An association of toxic substances with plants and plant products used for human or animal consumption has been made since Biblical times, with ergotism representing one of the first suspected effects of such toxic component. In the mid and late 50's of the 19th Century, there were reports of disease of cattle and swine caused by feeding mouldy corn. More than 100,000 young Turkey poults died due to consumption of Brazilian peanuts (Blount, 1961) that ultimately led to the discovery of Aflatoxin, a toxic metabolite produced by A. flavus. Aflatoxins have been defined as the chemical pollutant of biological origin and their worldwide occurrence in the environment (Stoloff, 1982) have led to establish maximum tolerance limits in different countries of the world. The safe limits are different in different countries and also vary with the commodity. Limits prescribed in different countries vary from 0 to 50 ug/kg for foods and from 0 to 1000 ug/kg for feeds (FAO, 1979).

In India, the safe levels were initially established along the guidelines issued by the Protein Advisory Group (PAG) of the United Nations System. They suggested that the level of aflatoxin in protein rich food should not exceed 30 ug/kg of food ingested. Since 1973 new regulations or amendments to earlier ones have generally set lower tolerance, e.g. European Economic Community (EEC). Food and Drug Administration (FDA), in 1965 declared an action guideline of 30 ppb for total aflatoxin in food. This was further reduced to its current level of 20 ppb in 1969. FDA fixed 20 ppb as a safe level for aflatoxin which was liberalised to 100 ppb during 'aflatoxin
Occurrence of Aflatoxin contamination may occur at various stages from sowing to the ultimate consumption of a food commodity. Rainfall, humidity, temperature, aeration, insect pests, microbial competition, geographic location, etc. are important environmental factors in determining aflatoxin contamination. However, occurrences of aflatoxins contamination are worldwide.

Cereal Grains

Site for Aflatoxin contamination: Insect pests, drought, flood, irrigation, fertilization, crop rotation may influence the extent and severity of fungal attacks on the crops. The incidence of aflatoxins contaminations were reported to be significantly higher in the corn damaged with insects which provides an opening for the entry of *A. flavus* (Lillehoj, et al., 1975,a,b; McMillan, et al., 1978). Drought conditions were also considered to be a stress factor to cause damage leading to *A. flavus* infection (Lillehoj, et al., 1976). Mould contamination and aflatoxin production were observed in flooded areas of Bhagalpur (India) by Sinha (1987). Aflatoxin contamination in maize was found to occur in field during sowing (Anderson, et al., 1975), prior to harvest (Stoloff, 1976) or after harvest (Christensen, 1957). Faulty storage was found to influence the infestation of *A. flavus* and aflatoxin contamination (Krishnamachari, et al., 1975a). The post-harvest drying operation was also
considered as a frequent site of aflatoxin contamination in the crops.

(i) **Maize:** Maize, of all cereal grains, are more prone to *A. flavus* attack and Lillehoj, et al. (1974) reported that the production of aflatoxins were high since they were enriched with Mn, Cd, Cu, Cr. Aflatoxin level in corn might vary from year to year and from region to region, even in the same country (Stoloff, 1982). Aflatoxins were found in corn samples at all stages of development (Anderson, et al., 1975).

India represents tropical as well as temperate regions. Droughts and floods are more frequent due to irregular monsoons. However, no systematic studies of aflatoxin in India have been conducted. Krishnamachari, et al. (1975a,b) reported an outbreak of epidemic jaundice in India in 1974. Over 200 villages of Banswada and Panchmahals District of Rajasthan and Gujarat State were affected. The staple food of these people was locally grown corn. The reports mentioned more than 100 people died due to consumption of visibly contaminated corn. Aflatoxin levels ranged from 0.25-15.6 mg/kg and *A. flavus* were isolated from corn samples. Further studies by Tandon et al. (1977) revealed that 1000 individuals were affected and 97 people died. This was accompanied by aflatoxicosis in dogs fed with left-overs from the human food (FAO., 1979). Aflatoxin contamination have been detected in corn samples by Afzal, et al. (1979) and Sinha (1987).

A systematic survey of aflatoxin has been conducted in U.S.A. by Shotwell, et al. (1969a,b; 1971, 1973, 1977a,b, 1981a,b, 1982) and Hesseltine, et al. (1981). In 1983, corn harvested in
Virginia had average aflatoxin levels of $117\pm360$ ug/kg (Failla, et al. 1986). Aflatoxin contamination has been detected in 8 types of corn products tested in Brazil, of which popcorn appeared to have the highest proportion ranging from 20-47 ug/kg (Soares and Rodriguez-Amaya, 1989).

Aflatoxin have been recovered in corn samples from France (Lafont and Lafont, 1970); Uganda (Alpert, et al., 1971); Swaziland (Martin and Gilman, 1976); Egypt (Girgis, et al., 1977); Philippines and Yugoslavia (FAO., 1979); Soviet Union, U.S.A., Gautemala, Kenya (Van Egmond, 1987).

ii) Rice: India is the second largest producer of rice, which is a staple diet in many States of India. In an epidemiological survey conducted in coastal area of Karnataka State, it was observed that 6\% samples contained aflatoxin (FAO., 1979). Aflatoxins have also been detected in 50\% Indian rice samples processed by Patel, et al. (1981). Tulpule, et al. (1982) detected aflatoxin at levels of 30-130 ug/kg in cyclone affected rice and parboiled rice.

