. Bovine serum albumin (BSA) is the most commonly employed carrier (large molecules to which haptens can be covalently attached). Once the hapten attached to the carrier protein is introduced into an animal, a specific antibody response to the hapten as well as the carrier occurs. Conjugation of a mycotoxin to a protein is complicated by the functional groups present in the molecule.

Good quality polyclonal antibodies to aflatoxins can be produced by multiple site immunization of rabbits with the haptens using a mixture of hapten and complete Fruends' adjuvant. Antibodies having sufficient titre are produced in about 8 weeks, after the initial immunization. Booster injections are essential if the titre is low. When rabbits are

immunized with aflatoxins, conjugated to a protein utilizing cyclopentane portion of the molecule, such as carboxymethyloxime of aflatoxin B_1, the antibodies generally recognize dihydrofuran portion of the molecule. When conjugates are prepared utilizing the dihydrofuran portion of the aflatoxin molecule, such as aflatoxin B_2, the antibody recognizes the cyclopentane ring. Because of this diversity in antibody specificity, different antibody preparations should be used for analysis of different aflatoxin metabolites.

An alternative to polyclonals is the development of monoclonal antibodies. Very often they can give more reproducible results. However their production is very costly, time consuming, and requires sophisticated facilities.

The effectiveness of the antibodies used in different immunological tests is dependent upon their specificities and titres. Specificity is governed by the cross reactivity of the antibody with different toxin analogs. High titred antibodies are efficient and economical. The specificity of antibody is determined by measuring its ability to bind to different structurally related analogs in a competitive ELISA. However, antibody titres are measured by the amount of a specific marker ligand bound to the antibody.

Carboxymethlyoxime aflatoxin derivatives used to tag carrier protein. The carbonyl group of aflatoxin (e.g. aflatoxin B_1) is converted to carboxymethyloxime, to facilitate conjugation to a protein. This can be achieved by refluxing aflatoxin B_1 with ethylhydroxylaminohemi-HCl for four hours in the presence of pyridine.

4.5.1 Choice of animals

Any warm blooded animal can be used for antibody production e.g., Rabbits, chickens, guinea pigs, rats, sheep, goats and horses. When small animals such as rats and mice are used, only small quantity of serum can be obtained. Although large animals such as goats and horses can provide large volumes of serum, large amounts of antigen are required for immunizing these animals. The rabbit is the most commonly used animal for antibody production.

4.5.2 Immunization

Injection of an antigen into an animal is accomplished either by intramuscular or subcutaneous injections or by a combination of both.

For injection the antigen preparation should be emulsified with an adjuvant (1:1 proportion). The most commonly used adjuvant is Freund's adjuvant which consists of paraffin oil and an emulsifier, mannide monooleate (incomplete). Complete adjuvants, in addition to these two components, contain heat-killed Mycobacterium tuberculosis, or *M. butyricurn* or a similar acid-fast bacterium. Emulsification with adjuvants results in very slow release of antigen, thereby stimulating excellent immune response. Antigen concentration required may vary from 100 $\mu g\ mL^{-1}$ to 500 $\mu g\ mL^{-1}$. A normal immunization schedule followed for rabbits is given below.

- Four subcutaneous injections (multiple sites) at weekly intervals (for first injection use Freund's complete adjuvant and for the 2nd, 3rd and 4th use incomplete adjuvant).

- non-immunization rest period of six to eight months
- Booster subcutaneous injections after this rest period results in the production of high titre antibodies for aflatoxins

4.5.3 Blood collection and serum preparation

Blood is collected from rabbits by making an incision in the marginal vein of the ear. It is preferable to collect the blood in sterile containers. The blood is allowed to clot at room temperature for 2 to 3 hours (this can also be done by exposure at 37^{0}C for 30 min). After overnight refrigeration the serum is collected with a pasteur pipette and then centrifuged at 5000 rpm for 10 minutes.

Note: It is important to starve rabbits for at least 24 h before blood collection to minimize concentration of lipids

4.6. Storage of antisera

- For long term storage of antisera at 4^{o}C it is essential to add either glycerol (1:1) or sodium azide to a concentration of 0.02%.
- In lyophilized form antisera can be stored at -20°C indefinitely for many years without losing potency.
- Antisera can be stored at –70^{o}C
- It is advisable to store serum in small aliquots of 1.0 mL or less.
- Antisera should not be frozen and thawed repeatedly. This leads to aggregation of antibodies thereby affecting antibody activity by steric interference of the antigen combining site or by generating insoluble material which may sediment during centrifugation.

