Environment Conservation Journal 9 (1&2) 111-127, 2008
(ISSN 0972-3099)

Recent progress in Aflatoxin analysis and their inactivation

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Abstract

Aflatoxin is the secondary metabolite of low molecular weight produced by naturally occurring fungi mainly *Aspergillus flavus* and *Aspergillus parasiticus*. Rapid progress in the area of aflatoxin analysis and detoxification method has been made during the last few years. Simplified method, cleanup protocol and chromatographic methods have been continuously developing to make these methods more sensitive and reliable. In this review we are discussing different aflatoxin analysis methods as well as different methods of aflatoxin detoxification.

Keywords : *Aflatoxin, Aspergillus flavus, Aspergillus parasiticus*

Introduction

Cereals, especially maize and groundnut are the major sources of carbohydrates and proteins in Asia and are important as export products in some Asian countries. Grain quality is critically monitored to meet international standards in export. Aflatoxin development in many stored cereal grains has constantly hampered the availability of good quality grains in Asian countries. The most important group of toxigenic Aspergilli are the Aflatoxigenic molds, *Aspergillus flavus, Aspergillus parasiticus* and the recently described but much less common species *Aspergillus nomius* all of which are classified in Aspergillus section Flavi (Gams *et al.*, 1985). Although these three species are closely related and share many similarities, a number of characteristics may be used in their differentiation. *A. flavus* is widely distributed in nature but *A. parasiticus* is less wide spread, the actual extent of its occurrence being complicated by the tendency for both the species reported indiscriminately as *A. flavus*.

A. flavus Link ex Fries and *A. parasiticus* Speare have been identified as the major pests that produce aflatoxin and deter the quality of grain when stored. They especially affect oilseed. Edible nuts and cereals in subtropical regions throughout the world are spoiled due to inadequate storage conditions. The main cause of disease in the human is their secondary metabolite i.e. Aflatoxins which are carcinogenic and result in liver cancer. Liver cancer takes time to develop but the aflatoxin acts as an immunosuppressant so that affected individuals become susceptible to wide range of diseases. In the last decade, aflatoxin levels on product were found to exceed an acceptable level limit of 20 ppb stipulated in most export specifications. Aflatoxin contamination has affected maize, groundnuts, peanuts and cottonseeds in several countries including Thailand and India; and coconut in the Philippines, Sri Lanka and other Pacific countries. Aflatoxicosis, both in humans and animals, has been more prevalent in areas where maize and groundnut constitute a major part of the diet. Many private and government organizations have embarked on aflatoxin research. However, aflatoxin control is still an intricate problem.

Aflatoxins have been widely detected in cereals, oilseeds, fermented beverages and milk which form basic human diet (Bilgrami *et al.*, 1983; Bullerman, 1986). Maize has been reported to be the most susceptible followed by groundnut to aflatoxin contamination. Bhat *et al.* (1997) reported that about 26% maize and 21% of groundnut samples collected from 11 states exceed the permissible limit of 30ppb aflatoxin per kg of material. Prevention of Food adulteration act (1954), amended in 1986, U.S. Food and Drug Administration (FDA) and the Codex Alimentarius Commission (1989) have recommended a permissible limit of aflatoxin of 30ppb/kg, 20ppb/kg and 5ppb/kg respectively.

A survey from thirty countries concluded that aflatoxins are the leading toxins present in agricultural commodities, compared to the presence of other mycotoxins (Hesseltine, 1986). Aflatoxin in animal feed presents two problems; first deleterious effect on the health of the animal ingesting the contaminated feed and on the health of humans consuming aflatoxin residue in foods derived from such animals. There is great variation among strains of *A. flavus* in the quantity of aflatoxins produced (Cotty, 1989), this quantity is independent of a strain's ability to infect and colonize developing cottonseed. Simultaneous inoculation of wounded 28-to-32-day-old cotton bolls with toxigenic and aflatoxigenic strains of *A. flavus* led to lower levels of aflatoxin B_1 in the cottonseed at maturity than in bolls inoculated with the toxigenic strain alone. Less B_1 was detected when the aflatoxigenic strain was introduced into the wound 1 day before inoculation with a toxigenic strain than when atoxigenic and toxigenic strains were co-inoculated (Cotty, 1990). In this review we are discussing some important methods for analyzing aflatoxin and methods of inactivation by physical, chemical as well as biological means.