Aflatoxin level upto 200 ug/kg was detected in one of the five mouldy rice samples by Tung and Ling (1968). Lucas, et al. (1971) analysed South Vietnamese rice samples and found that out of 139 samples, 13\% were aflatoxin positive. Van Rensburg, et al. (1975) conducted a survey in Mozambique and detected 3.4 ug/kg of aflatoxin in home storage rice. In Dominican Republic, two rice bran samples were reported to contain aflatoxin at the levels of 15-50 ug/kg. In Phillippines and Indonesia, the rice samples were found to
contain aflatoxin at the levels of 20 and 15 ug/kg, respectively. From Columbia, 20 ug/kg aflatoxin has been detected in 83% of rice samples (FAO., 1979). Soares and Rodriguez-Amaya (1989) tested 52 Brazilian polished rice samples, 2 samples had AFB₁, B₂ and G₁ at the levels of 38, 15 and 20 ug/kg respectively.

(iii) Wheat: Wheat is cultivated in many temperate regions of the world, viz. North America, Europe, China, India, Argentina and Australia. However, the incidence and level of aflatoxin was found to be low. Patel, et al. (1981) reported more than 50% of the wheat samples of some feed in India contained aflatoxin. A study in Yugoslavia and Greece showed the samples had aflatoxin levels of about 6.4 ug/kg and 5-50 ug/kg, respectively (FAO, 1979).

(iv) Sorghum: Sorghum is cultivated in Africa, Asia, North America and Australia. Aflatoxins have been reported by Tripathi (1973) in the heads of Indian sorghum which were heavily infested with the mould. A systematic survey was carried out in Uganda (Alpert, et al., 1971) and United States (Shotwell, et al., 1969a). Aflatoxin contamination was low in U.S. sorghum while in Uganda it was detected in the range of 100-1000 ug/kg.

Oilseeds

The major oilseeds include groundnuts, cottonseed, soyabean, copra and others like linseed, niger, safflower, sesame etc. High levels of aflatoxins have been detected from groundnut and groundnut cake collected during the years 1965-67 from Gujarat, Andhra Pradesh and Tamil Nadu (Patel, et al., 1981; FAO., 1979). Cotton-
seeds and soyabean samples from India were found to contain aflatoxins at the level of 50 and 180 ug/kg, respectively. Significant levels of aflatoxins have also been detected in copra from India, Finland, Philippines (FAO., 1979). Patel, et al., (1981) detected aflatoxin in cakes of linseed, niger, safflower and sesame.

The crude oil of different seeds like groundnut oil, coconut oil, olive oil had detectable levels of aflatoxins (FAO., 1979). The refining treatment reduces or eliminates aflatoxin from oil.

Pulses, Root Crops, Treenuts, Tubers, Vegetable Products and Milk Products

Incidence of aflatoxin contamination is not significant in these commodities. Aflatoxins have been detected in pulses, like mung, beans, legumes, kidney beans, black beans (FAO., 1979), and treenuts, like pistachionuts, almonds, walnuts, brazlnuts, pecans, hazelnuts, filbert (Fuller, et al., 1976).

Root crops like cassava, sweet potatoe, yams, taro, carrots, raddish and onion showed the presence of aflatoxin (FAO., 1979). It was also found in vegetable products like beverages (coffee beans, cocoa beans), spices, fermented foods, alcohol, wine etc.

Animal products like milk (FAO., 1979; Stoloff, 1976); animal tissues (Newberne and Butler, 1969); fish and fish products (FAO., 1979) have also been found to contain aflatoxins.

Airborne Aflatoxin

Agricultural workers account for more than half of the working populations of the world. Even during manual winnowing of
rice, maize, wheat, barley, etc., large amount of dusts is released into the working environment. During threshing and milling, large amount of dusts containing husk, germ and starch particles are also released. Asthma, abnormalities in upper respiratory tract, eye irritation, chronic bronchitis and related diseases are observed among agricultural workers. Ramazzini (1713) had described the disease of workers inhaling "foul and mischievous powder" from handling food, fodder and fiber crops. So, when the contaminated cereals were processed to convert them into edible form, along with the grain dust, husk, etc., the toxins produced by the moulds were also released into the atmosphere (Sorenson, et al., 1981, 1984; Burg and Shotwell, 1984).

Fabbri, et al. (1979) collected total and respirable airborne corn dust samples at the flow rate of 15-20 litre per minute for 30-60 minutes at different work sites. The maximum 55 mg/m$^3$ of total dust was found at the dextrin packing area and the minimum of 3.8 mg/m$^3$ from feed packing area. The total airborne dust was found at the range of 3.8-47.6 mg/m$^3$ from starch packing department. Burg, et al. (1981) collected airborne total and respirable corn dust samples using high volume total and Anderson sampler. The total dust content generated from Bournier divider and Bournier divider inlet were 8.51 and 1.74 mg/m$^3$ respectively. The dust concentration collected by Anderson sampler had significantly higher aflatoxin levels on the first stage than in the succeeding stages (16.3-0.4 mg/m$^3$). Shotwell, et al. (1981a) collected airborne corn dust samples that ranged in weight from 7 mg to 11.8 g, with a high
volume sampler. Burg and Shotwell (1984) collected total dust samples from the elevator, loading and unloading departments. The elevator department had maximum of 11,100 mg/m³ of dust, while loading the trucks from elevator had maximum 11,500 mg/m³, and unloading the trucks at elevator had 3490 mg/m³ of total dust.