5. Enzyme-linked immunosorbent assay (ELISA)

5.1 Principles

It is by far the most widely used serological test for the detection of aflatoxins, because of its simplicity, adaptability and sensitivity. The test departs from the classical serological procedures in which immuno-precipitin reactions are used. Immuno-specificity is recognized through the action of the associated enzyme label on a suitable substrate rather than by observing the formation of an insoluble antigen-antibody complex.

The basic principle of ELISA lies in immobilizing the antigen onto a solid surface, or capturing antigen by specific antibodies, and probing with specific immunoglobulins carrying an enzyme lable. The enzyme, retained in the case of positive reaction, is detected by adding the suitable substrate. The enzyme converts substrate to a product which can be easily recognized by its colour.

5.2 Choice of enzyme lables

The two enzyme lables which are widely used are alkaline phosphatase (ALP) and horseradish peroxidase (HRP). Galactosidase, urease and penicillinase (B-lactamase) have subsequently been introduced. ALP and its substrate, p-nitrophenyl phosphate, are very expensive and are not readily available in developing countries. HRP and its substrate, tetramethyl benzidine, though less expensive than ALP, are also not readily available in developing countries.

Penicillinase has several advantages over the ALP system. They are listed below:

- It is less expensive than ALP (typical cost is a tenth of that of the ALP system for enzyme and substrate components).
- Penicillin and penicillinase, suitable for ELISA, are available in some developing countries.
- P-nitrophenol, a hydrolyzed product of the ALP substrate, p-nitrophenyl phosphate, is more hazardous than penicilloic acid.
- The substrate for penicillinase has longer shelf-life than the other enzyme systems.
- Visual reading of results is easier for penicillinase substrate than for the ALP system.
- Penicillinase is not known to occur naturally in higher plants and animals. However it is abundant in bacteria.

Penicillinase breaks down penicillin into penicilloic acid, and this is detected either by the rapid decolourization of a starch-iodine reagent or by utilizing acid-sensitive pH indicators.

5.3. Conjugation of AFB1-BSA with penicillinase, horseradish peroxidase or alkaline phosphatase enzymes

5.3.1. Materials

- AFB1-BSA (Sigma A 6655)
- Phosphate buffer (PBS)

Na_2HP04	:	2.38 g
KH_2P04	:	0.4 g
KCl	:	0.4 g
NaCl	:	16 g
Dist. water	:	2L

pH of the solution is generally 7.4.

- Glutaraldehyde (25% Sigma G 5882)

- Penicillinase (PNC). Generally supplied as lyophilised powder from Sigma (P 0389) or from Hindustan Antibiotics Ltd., Pimpri, India, ELISA grade.
- Horseradish peroxidase (HRP) Sigma P 8375.
- **Alkaline phosphatase (ALP). Type VII T (Sigma - P-6774)**
- Bromothymol blue (BTB)
- Sodium penicillin-G (K-penicillin-G, a procaine penicillin, can also be used)
- p-nitrophenyl phosphate tablets, 15 or 40 mg (Sigma N.2640)
- Diethanolamine
- HRP substrate : Kirkegaard and Perry (50-76-05)
- HRP stopping solution: 10% H_2SO_4.
- Dialysis bag (0.6 cm dia.)
- Micropipettes
- Bovine serum albumin. Highly pure chemical should be used (Sigma A 7638).

5.3.2. Conjugation of AFB1-BSA with penicillinase by single bridge glutaraldehyde method

- Take AFB1-BSA, 500 μg mL^{-1}, (make an appropriate dilution in PBS) in a dialysis bag and add 500 μg of penicillinase. Solutions of AFB1-BSA and penicillinase can be made at higher concentrations and mixed to get AFB1-BSA 1 mg mL^{-1} and 1 mg mL^{-1} penicillinase.
- Dialyse against PBS in a beaker for 1 h at room temperature.
- Transfer the dialysis bag (containing AFB1-BSA and enzyme) into a beaker containing PBS with 0.06 % glutaraldehyde (mix 1 mL of 25% glutaraldehyde in 400 mL PBS to get 0.06% glutaraldehyde) and dialyse for 3-4 h at room temperature.
- Replace the buffer containing glutaraldehyde with 500 mL PBS containing sodium azide (0.02%) and dialyse for 18 h at 4°C with at least three changes of buffer (for each change replace with 500 mL PBS containing azide).
- Transfer the conjugate into a new glass or plastic vial and add bovine serum albumin at 5 mg mL^{-1} concentration. Store in small aliquots (100 μL) at 4°C. Do not freeze the conjugate. If stored properly the shelf life of conjugated AFB-BSA should exceed 1 year.