1.0 Sampling and sample preparation for aflatoxin analysis

Sampling is important and usually the largest source of error. This is because a small percentage of the kernels and contaminated kernels have high level of Aflatoxin and other fungal toxin (Whitakar and Wiser, 1969). With small samples, it is difficult to get contaminated kernel in the sample. Studies have shown that less than 1 kernel per 1000 (0.1%) is contaminated in a lot or raw shelled peanuts. The sample has to be selected in such a way that every kernel in the lot has an equal chance of being chosen. The sample should be the accumulation of many small incremental portions taken at many different locations throughout the lot (Whitaker, 2000). The sample is ground in mill. The kernels are broken into many small pieces. Then a small sub sample of ground product is removed from the sample. The aflatoxin and other fungal toxin in replicated subsample will also vary about the sample concentration. Sample error is reduced by increasing sub-sample size and grinding the sample into finer particles.

1.1 Detection of Aflatoxin in food commodities

There are various methods for determination of aflatoxin in food commodities. Some of the important methods are:

A. Thin Layer Chromatography (TLC)

Aflatoxin has fluorescent properties i.e. Aflatoxin B_1 and B_2 give blue and Aflatoxin G_1 and G_2 give green fluorescence under U.V. light at 360 nm. This property of fluorescence has been utilized for the detection of different types of aflatoxin by TLC. The first thin layer chromatographic (TLC) separations of aflatoxins were proposed simultaneously by Coomes and Sanders (1963). They used paper chromatography and reported that the system did not resolve aflatoxins B_1 and B_2, the least detectable amount of B ($B_1 + B_2$)

was about 0.2 µg. Broadbent *et al.* (1963) used glass plates coated with neutral alumina and reported that B_1 and B_2 were not resolved but as little as 6×10^{-3} µg of B could be detected, about a 30 fold increase in sensitivity over that attainable with paper chromatography for development. The two dimensional thin layer chromatography was proposed by Peterson and Ciegler (1967) to yield improved separation of aflatoxins from impurities in plant extract and for better separation of aflatoxin B_2 from G_2. Neshiem (1968) has investigated the effect of variations in developing solvents, commercial silica gels, calcium sulfate binders, gel thickness, humidity and vapor phase composition, all of which influence the separation of aflatoxins in thin layer chromatography. TLC method has detection limit of 50-100 ppb. In TLC method compare the fluorescence intensities of the spots at the Rf of B_1 in the sample with those of the B_1 standard spots and determine which of the sample spots matches one of the standards and record the corresponding aliquot volumes. If the sample spot intensity lies between two adjacent standard spots the average liquor volume of the standard spots is recorded. If the spots of the smallest volume of sample are too intense to match the standards the sample extract should be diluted and re-chromatographed. Stroka and Anklam (2000) developed miniaturized and low power consuming detector cell for the densitometeric measurement of aflatoxin on TLC plates. A UV-light emitting diode (UV-LED) with a peak emission wavelength of 370nm was used for the fluorescence excitation, while photo diode with peak sensitivity of 440 nm in combination with a 418 nm cut off filter was applied for detecting the fluorescence intensity. The resulting signal was further amplified by means of commonly used operational amplifier integrated circuit (OA) and directly converted into digital signal with simple analogue-digital converter (ADC). This signal was recorded at the serial (RS232) port of a portable PC and processed with spreadsheet program.

HPTLC-ELISA involves the separation of Mycotoxins in HPTLC, followed by blotting the chromatogram to nitrocellulose membrane coated with antibody, incubation with mycotoxin enzyme conjugate and finally incubation with substrate to develop the color. The only disadvantage of this technique is use of large amount of antibody.

B. High Performance Liquid Chromatography (HPLC)

The development of highly automated HPLC systems has afforded very precise, selective and sensitive quantification techniques for aflatoxin analysis. HPLC methods have been developed using both normal and reverse phase systems in conjunction with UV adsorption and fluorescence detection techniques. Reverse phase HPLC separations of aflatoxins are more widely used than normal-phase separations. Aflatoxin analysis using HPLC for separation and detection is quite similar to TLC because similar sampling and extraction procedures are used. The major advantages of HPLC over TLC are speed, automation, improved accuracy and precision. Both normal-phase and reverse-phase HPLC separations have been developed for aflatoxin analysis. Early experimental work by Seitz (1975) on HPLC separations revealed that aflatoxins could be separated on normal-phase columns and detected with either a UV detector or a fluorescence detector. Seitz (1975) noted that the fluorescence detector had limited usefulness for aflatoxin B_1 and B_2 with normal phase separations. Panalaks and Scott (1977) developed a silica-gel packed flow cell for fluorometric detection of B_1, B_2, G_1, and G_2 with normal phase aflatoxin separations. A silica-gel packed cell was used by Pons (1979) and Thean *et al.* (1980) in two different HPLC methods for determination of aflatoxins. The major disadvantage of the packed cell is lack of stability. The cell needs to be repacked often and the detector signal weakens with time. The advantages of a packed cell