Stoloff (1982) reported that the aflatoxin content was reduced during the processing of corn meal, corn flour, tortillas, ground and dry milled corn. Therefore, it might be disseminated into the surrounding environment causing great risk to aflatoxin exposure via inhalation to occupational workers. The acute inhalational exposure to aflatoxin had detrimental effects on the exposed respiratory tract cells (Goldbatt, 1969). These reports provided the first experimental evidence of a health hazard of inhaled aflatoxin. There were coincidences of liver, intestine and kidney tumours in experimental animals which had developed from absorption of the toxin either from the lung or during subsequent passages through alimentary tract. There were several reports available on cytotoxicity and carcinogenicity of \( \text{AFB}_1 \) towards respiratory tract (Coulombe, et al., 1984; Legator 1965). Baxter, et al. (1981); Sorenson, et al. (1981, 1984); and Hayes, et al. (1984), in their epidemiological studies have indicated that there exists, throughout the world, a risk to occupational workers exposed to aflatoxin via respiratory tract due to their occupations. Agricultural workers exposed for a long time to 0.4 ppb to low level of aflatoxin exposure produces significant number of liver cancers in less than a year (Lee, et al., 1968).
Aflatoxins have been detected in corn dust samples collected using high volume sampler by Burg, et al. (1981) at the level of 4560.0 ppb and 2555.0 ppb w/w from Bourner divider and Bourner divider inlet area, respectively. The level of aflatoxin decreased significantly from 4540.0 to 926.0 ppb with Anderson sampler. From the storage bin, aflatoxin concentrations ranged from a trace to 204.3 ppb w/w showing that aflatoxin concentration varied considerably. The average of 138 ppm aflatoxins were collected. About 5-17% of the particles were less than 7.0 um in diameter. Burg and Shotwell (1984) estimated aflatoxin in airborne and settled dust. Settled dust had 173-660 ng/g of aflatoxin and airborne dust had highest aflatoxin level of 13000 ng/m³ at the elevator department. The unloading area near elevator had aflatoxin level ranging from 31.1 to 1840 ng/m³, while the loading of corn bags in the cars near elevator had airborne aflatoxin at the level of 506 to 2850 ng/m³. They reported that airborne dust usually contains higher aflatoxins level than the bulk corn from which they were generated.

Emanuel, et al. (1975) reported the lung disease due to inhalation of fungal toxins when workers were exposed to large amount of mouldy silage. Dvořáčková (1976) and Dvořáčková and Polster (1984) reported lung cancer in occupational workers exposed to aflatoxin contamination. Deger (1976) reported death of one biochemist and similar type of illness in another one due to possible aflatoxin contaminated dust inhalation. Sorenson, et al. (1981) in Georgia feed mill near Tifton collected the sample which contained 130 ppb aflatoxin B₁. The contaminated particles were found to be
less than 7.0 μm in diameter.

Rati and Rammlingam (1979) found 72% of toxigenic strain of A. flavus from Manasa Gangotri air samples taken from outdoor and indoor air of a poultry shed. The incidence was found to be greater in winter. Krishnachari, et al. (1975a) isolated toxigenic strain of A. flavus from contaminated Indian corn samples.

**Structure and Metabolism of Aflatoxin**

Since the discovery of aflatoxin, intensive study has been carried out on this toxin for its carcinogenic, mutagenic, teratogenic properties. Nesbitt, et al. (1962) studied the aflatoxin on alumina chromatoplates and found two distinct components of aflatoxin. They were named as aflatoxin B and aflatoxin G denoting their fluorescent color. de Iongh, et al. (1964) observed two fluorescent spots and designated them as B1 and B2. Hartley, et al. (1963) were the first group to isolate and characterise four closely related toxins. They were B1, B2, G1 and G2 in sequence of decreasing relative chromatography mobility. Infrared and ultraviolet absorption spectra showed that these compounds were closely related.

Allcroft and Carnaghan (1963) reported toxic materials from cow's milk when fed rations contaminated with aflatoxin B1 and B2. They observed blue-violet fluorescent. Allcroft, et al. (1966) isolated aflatoxin M1 and M2 with blue-violet fluorescent having lower Rf value than B1, B2, G1 and G2. Holzapfel, et al. (1966) reported that AFM1 and M2 are the 4-hydroxylated derivatives of B1 and B2 respectively. Dutton and Heathcote (1966) reported aflatoxin B2α and
aflatoxin $G_{2a}$ from *A. flavus* strain representing blue and green fluorescences. They were 2-hydroxy derivatives of aflatoxin $B_1$ and $C_1$, which was confirmed by them later in 1968.

Chemically, aflatoxins are highly oxygenated naturally occurring heterocyclic compounds and have closely related structure. Aflatoxins contain a coumarin nucleus fused to a bifuran. A pentanone structure is attached to coumarin nucleus in case of aflatoxin of B series. This is substituted by a six membered lactone in aflatoxins of G series. According to Wogan, et al. (1971), the furofuran ring attached to the coumarin nucleus was essential for toxic and carcinogenic responses. Compounds lacking the furofuran portion were observed to be inactive (Ayres, et al., 1971).

**Metabolism and Biosynthesis of Aflatoxin:**

**Metabolism:** Metabolism by *A. flavus* group may be characterized as primary and secondary metabolites. Primary metabolites are essential for the growth and multiplication of the fungi, e.g. protein, nucleic acid, carbohydrates, lipids, etc. while the secondary metabolites are produced at the exponential growth phase of the fungi. They are antibiotics, quinones, mycotoxins, etc. Some of these secondary metabolites may have beneficial, and some may have harmful effects on man.

**Biosynthesis:** The precursors and intermediates in biosynthesis of aflatoxin by the *Aspergillus flavus* and *Aspergillus parasiticus* have been reported in the literature. The intermediate metabolites were sterigmatocystin, averufin, norsolorinic acid by
A. parasiticus (Bennett, et al., 1980) and averufanin, asperentin, o-methyl sterigmatocystin etc. by A. flavus (Grove, 1972).

Several pathways have been proposed for aflatoxin biosynthesis by Maggon, et al. (1977); Gupta, et al. (1977); Hsieh, et al. (1976), etc.

Regulation of Aflatoxin Biosynthesis:

The genetic and environmental factors influence the production of aflatoxin by A. flavus and A. parasiticus strains. The biosynthesis of aflatoxins are regulated by the products of primary metabolism of the moulds. Trace elements, especially zinc, have been shown to influence the aflatoxin production on synthetic media (Reddy, et al., 1971) as well as on agricultural products such as maize, soyabean, coconut, etc. (Lillehoj, et al., 1974). Sugars like sucrose, glucose, fructose, mannose, etc. induced aflatoxin production in a peptone basal medium (Abdollahi and Buchanan, 1981).