5.3.3. Conjugation of AFB1-BSA with alkaline phosphate by single bridge glutaraldehyde method

Procedure is similar to that described for penicillinase.

5.3.4. Conjugation of AFB1-BSA with horse radish peroxidase (HRP) utilizing periodate oxidation method

- Take HRP (2.0 mg) in 0.2 ml of citric acid /sodium citrate buffer (0.1M, pH 5.0) and warm to 37° C
- Add 26 μL of sodium periodate (30 mg/ml) and keep for 5mts.
- Add 13 μL of 1% ethylene glycol and immediately dialyse the oxidised enzyme against 1 L of citric acid/sodium citrate buffer (0.001M pH 5.0 for 1 h)
- React the oxidised HRP with AFB1-BSA. Nearly 239 μl of 2.0 mg oxidized HRP was added to 500 μg of AFB1-BSA suspended in 1.0 ml of 0.1 M sodium carbonate buffer, pH 10.0 and held at 4°C for 2 days.
- Add 10 μL of sodium cyanoborohydrate solution (10 mg/mL) and react for another 2h at 4°C.
- Dialyse the reaction mixture (AFB1-BSA conjugated to HRP) against PBS and store at 4°C after the addition of glycerol (1:1 v/v)

5.4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Two types of ELISA have been used in the analysis of aflatoxins (i) **indirect ELISA** and (ii) **direct ELISA**. Both forms are heterogeneous competitive assays which involve the separation of free (unreacted) toxin in liquid phase from the bound toxin in solid-phase.

5.4.1. ELISA General

Materials

- ***ELISA-plates*** (Nunc-Maxisorp). Nunc also market individual strips containing 16 wells (2 rows of 8 wells).
- ***Micropipettes*** (10-100 μL and 100-1000 μL). Several brands are available. Those which provide adjustable volumes are preferable. (Some commonly used brands are: Eppendorf, Finipette, Gilson, etc.).
- ***Repeatable micropipette*** (100 or 200 μL volume): It is possible to dispense desired volumes of liquids repeatedly from a reservoir attached to a micropipette. From some manufacturers it is possible to purchase micropipettes which hold four, eight or 12 micro tips thus permitting dispensing of volumes simultaneously into several wells.
- ***ELISA plate reader*** - manual or automatic. Several ELISA plate readers are available ranging in price from $ 3500 to $ 15,000. Some of the popular brand names are Dynatech, Flow Laboratories, Biorad, Biotek and Immuno-reader. If funds are available, it is preferable to purchase an automatic ELISA reader which provides several wavelengths, and which can print out the readings and can also be interfaced with a computer.

Helpful hints

- Do all incubations in a humid chamber. A small rectangular plastic box with moist paper towels covering the bottom of the box is adequate.
- Take care not to contaminate the glassware intended for storing penicillin-BTB solution with buffering salts because the reaction with penicillin and penicillinase is detected by changes in pH due to production of penicilloic acid.
- New plates should be used as supplied by manufacturers. Do not wash or rinse them prior to use. As far as possible use recommended plates. If particular brand is not available use plates of equal quality and specificity.
- Always include appropriate controls, especially toxin standards, and samples devoid of toxins.

Precautions for handling pure aflatoxin

- Aflatoxin B_1 is highly carcinogenic and handle it very carefully.
- Use gloves when opening the vial containing the toxin
- Open the bottle under fume hood. Use a nose mask. Aflatoxin may disseminate to surroundings due to electrostatic charge on particles.
- Use nose mask while preparing different dilutions of the pure toxin.
- Rinse all glassware exposed to aflatoxin carefully with chloroform, then with a bleach and then wash thoroughly.
- Swab accidental spills of aflatoxin with bleach and then with water.