method are that no derivative is necessary for detection and the mobile phase can be recycled. Reverse-phase HPLC separations of aflatoxins are more widely used than normal-phase HPLC separations. However, the fluorescence intensities of B_1 and G_1 are diminished in reverse-phase solvent mixtures so the derivatives B and G are diminished in reverse-phase solvent mixtures so the derivatives B and G are generally prepared before injection. Analysts should be aware that derivatives B and G are not stable in methanol, which should be used with caution, especially in the injection solvent. Acetonitrile-water mixtures do not degrade B and G rapidly and are preferred to methanol-water mobile phases. Several reverse-phase methods have been published (Stubblefield and Shotwell, 1977; Hutchins and Hagler, 1983 and Tarter *et al.*, 1984). Stubblefield and Shotwell (1977) found that M_1 and M_2 as well as B_1, B_2, G_1 and G_2 could be resolved and detected with a UV detector at 350 nm using reverse-phase chromatography. The methods developed by Hutchins and Hagler (1983) and Tarter *et al.* (1984) all use TFA derivatization and apparently compare favorably with other methods

According to the procedure described by Sharma and Marquez (2001) a 50gm sample was extracted with 100 ml of methanol-water (60:40 v/v) for 1 minute and then filtered on Whattman Filter paper no. 4. The sample extract was concentrated to a volume of 30ml and was then diluted with 40ml PBS (pH 7.4). It was cleaned up on an immunoaffinity column by gentle syringe pressure at flow rate of 5ml/min and then the column was washed with distilled water (20 ml). Elute from each column containing the analytes were evaporated to dryness under nitrogen in 2 ml vials, the residue were derivatised with hexane/trifluoroacetic acid (300µl each) mixed and incubated for 10 min at 40°C. The solvent were evaporated and finally the residues were dissolved in Acetonitrile and distilled water (60 and 180 µl respectively). Aflatoxins were separated isocratically on Perkin-Elmer HPLC chromatograph, connected to reverse phase C18 column particle size 5 µm, LC-10 flourescence detector (Perkin Elmer) and LCI-100 computing integrator. Measurements were made by peak area. The mobile phase was 60% water, 22% acetonitrile and 18% methanol (filtered through 0.2 µm Millipore filter) at flow rate of 1 ml/min and detection was observed by fluorescence with excitation at 370 nm (λ_{em}) and emission cut off at 418 nm(λ_{em}). Quantification of each toxin was performed by measuring peak areas at their retention time and comparing with their relevant standard calibration curve. The identity of each toxin was confirmed in all the analyzed samples by injecting sequentially sample extract and comparing the peak area ratio with their corresponding standard. HPLC has partly superceded TLC in the analysis of food for mycotoxin. Separation is usually much better than those obtained with one dimensional TLC. HPLC methods generally provide good quantitative information and the equipment employed in HPLC systems is generally automated rather easily.

Otta *et al.* (2000) developed rapid, reproducible and cost effective Over Pressure Layer Chromatography (OPLC) method for quantitative determination of Aflatoxin B_1, B_2, G_1 and G_2 in different foods. Using OPLC one can analyse ten samples simultaneously. Chiavaro *et al.* (2001) studied the effect of succinyl-β-cyclodextrin (β-CD-Su), Dimethyl-β-cyclodextrin (DINEB) and β-cylodextrin (β-Cd) on the fluorescence of B_1, B_2, G_1, G_2 and M_1. β-CD-Su promoted the largest fluorescence enhancement for AFB_1 and AFM_1 while DIMEB showed better result for AFG_1. On the basis of the Fluorescence enhancement, a RP-HPLC method for detecting aflatoxin B_1, B_2, G_1, G_2 and M_1 was developed using cyclodextrins directly dissolved in the LC elutent. Aflatoxin B_1, B_2, G_1 and G_2 were resolved using methanol: water as mobile phase to which β-CD-Su or β-Cd was added. Chromatographic response of AFB_1 and AFG_1 achieved using β-CD dissolved in the mobile phase were enhanced respectively, 8 and 12 times and 10 to 15 times with β-CD-Su. Blesa *et al.* (2003), developed method based on solid-solid phase dispersion (MSPD) extraction to determine