Methods to Screen Aflatoxigenic Strain

Fungi Producing Aflatoxin:

The aflatoxins are a family of structurally related, highly toxic and carcinogenic mycotoxin produced by A. flavus Link ex Fries (Borut and Joffe, 1965; Diener and Davis, 1969; Stoloff, 1980), and A. parasiticus Speare (Bennett and Goldblatt, 1973; Stoloff, 1930).

Screening Methods for Aflatoxigenic Strain: To detect aflatoxin-producing moulds, a number of screening methods have been developed.
(i) Physicochemical assay.

(ii) Direct application of a small agar plug from a pure mould culture on the TLC plates to observe fluorescence (Filtenborg and Frisvad, 1980).

(iii) To detect the fluorescent of aflatoxin in (a) liquid culture (Lee and Townsley, 1968) or (b) on agar plates (de Vogel, et al., 1965; Hara, et al., 1974; Davis, et al., 1987).

Many liquid media (Mateles and Adye, 1965) and complex synthetic media (Adye and Mateles, 1964; Davies, et al., 1967; Reddy, et al., 1971; 1979; Payne, 1983) have been devised to detect aflatoxin production by toxigenic strain of *A. flavus* and *A. parasiticus*. However, these methods are time consuming and tedious. Hara, et al. (1974), Davies, et al. (1987); Wei, et al. (1984) have developed a simple and quicker medium to screen large number of toxigenic strains of *A. flavus* and related group.

Bothast and Fennell (1974) developed Aspergillus Differential Medium (ADM) to differentiate between *A. flavus* and related group from the other species of *Aspergillus* by producing a bright yellow orange reverse pigmentation and non-sporulating colonies when incubated at 28°C for 3 days. This medium was modified by Hamsa and Ayres (1977) and was again modified by Pitt, et al. (1983) for rapid detection of *A. flavus* and *A. parasiticus* and termed as *Aspergillus Flavus Parasiticus* Agar (AFPA) medium. This medium has been incorporated with peptone and ferric ammonium citrate instead of tryptone and ferric citrate used for *Aspergillus Differential Medium*
(ADM). The persistent orange, yellow reverse pigmentation was observed at temperature of 30±1°C after 42 hrs incubation.

The Cz agar slant containing 0.05% anisaldehyde has been considered as a key medium to differentiate between toxigenic and non-toxogenic strains when inoculated with conidia of A. flavus group and incubated at 30°C, the conidia of toxigenic strain becomes pink in color when observed daily for a month (Wei, et al., 1984).

de Vogel, et al. (1965) have described a complex screening medium to identify the toxigenic strain. The toxigenic strain produces a bright blue fluoroscence when inoculated with mycelial plug in the centre of the plate after 48-72 hrs incubation.

Hara, et al. (1974) developed Aflatoxin Producing Ability (APA) medium for the qualitative study of toxigenic strain. The APA medium contains corn steep liquor, mercuric chloride and ammonium dihydrogen phosphate. The strain was inoculated in the centre of the agar medium and the plates were incubated at 28°C in the dark and observed through 7th to 10th day for the presence or absence of the blue fluorescent surrounding the colony. The intensity of the fluorescent was determined subjectively and the toxins were extracted with chloroform and processed for TLC.

Lin and Dianese (1976) reported a Coconut Agar Medium (CAM) which produces reverse orange yellow pigmentation and blue fluorescent surrounding the colony when the centre of the agar plate was inoculated with conidia of A. flavus and related group and incubated upside down at 28°C for a week. CAM produced an orange
yellow pigmentation prior to the appearance of blue fluorescence. Thus, visual observation of orange yellow pigmentation suggests the toxicity of the strain. The characteristic blue fluorescence surrounding the colony was observed under long wave U.V. This CAM was improved by Davis, et al. (1987). They obtained excellent results in 2 to 5 days when incubated at 28°C.

Yabe, et al. (1987) have developed a simple screening method for aflatoxin-producing moulds by UV photography. The toxigenic moulds were identified as grey or black colonies while non-toxic strain as white under UV photographs.

Yabe, et al. (1988) developed a tip culture method which analyses aflatoxin quantitatively or semi-quantitatively.

**Estimation of Aflatoxin by Different Methods**

Aflatoxins can be estimated by either of the following methods -

(i) Physicochemical assay
(ii) Biological assay
(iii) Immunological assay

Societies like Association of Official Analytical Chemists (AOAC), American Oil Chemists Society (AOCS), American Association of Cereal Chemists (AACC) and the International Union of Pure and Applied Chemistry (IUPAC) have developed analytical methods. The methods developed by AOAC have been adopted by FDA and Governments of various countries. AOAC has evaluated two official first action methods for aflatoxin analysis - Contaminated Batch (CB) and
Best Food (BF) method. These methods have been confirmed for their reliability.

(i) Physiochemical Assay:

(1) **Sampling:** The environmental mycoflora and their mycotoxins can be collected by different methods which may be qualitative (Petriplate exposure method), quantitative (Midget impinger and Andersen-6-stage viable sampler), or gravimetric method (Cone sampler for total dust and Hexhlet sampler for respirable dust).

(2) **Extraction:** The extraction procedure differs according to the sampling technique and commodity. The extraction was performed by using different solvents either alone or in combination, and it may be in single-phase or in multi-phase system, e.g. methanol (Sargent, et al., 1961; Tropical Product Institute [T.P.I.] 1965; Thean, et al., 1980); chloroform (de Iongh, et al., 1964; Pennington, 1986); methanol: water: hexane (Nesheim, et al., 1964); chloroform: water (Eppley, 1966; Kamimura, et al., 1985), etc.