5.4.2. Preparation of groundnut seed extracts:

First make the seed into powder using a blender. Then triturate the seed powder in 70% methanol (v/v-70 ml absolute methanol in 30 ml distilled water) containing 0.5% KCl (proportion used is 100 ml for 20 gm seed) in a blender, until the seed powder is thoroughly ground. Transfer the extract to a conical flask and shake it for 30 min at 300 rpm. The extract was filtered through Whatman No. 41 filter paper and diluted 1:10 in PBS-Tween (1 ml extract and 9 ml of buffer). To estimate lower levels of AFB_1 (< 10

µg/kg), prior to ELISA a simple liquid-liquid clean up and concentration (5:1) procedure is to be adopted. For clean up, 20 ml of methanol extract, 10 ml distilled water and 20 ml chloroform are to be mixed in a separating funnel. After vigorous shaking for one minute, collect the lower chloroform layer and evaporate to near dryness in water bath at 60°C. To the residue add 4 ml PBS-Tween containing 7% methanol and use for analysis by ELISA.

5.4.3. Indirect Competitive ELISA

Materials

- ***Carbonate buffer (coating buffer)***

  Na_2CO_3 : 1.59 g
  $NaHCO_3$: 2.93 g
  Distilled Water : 1.0 L
  pH of buffer should be 9.6. No need to adjust the pH.

- ***Phosphate buffer (PBS)***

  Na_2HP0_4 : 2.38 g
  KH_2P0_4 : 0.4 g
  KCl : 0.4 g
  NaCl : 16.0 g
  Distilled water : 2.0 L

- ***Phosphate-buffered saline with Tween*** **(PBS-Tween):** PBS: l L; Tween-20: 0.5 mL

- ***Albumin bovine serum*** **(Sigma A 6793):**
  Dissolve 200 mg BSA in 100 ml PBS-Tween

- ***Substrate buffer for alkaline phosphatase system:*** p- nitrophenyl phosphate (PNPP) should be stored at -20°C. It is preferable to buy the chemical in tablet form (5, 15 or 20 mg tablets available). Prepare 10% diethanolamine (v/v) in distilled water. Adjust pH to 9.8 with conc. HCl. This solution can be stored but pH should be adjusted to 9.80 prior to use. Prepare 0.5 mg ml^{-1} PNPP in 10% diethanolamine, pH 9.80 (for each 15 mg tablet 30 ml solution is required). Ensure that the PNPP solution does not turn yellow. This may sometimes happen because of ALP contamination from skin.

  Caution:
  - Use gloves to avoid ALP contamination. Diethanolamine is toxic and harmful to eyes. Take all the necessary care to avoid contact with skin.
  - PNPP when reacted with ALP becomes p-nitrophenol. It can cause corrosion. Therefore plates must be handled with the utmost caution after adding the PNPP.

5.4.4. Procedure

In the case of indirect competitive ELISA, AFB1-BSA is adsorbed to the plate surface. Competition is between enzyme-labelled or unlabelled antibody with the toxin present in the sample or in the standard

- Prepare AFB_1-BSA conjugate in carbonate coating buffer at 100 ng/mL concentration and dispense 150 µl of the diluted toxin-BSA to each well of ELISA plate. Incubate the plate in a refrigerator overnight or at 37°C for at least 1 h.
- Collect the toxin and store in a large glass bottle for disposal.
- Wash the plates in three changes of PBS-Tween, allowing 3 min for each wash.
- Add 0.2% BSA prepared in PBS-Tween at 150 µl per each well of ELISA plate. Incubate at 37°C for 1 h.
- Prepare a suitable dilution of antiserum in a tube. This dilution of antiserum (for each polyclonal antiserum this should be pre-determined) in PBS-Tween containing 0.2% BSA and incubate for 30 min at 37°C.
- Wash the blocked plates in three changes of PBS-Tween, allowing 3 min for each wash.
- Preparation of aflatoxin B_1 standards:
  Prepare healthy groundnut seed extract as mentioned in the section 5.4.2.
  Dilute aflatoxin B_1 standards (using 1:10 diluted groundnut extract) at concentrations ranging from 25 ng to 10 picogram in 100 µl volume.
- Add 50 µl of antiserum to each of the dilution of aflatoxin standards (100 µl) and groundnut seed extract (100 µl) intended for analysis. Incubate the plate containing the mixture of aflatoxin samples (100 µl) and antiserum (50 µl) for 1 h at 37°C to facilitate reaction between the toxin present in the sample with antibody. Entire process is done in the ELISA plate and there is no need to preincubate the toxin and antibody mixture in separate tubes.
- Wash the plate in three changes of PBS-Tween allowing 3 min for each wash.
- Prepare 1:4000 dilution of goat anti-rabbit IgG, labelled with alkaline phosphatase, in PBS-Tween containing 0.2% BSA. Add 150 µl to each well and incubate for 1 h at 37°C.
- Wash the plate in three changes of PBS-Tween allowing 3 min for each wash.
- Add 150 µl substrate solution (p-nitrophenyl phosphate prepared in 10% diethanolamine buffer, pH 9.8) and incubate for 1 h at room temperature or for shorter intervals depending on the development of yellow colour (in wells where low aflatoxin concentration were used for competition). Measure absorbance at 405 nm in an ELISA reader (preferably automatic).
- Using the values obtained for aflatoxin B_1 standards draw a curve, using with the help of a computer, taking aflatoxin concentrations on the "X" axis and optical density values on the "Y" axis.