aflatoxin B_1, B_2, G_1 and G_2 from peanut. The method used 2 gm of peanut sample, 2 gm of C18 bonded silica as MSPD sorbent and Acetonitrile as eluting solvent. The limit of quantification ranges from 0.125 to 2.5 ng/g for the four studied aflatoxin using Liquid Chromatography. Calleri *et al.* (2007) developed fully automated HPLC method with fluorescence detection for the determination of AFB_1 in aqueous solution by using anti-aflatoxin B_1 immunoaffinity monolithic disk. Polyclonal anti AFB_1 is covalently immobilized in batch on an epoxy activated monolithic Convective Interaction Media (CIM) disk (12mm x 3mm i.d) by a one step reaction. 0.96 mg of antibody were immobilized. The CIM disk was coupled through a switching valve to reverse-phase column Chromolith Performance RP-18e. The total analysis time with integrated system is 46 min and the retention time of AFB_1 is approx. 29 min.

C. Radio Immuno Assay (RIA)

The RIA involves the incubation of specific antibody simultaneously with unknown sample or known standard and constant amount of labeled toxin. After separation of the free and bound toxin, the radioactivity in those fraction is then determined. The toxin concentration of the unknown sample is determined by compairing the results to a standard curve, which is established by plotting the ratio of radioactivities in the bound fraction and free fraction with log concentration of unlabeled standard toxin. RIA can detect 0.25 to 0.5 ng of purified mycotoxin. The sensitivity of RIA can be improved by a simple cleanup procedure after extraction or by using radioactive markers of high specific activity (^{125}I-labeled mycotoxin) (Chu, 1991).

D. Enzyme Linked Immunosorbent Assay

There are two methods of ELISA used in the analysis of Aflatoxin. One type, direct ELISA involves the use of an aflatoxin-enzyme conjugate and other system, indirect ELISA involves the use of a protein-aflatoxin conjugate and a secondary antibody to which an enzyme has been conjugated (Chu, 1986). In the direct competitive assay specific antibodies are first coated to a solid phase, including polystyrene microtiter plate (Chu, 1991). The sample solution or standard toxin is generally incubated simultaneously with enzyme conjugate or incubated separately in two steps. After washing, the amount of enzyme bound to the plate is then determined by incubation with a substrate solution containing hydrogen peroxide and appropriate oxidizable chromogen. The resulting color is measured spectrophotometrically or by visual comparison with the standard. In this assay, toxin in the sample and toxin-enzyme conjugate compete for the same binding site with the antibody coated to the solid-phase. Because the toxin-enzyme and antibody concentration are constant, the color intensity as a function of enzyme is inversely proportional to the toxin concentration in the testing sample. In the indirect ELISA, an aflatoxin protein conjugate is first prepared and then coated to the microtiter plate before assay. The plate is then incubated with specific rabbit antibody in the presence or absence of the homologous aflatoxin. The amount of antibody bound to the plate coated with aflatoxin conjugated protein is then determined by reaction with goat anti-rabbit IgG enzyme. Thus toxin in the sample and toxin in the solid-phase compete for the same binding site with the specific antibody in the solution. The indirect ELISA has been used for the analysis of a number of mycotoxins (Chu, 1991). Degan *et al.* (1989) developed method to analyse Aflatoxin involving the use of Europium ion (Eu) labeled antibody. Kolosovo *et al.* (2006), developed direct competitive ELISA for detection of Aflatoxin B_1 and an ELISA kit has been designed. This immunoassay was highly specific, sensitive, rapid, simple and suitable for aflatoxin monitoring. AFB_1 concentration determinable by ELISA ranged from 0.1 to 10 µg/l.

E. Immunochromatographic technique

A rapid diagnostic method has been developed by Xiulan *et al.* (2005) in which they prepared an antibody-colloidal probe (conjugate) specific to aflatoxin B_1 (AFB_1). Nanogold – labeled probe was used to develop an immunochromatographic (IC) method for Aflatoxin B_1 analysis. With this method analysis can be completed within 10 min. reducing the detection time 6-10 times comparative with ELISA. Shim *et al.* (2007) developed an Immunochromatography (ICG) strip test using a nanocolloidal gold-antibody probe and optimized for the rapid detection of aflatoxin B_1. A monoclonal antibody specific to AFB_1 was produced from the cloned hybridoma cell (AF78), coupled with nanocolloidal gold and distributed on the conjugated pad of the ICG strip test. The visual detection limit of the ICG strip was 0.5 ng/ml. They analyzed 172 grain sample by ICG and compared with HPLC and showed good agreement with those obtained by HPLC. These strips are a potential screening tool for the detection of AFB_1 in samples and could be applied to the preliminary screening of mycotoxin in food and agricultural products, generating result within 15 minutes.