(3) **Precipitation:** Precipitation step was applied to remove the interfering substances like protein, pigments, residual salts, etc. Precipitation was usually done with lead acetate or by ammonium sulphate (AOAC, 1975a).

(4) **Clean up:** Interfering substances like lipids, carbohydrates and pigments were removed by adopting clean up procedure. Initially, different liquid partition systems have been employed, viz. methanol: water: petroleum ether (Coomes and Sanders, 1963); methanol: water: chloroform and methanol: water: hexane (T.P.I.,
This was further replaced by column chromatographic system using neutral alumina, celite, florisil etc. Silica gel column for chromatographic technique was proposed by Pons, et al. (1968). The other clean up procedure developed were Sep-pak cartridges (Thean, et al., 1980); silica Sep-paks (Kozloski, 1986); Hyflo Super Gel with two partitions to chloroform (Soares and Rodriguez-Amaya, 1989) etc.

(5) Separation:

(a) Thin Layer Chromatography (TLC): Aflatoxins in purified extracts were separated by TLC (Shannon, et al. 1983; Pennington, 1986; Soares and Rodriguez-Amaya, 1989). The first chromatographic separation of aflatoxins was proposed simultaneously by Coomes and Sanders (1963) and Broadbent, et al. (1963). Broadbent, et al. (1963) used glass plates coated with neutral alumina and chloroform: methanol: as solvent system. de Iongh, et al (1962; 1964) were the first to use silica gel (Kiesel gel G) coated TLC plates and chloroform: methanol as the solvent system. Subsequently, various aflatoxin assay procedures using different solvent systems have been developed by T.P.I. (1965); AOAC (1975b) and others.

The aflatoxin in corn dust was measured by Shotwell, et al. (1981a) with 1 or 2-dimensional TLC. The limit of aflatoxin detection was about 9 ng/g for 0.1 g samples. Kamimura, et al. (1985), Kozlaski (1986) used High Performance Thin Layer Chromatography (HPTLC) to detect aflatoxin in different commodities.

Aflatoxin can be detected upto 0.05 ppb level in foods and
feeds by TLC. It is less time consuming and widely accepted procedure till today.

(b) **High Pressure Liquid Chromatography (HPLC):** HPLC is an accurate method for resolution and quantitation of aflatoxin. Pons, Jr., 1979 quantitated aflatoxin by normal phase HPLC and Takahashi, 1977; Haghighi, et al., 1981 by reverse phase HPLC. HPLC has been accepted as official method for aflatoxin analysis (AOAC, 1980). Rapid HPLC method to detect aflatoxin in corn and peanut has been developed by De Vries and Chang (1982). This method was almost equivalent to CB method.

The HPLC assay method is sensitive to detect the aflatoxin at the level of 1-2 ng/g of aflatoxin B$_1$, B$_2$, G$_1$ and G$_2$.

(c) **Liquid Chromatography (LC):** LC comprise of a normal phase column with a silica gel packed flow cell for fluorescence detection (Panalaks and Scott, 1977) and trifluoroacetic acid (TFA) derivatization of the toxins and a reverse phase column with fluorescence detection (Tarter, et al., 1984) and post column derivatization with iodine in water. Park, et al (1990) studied TFA derivatization of the toxins and a reverse phase column with fluorescence detection. This has been adopted as official first action method to detect aflatoxin level at 13 ng of total aflatoxin/g in corn and peanut butter.

(6) **Quantitation:** After separation of Aflatoxin, they can be quantitatively measured on the basis of their fluorescent intensity.

(a) **Visual comparisons:** Aflatoxins were diluted for its
assay (Andrellos and Reid, 1964), and quantitate with visual comparison of intensity of the fluorescent on TLC plates using standards (Pons, Jr., et al., 1966). However, this method was not precise, and varied considerably (Beckwith and Stoloff, 1968). It is regarded as a semi-quantitative estimation.

(b) **Spectrophotometric Measurement:** In this method, the absorbance of extinction coefficient of aflatoxin was measured in methanol at 363 nm. Aflatoxin B₁ was detected at the levels of 2.5 to 12.5 ng/kg in groundnut (Nabney and Nesbitt, 1965).

(c) **Fluorodensitometric Measurement:** Kamimura, et al. (1985) quantitated aflatoxin by fluorodensitometric measurement method and reported it to be more accurate, sensitive and precise. Dickens, et al. (1980) have developed densitometric equipment for rapid quantitation of aflatoxin on TLC plates.

(d) **Fluorometric Measurement:** In this method the acid treatment of aqueous solution of aflatoxin leads to chemical transformations which gives excellent stability and higher intensity of fluorescent (Scoppa and Marafante, 1971). The detection limit of aflatoxin was of 1 ng (Velasco and Whitaker, 1975).

(e) **Laser-induced Fluorescence Analysis:** Comparatively this is a newer technique for aflatoxin assay. Diebold, et al. (1979) detected aflatoxin contamination at the level of 0.1 ug/kg in corn using TLC and HPLC followed with laser-fluorometry.