Calculations:

AFB_1 (µg/kg) : $\frac{AxDxE}{G}$ or $\frac{AxE}{CxG}$

A = AFB_1 concentration in diluted or concentrated sample extract (ng/ml)

D = Times dilution with buffer

C = Times concentration after cleanup

E = Extraction solvent volume used (ml)

G = Sample weight (g)

5.4.5. Direct competitive ELISA

The antibody is coated on to the wells of the ELISA plate. The test sample and the enzyme labelled aflatoxin B1-BSA are added to the wells. Competition between enzyme-labelled AFB1-BSA and toxin derived from the sample or standards. If toxin is present in the sample, it will compete with the labelled toxin for binding to the antibody. During washing procedure any unbound labelled enzyme will be washed away. On the addition of substrate, a colour will develop, the intensity of which is proportional to the amount of AFB1-BSA-enzyme bound to the well; i.e., the colour intensity decreases with increasing concentrations of the toxin in the sample.

Materials

- Carbonate buffer (coating buffer): section 5.4.3
- Phosphate buffer (PBS): section 5.4.3
- Phosphate-buffered saline with Tween (PBS-Tween): section 5.4.3
- Bovine serum albumin (Sigma A 6793): section 5.4.3
- AFB1 antiserum
- Aflatoxin B_1 standards in healthy groundnut seed extract: section 5.4.3
- AFB1-BSA-labelled with PNC
- AFB1-BSA-labelled with HRP

- Substrate buffer for penicillinase system*:* Dissolve 15 mg bromothymol blue (BTB) in 50 ml of 0.01 M NaOH. Neutralise the alkali by adding conc. HCl drop wise. Make up the volume to 100 ml. Incorporate sodium penicillin-G (Potassium penicillin-G and procaine penicillin are alternatives) at 0.5 mg ml^{-1} and adjust the pH to 7.2 using either HCl or NaOH. Store the mixture at 4°C. **It is absolutely essential to adjust the pH to 7.2 before use.**
  Note: BTB solution alone is stable for several months at 4°C but with penicillin it is stable for only a few days.

- Substrate buffer for horseradish peroxidase system : Kirkegaard and Perry: TMB Microwell Peroxidase Substrate (50-76-05)

5.4.6 Procedure utilising PNC/HRP

- Coat each well of an ELISA plate by using 150 µL of appropriate dilution of AFB1 antiserum in coating buffer (Use the antiserum produced at ICRISAT at 1:20,000 dilution). Incubate the plate in a refrigerator overnight or at 37°C for at least 1 h.
- Wash the plates in three changes of PBS-Tween, allowing 3 min for each wash.
- Dilute aflatoxin B_1 standards (using 1:10 healthy groundnut extract) at concentrations ranging from 100 ng to 10 picogram in 100 µl volume. Add 100 µL of AFB1 standards at concentrations ranging from 100 to 0.09 ng/ml in two rows.
- For samples to be analysed, use 100 µl of 1:10 diluted sample.

For PNC-labelled conjugate

- To 100 µl of the sample add 50 µl of appropriately diluted AFB1-BSA labelled with PNC and incubate at 37°C for ½ hr.