(7) **Confirmatory Test:** The detection of toxin needs confirmatory test for its identification as some other compounds may have
identical fluorescence spectra. Confirmatory test was developed by Andrellos and Reid (1964). Later, the method was modified by Pohland, et al. (1970) and the modified procedure was adopted as official first action method. Przybylski (1975) modified again this method to make it more specific by converting AFB\textsubscript{1} and AFG\textsubscript{1} into their respective hemiacetate AFB\textsubscript{2a} and AFG\textsubscript{2a} by adding trifluoroacetic acid directly on TLC plates. This improved method was adopted as official first action method.

a) Crisan and Mazzucca (1967) suggested a test based on the formation of oximes and 2,4-dinitrophenylhydrazine with the carbonyl group in cyclopentenone ring of AFB\textsubscript{1} and AFB\textsubscript{2}.

b) Ashoor and Chu (1975) demonstrated reduction of AFB\textsubscript{1} and AFB\textsubscript{2} with sodium borohydride and quantitatively yielded new fluorescent which had higher R\textsubscript{f} value.

c) Mucke and Keirmeir (1971) reported a di-O-anisidinetetrazolium chloride with AFB\textsubscript{1} and AFB\textsubscript{2} yields blue-violet product with AFG\textsubscript{1} and a brown colored product with AFG\textsubscript{2}. The detection limits of toxins were 0.03 ug and 0.09 ug respectively.

d) Haddon, et al. (1977) have reported two methods of Mass spectral confirmation of aflatoxin.

e) Davis and Diener (1980a) used iodine derivative for AFB\textsubscript{1} in HPLC analysis. The AFB\textsubscript{1} were detected at a level of 0.2 ng.
(ii) Bioassay:

The aflatoxin can be assayed by biological systems based on the histopathological changes in their organs. A number of biological assay systems have been proposed, like chicken embryo (AOAC, 1975h); duckling (Newberne and Butler, 1969); rainbow trout (Ciegler, et al., 1981); micro-organisms (Reiss, 1975); cell culture (Legator and Withrow, 1964); rats and mice (Dickens and Jones, 1965); guinea pigs (Butler, 1966), etc. But the bioassays are less specific, less sensitive and more time consuming than the physico-chemical assays.

(iii) Immunological Assay:

Recently, the development of immunological methods like Radio-Immuno Assay (RIA) and Enzyme Linked Immunosorbent Assay (ELISA) for the detection of aflatoxin against specific antibody are more dependable, sensitive, specific, simple and economic. Both the techniques are based on the competition of binding between the unlabelled and the labelled toxins in the assay system for the specific binding sites of antibody molecules.

Toxins are non-immunogenic. Therefore, toxins are first conjugated to a protein or a polypeptide carrier, which are used for immunization. Using this approach, the antibody was produced against aflatoxin by Chu (1983; 1984a,b). The animal, especially rabbit, was immunized with aflatoxin-ovine serum albumin conjugate (afla.B₁-BSA) to produce specific antibody against AFB₁. Gaur, et al. (1980) have tested goat and rabbit antiserum and observed
rabbit's antiserum was superior to test both qualitatively and quantitatively.

A number of immuno-assays have been described for \( \text{AFB}_1 \) - Polyclonal antibodies (Pestka, et al. 1980; El-Nakib, et al., 1981; Pestka and Chu, 1984; Ram, et al., 1986); Monoclonal antibodies (Groopman, et al. 1985; Lubet, et al., 1983; Sun, et al., 1983); Monoclonal Affinity Chromatography (Ramakrishna, et al., 1990); High Affinity Monoclonal antibody (Kaveri, et al., 1987); AFB-DNA adducts (Groopman, et al., 1985 and Haugen, et al., 1981).

**Radio-Immuno Assay:**

In RIA method, radioactive iodine (\( ^{135} \text{I} \)) is tagged and emits gamma-radiation as it decays. The amount of radioactive emitted is measured by a scintillation counter.

There are different types of RIA methods, e.g. ammonium sulphate precipitation method (Lee and Chu, 1981); double antibody technique (Langone and Vunakis, 1976); a solid phase RIA method (Sun and Chu, 1977); a dextran coated charcoal column (Thorpe and Yang, 1979), etc. Langone and Vunakis (1976) detected \( \text{AFB}_1 \) by RIA assay at the level of 0.06 ng.

**Enzyme linked Immunosorbent Assay:**

Aflatoxins can be detected by Direct Competitive ELISA or by Indirect Competitive ELISA method. Both types are heterogenous competitive assays.

1. **Direct competitive ELISA:** For direct competitive
technique, aflatoxin-enzyme conjugate was used (Biermann and Terplan, 1980; Pestka, et al., 1980; 1981a,b,c; 1982b). The specific antibody was first coated to a solid phase, i.e. microtiter plate which can rapidly quantitate AFB₁ at level of 25 pg per assay by the gluteraldehyde method (Pestka, et al., 1980) or by a direct coating method (Biermann and Terplan, 1980; Lawellin, et al., 1977). Other solid phases induced nylon beads and terisaki plates were applied by Pestka and Chu (1984).

The sample solution or the standard toxin was incubated simultaneously with enzyme conjugate (Pestka, et al., 1980; 1981b) or incubated separately in two steps as described by Biermann and Terplan (1980). After washing, the amount of enzyme bound to the solid phase was then determined by incubation with a substrate containing hydrogen peroxide and appropriate oxidizable chromogen. The resulting color was measured spectrophotometrically or by visual comparison with the standards.

Ram, et al. (1986) estimated the contaminated corn samples for AFB₁ by a direct competitive ELISA. The AFB₁ estimated in corn by this method was compared with TLC (CB Method) and the correlation coefficient was found to be 0.95. The AFB₁ in contaminated cottonseed samples estimated by ELISA method was compared with liquid chromatography (Pons Method) and the correlation coefficient was found to be 0.96.

(2) Indirect Competitive ELISA: In indirect competitive ELISA method, instead of aflatoxin-enzyme conjugate as used in
direct competitive ELISA which may pose an enzyme stability problem, a aflatoxin-protein (or polypeptide) conjugate was first prepared, then it was coated to the microplate before assay. In the presence or absence of the homologous aflatoxin, the plate was incubated with specific rabbit antibody. The amount of antibody bound to the plate coated with aflatoxin-protein conjugate was then determined by reaction with goat antirabbit IgG-enzyme complex by subsequent reaction with the substrate, Horseradish-peroxidase (HRP), and alkaline phosxphatase (Sashidhar and Narasinga Rao, 1988) conjugate for goat antirabbit-IgG were usually used.

The efficiency of Indirect ELISA has been tested for AFB by Fan, et al. (1984) and Martin, et al. (1984). The sensitivity of the indirect ELISA was comparable or slightly better than the direct ELISA technique. The advantage was that much less antibody was required in this technique. Recovery of about 80-97% has been obtained in corn meal and peanut butter added with AFB at level of 5 to 40 ppb (Fan, et al., 1984).

ELISA method has several advantages over other methods. It shows recoveries of about 30 to 50% higher than those obtained by TLC. Cleanup step was not necessary (Chu, et al., 1988). As low as 2-5 pg of pure toxin has been detected by ELISA (Pestka, et al., 1981a). Sensitivity of ELISA is approximately 10-100 times more than RIA method. The major advantage of ELISA over RIA method is that the radioactive substances are not used. All these reasons have led to an increased tendency of using ELISA (Zhu, et al. 1987; Autrup, 1990).
ELISA has been adopted as official first action by AOAC for screening AFB$_1$ at level of $\geq$15 ng/g in cottonseed products and mixed feed, and for corn and roasted peanut product at level of $\geq$20 ng/g (Park 1989).

Nowadays commercial ELISA kits like Biokits Total Aflatoxin Kits, Cambridge Life Sciences Aflasure B Kit, and May & Baker Quantitox B$_1$ Kit are available, and these kits have been tested for their specificity by 16 different Laboratories (Patey, et al., 1989).

**Fluorometric Iodine Method (FL-I-Method):**

The method as described by AOAC (1975a) based on addition of the aqueous aflatoxin solution and the fluorescence was determined for AFB$_1$ and AFG$_1$ (Davis and Diener, 1979).

**Improved Fluorometric-Iodine (FL-IM) Method:** This method is faster, more reliable and simple compared to FL-I-Method (Davis, et al., 1981).

**Screening Tests for Aflatoxin:**

Presumptive tests or screening test suggests the presence or absence of aflatoxin in agricultural commodities. The advantage is to study large number of samples in a short time. Several screening methods have been developed, e.g. Bright Greenish Yellow Fluorescence (TEGR) Method (Shotwell and Hesseltine, 1981b); Mini Column Method (Holaday and Lansden, 1975); Fluorometric Iodine Rapid Screen Method (FL-IRS Method) (Davis and Diener, 1979) and Fluorometric Analysis of Peanuts (FLAP) Method (Davis and Diener, 1980b).
Methods for Individual Commodities:

The AOAC has developed methods available for specific agricultural commodities like corn (AOAC, 1975e); groundnut (AOAC, 1975c,d); cottonseed (AOAC, 1975f); soyabean (AOAC, 1975g); coconut, cocoa beans (AOAC, 1975e); green coffee, pistachio nuts (AOAC, 1975g); dairy products like liquid milk, powdered milk, cheese, butter (AOAC, 1975i), etc.

Toxicity Due to Aflatoxin

Health hazards induced by aflatoxins are mainly caused by alimentary exposure or inhalation of airborne dust containing aflatoxin. They cause aflatoxicosis which may have acute or chronic effects on animals and human beings. There were evidences available on aflatoxin as a causative agent in human primary liver cancer (IARC, 1976; 1983; Linsell and Peers, 1977; Shank, 1977). The first evidence of aflatoxin being a carcinogenic was established by Lancaster, et al. (1961) when they reported multiple liver tumors in rats.

Dietary Intake and Level of Aflatoxin:

The contamination of a staple food is an important problem associated with low level dietary intake of aflatoxin for a long period which may result in chronic effects, and high level of aflatoxin consumed for a short period may lead to acute effects. The acute effects of aflatoxin following exposure to minute levels have been studied in experimental animals.
Studies in Animals:

The acute toxicity of aflatoxin varies with different species. The LD$_{50}$ dose of aflatoxin ranges from 0.3 to 17.9 mg/kg body weight which may result in cancer of liver, biliary proliferation, etc. or even death. Carnaghan, et al. (1966) fed chicken with 1.5 mg/kg of aflatoxin, noticed liver lesions like fatty changes, biliary proliferation and fibrosis after 4 weeks. Newberne and Butler (1969) fed rabbit, duckling, cat, pig, dog, sheep, guineapig, rats, mouse and hamster with varying doses of aflatoxin and observed liver lesions in these animals.

Aflatoxin B$_1$ at levels of 7.2 and 17.9 mg/kg body weight for males and females respectively appear to cause bilateral adrenal haemorrhages, petechial haemorrhages in many organs, particularly in the congested lungs and occasionally patchy necrosis in the myocardium and in kidney and spleen. Newberne and Wogan (1968) studied the carcinogenicity of aflatoxin towards males and females in rats and observed that males were more susceptible than females.

Wogan and Newberne (1967); and Wogan (1973) observed that 1.0 mg/kg body weight of aflatoxin B$_1$ resulted in the incidence of liver tumor in rats and mouse. In pigs, during a normal feeding period of 3-4 months, 300 ug/kg of aflatoxin resulted in the development of centrilobular necrosis and fibrosis of liver, growth depression (Krogh, et al., 1973). The Indian dogs fed mouldy corn had acute toxic hepatitis (FAO., 1979).

Effects of Aflatoxin in Humanbeings:

The degree of morbidity produced by aflatoxin in human-
beings depends upon the level of aflatoxin present in foods and its intake resulting either in acute or chronic effects.

(1) **Acute effects:** High level (mg/kg) ingestion of aflatoxin results in acute single or short-term effects.

 a) **Acute liver disease:** An epidemic of fatal hepatitis in India in 1974, reported the death of about 100 people due to consumption of mouldy corn. Microscopic examination revealed extensive bile duct proliferation with periductal fibrosis and cholestasis (Krishnamachari, et al. 1975a,b). Later, Tandon, et al. (1977) observed that the disease had a subacute onset of fever followed by jaundice and ascites or hepatosplenomegaly, vomiting.