- Wash the plates in three changes of distilled water-Tween, allowing 3 min for each wash.
- Add 150 µL of PNC substrate and keep the plate at room temperature for ½ h.
- Read the plate at 620 nm

For HRP-labelled conjugate

- To 100 µl of the sample add 50 µl of appropriately diluted AFB1-BSA labelled with HRP and incubate at 37°C for ½ hr.
- Wash the plates in three changes of PBS-Tween, allowing 3 min for each wash.
- Add 150 µL of HRP substrate and keep the plate at room temperature for ½ h.
- Stop the HRP reaction in the plate by adding 10 % H_2SO_4
- Read the plate at 450 nm

Calculate the toxin concentration as described in the section 5.4.3.

***Aspergillus flavus* seed infection by blotter plate method**

Collect the groundnut pod samples and draw the sub-sample lots. Shell the sub-samples and pick randomly 100 undamaged kernels (from each sub-sample) for *A. flavus* seed infection study by blotter plate method. First soak the kernels in sterile distilled water for 3-5 minutes, then surface sterilize the seed with 1% sodium-hypochloride solution for one minute followed by three washes with sterile distilled water. Then place the seed on sterile moist filter paper in the sterile petridish. Place all the petridishes in moist humid plastic boxes (45 x 25 cm) before they were incubated at 28°C for five days. On sixth day observe the plates and count the number of seed showing *A. flavus* seed infection.

Screening for resistance to A. flavus seed colonization and aflatoxins contamination

Select highly virulent, toxigenic strain of *A. flavus* and multiply it either on PDA or on groundnut seed for 7-10 days. Prepare the conidial suspension (1 x 10^6 spores/ml) to inoculate the seed. Take 100 fully matured undamaged sound kernels from each genotype meant for resistance screening. First soak the seed in sterile distilled water for 3-5 minutes, then surface sterilize the seed with 1% sodium hypochloride for 1 min. followed by three washes with sterile distill water. Then dip the seed in *A. flavus* spore suspension for 1-2 min. then place the seed on moist blotter in a petridish. Place all the petridishes in humid plastic box and incubate the plastic boxes at 280C for 5-7 day. After incubation record the number of seed colonized with *A. flavus*. Precaution should be taken not to damage the seed coat while doing this exercise.