 Ling, et al. (1967) reported death of 3 children from Taiwan due to intoxication caused by consumption of black and mouldy rice containing AFB₁ up to 200 and 7700 ug/kg. The acute necrosis was observed in the liver sample. Another report from Uganda mentioned a 15 year old boy who developed abdominal pain and died within 2 days due to aflatoxin poisoning (Serck-Hansen, 1970). Ngindu, et al. (1982) reported the outbreak of acute hepatitis in Kenya where 12, out of 20 patients, died of hepatitis due to consumption of maize containing 12,000 ppb of aflatoxin. Liver tissue contained 89 ppb AFB₁. Surveys have established a positive correlation between the prevalence of acute liver disease and the predominantly high levels of aflatoxin in food from the same geographic region.

 b) **Reye's Syndrome:** The Reye's syndrome (Encephalopathy and Fatty Degeneration of Viscera [EFDV]) and its association
with aflatoxin have been established by Bourgeois, et al. (1971) and Becroft and Webster (1972). Children were found to be more prone to Reye's syndrome. Bourgeois, et al. (1969) and Olson, et al. (1971) reported Reye's syndrome in a Thai boy who consumed leftover cooked rice containing 10 mg/kg aflatoxin for 2 consecutive days. He suffered from fever, vomiting, coma, convulsion and ultimately died.

In Thailand, the high incidence of Reye's syndrome and liver cancer have been observed. Shank, et al. (1972b) reported Reye's syndrome in 22 out of 23 autopsy specimens from Thai children. Aflatoxin $B_1$ and $B_2$ at levels of 1 and 4 ug/kg were found in the specimens. From New Zealand, Becroft and Webster (1972) observed aflatoxin $B_1$ and $G_1$ in liver extracts of 2 patients suffering from Reye's syndrome. Dvorachová, et al. (1977) reported the death of children with EFDV syndrome in Czechoslovakia. Chaves-Carbello, et al. (1976) and Hogan, et al. (1978) found aflatoxin in liver of the patient suffering from Reye's syndrome.

(ii) Chronic Effects: The low level of aflatoxin (ug/kg) exposure to man for a long period results in chronic effect.

(a) Liver Cancer: In survey conducted in Uganda by Alpert, et al. (1968), they reported a strong association of aflatoxin intake and human liver cancer. Kraybill and Shimkin (1964) and Alpert and Davidson (1969) had suggested that dietary aflatoxin may be a causative factor in human cancer. Alpert, et al. (1971) found aflatoxin at detectable level in food (10.8-43%) and associated it with increased incidence of primary liver cancer (range 1.4-15.0
cases/100,000 total population/year). Peers and Linsell (1973) studied the relationship between aflatoxin intake and liver cancer incidence in Kenya. They observed males were more susceptible than females. Similar observations were made by Shank (1977). Peers, et al. (1977) carried out a study at Swallzand and reported the high aflatoxin intake related to the incidence of liver cancer. Phillips, et al. (1976) analysed urine of the person suffering from carcinoma of the liver and rectum and detected 520 ug/kg of aflatoxin B₁. Keen and Martin (1971) observed regional differences in liver cancer and also reported differences in levels of aflatoxin in different areas of Swaziland. Van Rensburg, et al. (1974) observed a relationship between primary liver cancer rate and aflatoxin intake in a high risk cancer area of Mozambique.

(b) Indian Childhood Cirrhosis (ICC): The incidence of Indian Childhood Cirrhosis was found to be associated with aflatoxin intake in infants. It was reported to be characterized by fatty infiltration of liver cells with degeneration, fibrosis and hepatomegaly, and in advanced stage it proceeded to jaundice, ascites and hepatic coma (Sen, 1987). The children exposed to aflatoxin through breast milk and dietary items may develop ICC (Amla, et al., 1970; 1974). The children suffering from Kwashiorkor were supplemented with protein diet of groundnuts that contained 300 ug/kg aflatoxin. Out of 18, 16 children developed hepatomegaly. Liver biopsies revealed lesions comparable to ICC (Amla, et al., 1971).

Yadgirli, et al. (1970) observed resemblance of the compound to aflatoxin isolated from urine and liver extracts of children suffering from ICC.
Toxicity Screening of Dust Samples by Microtox™

Various biological methods and assays have been developed for determining toxicity using algae, protozoa, fish, etc. ((Bringman and Kuhn, 1980; Marking and Kimule, 1974). All these bioassays are time consuming, expensive and require extensive preparations. Due to the increasing concern regarding the long-term effects of an ever-increasing number of chemicals in the environment, there is a need for simple, inexpensive, rapid and sensitive test for toxicity testing. The use of micro-organisms, especially bacteria as the assay agent, offers an opportunity to meet all the challenges.

Bulich and Isenberg (1980) have described a bacterial test system for determining toxicity by Microtox™. The toxicity screening is based on monitoring changes in natural light emission from the luminescent bacteria (*Photobacterium phosphoreum*) when challenged with a toxic agent. These bacteria have the capacity to emit visible blue light at 470 nm (Terpstra, 1963) and respond to the toxic substances by the loss of luminance output, which is indicative of metabolic inhibition. The toxicity endpoint can be measured as the effective concentration of a test sample that causes a 50% decrease in light output ($EC_{50}$). The toxicity screening using Microtox™ have been processed and compared with other conventional bioassays for monitoring the toxicity of a wide variety of chemicals (Dutka and Kwan, 1981; Qureshi, et al., 1982), complex effluent (Bulich, et al., 1981), distilled and tap water, pesticides (Chang, et al., 1981), mycotoxins (Yates and Porter, 1982).