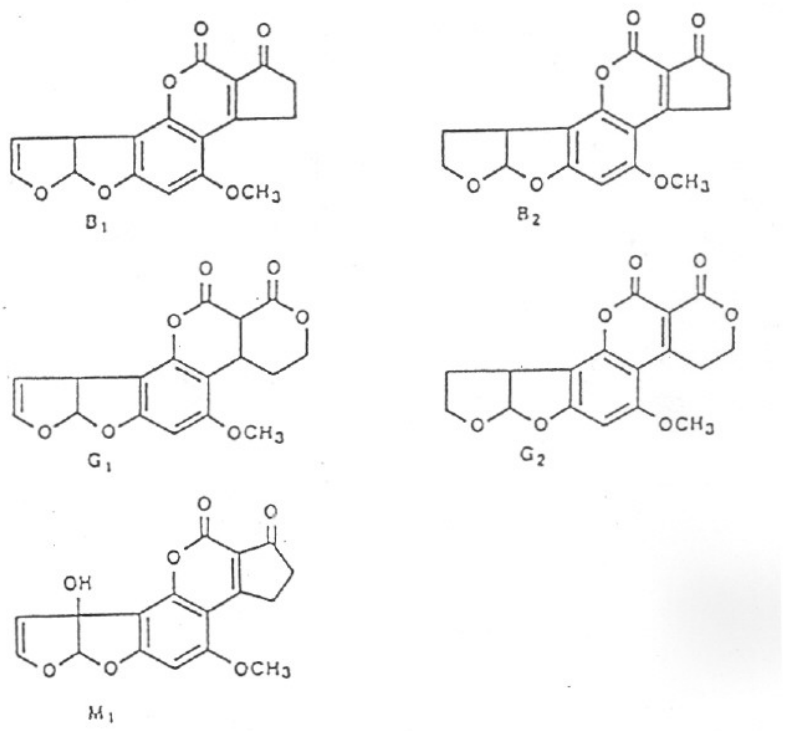

Figure 1: Structure of aflatoxin B_1 and related toxins

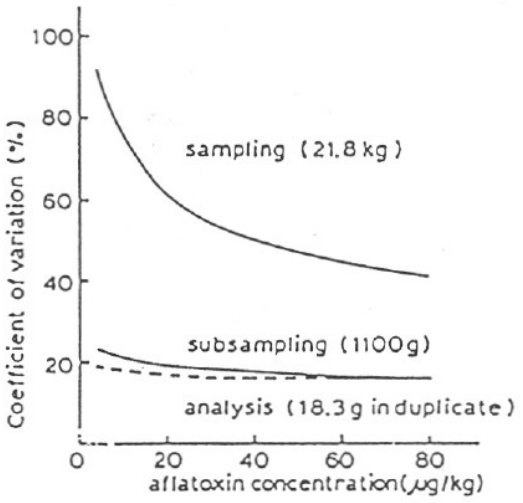

Figure 2: Relative contributions of the errors of the sampling and analysis to the total error (after Whitaker 1977, courtesy IUPAC)

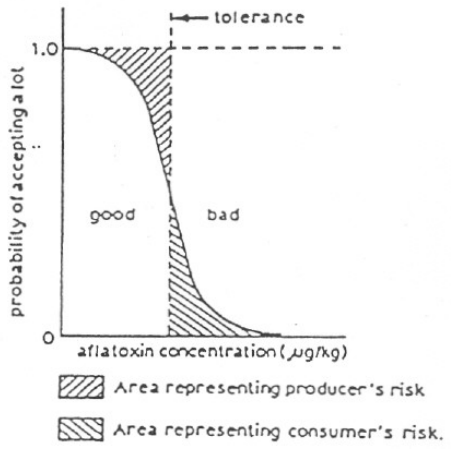

Figure 3: Relation of probability of acceptance of a lot versus the aflatoxin concentration (operating characteristic curve) (after Whitaker, 1977, courtesy IUPAC)

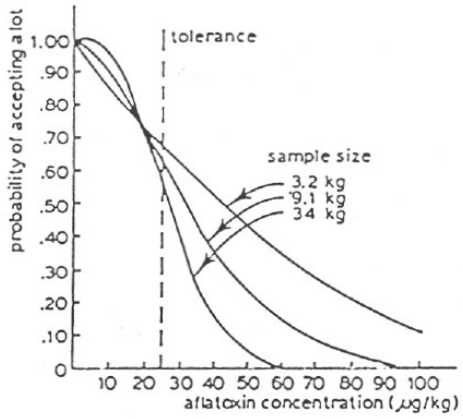

Figure 4: Effect of the sample size on operating characteristic curve (after Dickens, 1978; courtesy institut fur toxikologie, Zurich)

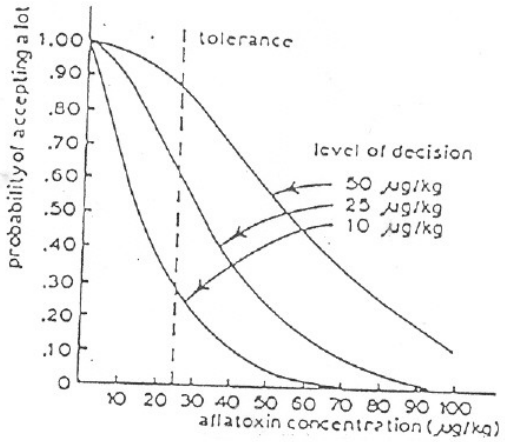

Figure 5: Effect of the level of the decision on the operating characteristic curve (after Dickens, 1978; courtesy institut fur toxikologie, Zurich)

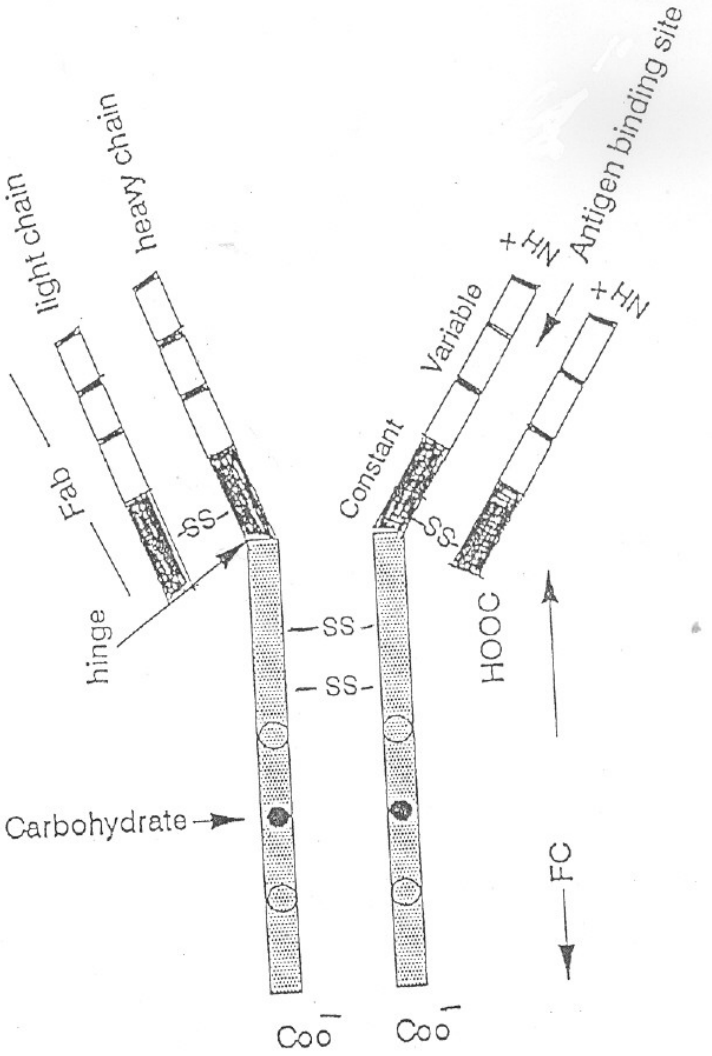

Figure 6: Structure of immunoglobulin (IgG)

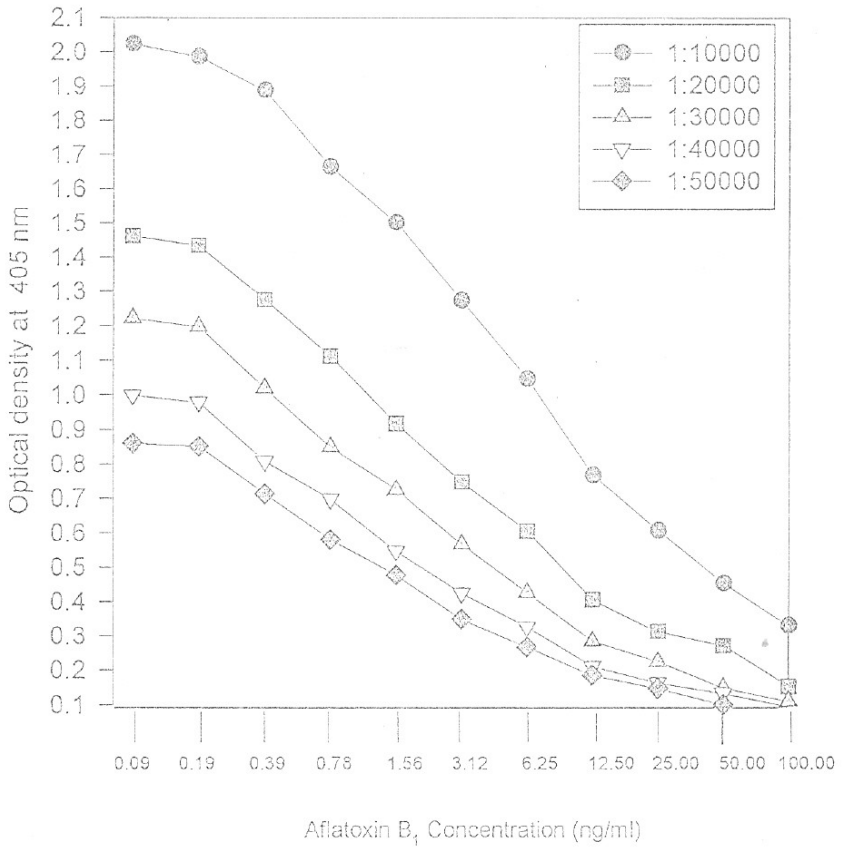

Fig 7: Effect of various antibody diltuions on the standard curve to estimate aflatoxin concentraion when a constant coating of aflatoxin B_1 - BSA is used.