F. Polymerase Chain Reaction

Manonmani *et al.* (2006) developed rapid method for assessment of aflatoxigenic fungi in food using an indigenously designed primer pair for the aflatoxin regulatory gene *aflR* in Polymerase Chain Reaction. They extracted DNA from 28 different fungal strains on PCR template. Positive amplification was achieved only with DNA from Aflatoxigenic *A. flavus* and *A. parasiticus.* The detection limit for mycelium was determined as 0.05 g and $\geq$ 100 cfu respectively. Some of the genes coding for enzymes involved in aflatoxin biosynthesis have been cloned and sequenced which aided in the characterization of the aflatoxigenic molds. It is estimated that at least 16 enzymes are involved in the bioconversion of norsolorinic acid to aflatoxin (Chang *et al.*, 1995, Yu *et al.*, 1995). Among these genes, the nor-1 gene codes for reductase that convert norsolorinic acid to averantin (Trail *et al.*, 1994). The ver-1 gene is involved in the conversion of versicolorin A to Sterigmatocystin and omt-1 gene codes for O-methyltransferase that converts sterigmatocystin (Skory *et al.*, 1992). In addition to these structural genes, a regulatory gene, aflR, codes for regulatory protein that activates the pathway genes. On the basis of cloned and sequenced gene involved in the aflatoxin biosynthesis, specific primer had been designed for Polymerase Chain Reaction or Multiplex PCR method for detection of aflatoxigenic fungi (Farber *et al.*, 1997). Yang *et al.* (2004) applied multiplex PCR for the detection of potential aflatoxin-producing molds in Korean fermented foods and grains. Three genes, avfA, omtA, and ver-1, coding for key enzymes in aflatoxin biosynthesis were used as aflatoxin-detecting target genes in multiplex PCR. DNA extracted from *Aspergillus flavus, Aspergillus parasiticus, Aspergillus oryzae, Aspergillus niger, Aspergillus terreus, Penicillium expansum* and *Fusarium verticillioides* were used as PCR template to test specificity of the multiplex PCR assay. Positive results were achieved only with DNA that was extracted from the aflatoxigenic molds *A. flavus* and *A. parasiticus* in all three primer pairs. This result was supported by aflatoxin detection with direct competitive enzyme-linked immunosorbent assay (DC-ELISA).

G. Tandem Mass Spectrometry

The tandem MS method (Plattner, 1986) has been used extensively in the last few years. This method involves one stage of mass separation to select the compound of interest from the sample matrix (molecular ion, protonated molecular ion or molecular anion) and a second stage of analysis after colloisionally

activated dissociation (CAD) by collision with a target gas such as argon. Thus both the parent ion and the activated dissociated ion (daughter ions, or secondary ions) are analyzed. In contrast to selection ion mode (SIM), the monitoring system of tandem mass spectrometry is called "multiple reaction monitoring" (Plasencia *et al.*, 1990). This method has also been used in conjunction with Gas Chromatography (GC) (Plattner, 1986). Fast atom bombardment/tandem MS has been used for the detection of aflatoxin (Uyakual *et al.*, 1989). Lattanzio *et al.* (2007) developed liquid chromatography/tandem mass spectrometry method for simultaneous determination of aflatoxin B_1, B_2, G_1, G_2, Fumonisin, ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in maize.

2.0 Inactivation Method to Control Aflatoxin

Decontamination process must be technically and economically feasible. The FAO requirement for acceptable decontamination process is that the process must destroy, inactivate or remove aflatoxins, not produce, nor leave toxic (carcinogenic and mutagenic) residue in the food commodities and should destroy fungal spores and mycelium that could proliferate and produce new toxins under favorable conditions. Many detoxification methods of aflatoxin have been recommended and include: mechanical separation of contaminated seed, heat treatment, extraction using solvents, detoxification using solvent, detoxification using chemical agents and added sorbents. Some physical, chemical and biological methods to detoxify aflatoxin in feedstuffs are reviewed here.

Botanicals

Krishnamurthy and Shashikala (2006) studied the effect of *Withania somnifera, Hyptis suaveolens, Eucalyptus cimodora,* peel powder of *Citrus sinensis, Citrus medica* and *Punica granatum*, neem cake and Pongamia cake on Aflatoxin B_1 production by *A. flavus.* All tested plant material was significantly effective in inhibiting aflatoxin B_1. Neem cake showed maximum inhibition (98.22% at 10% and 87.07% at 5%) followed by *H. suaveolens* (84.38% at 5% and 78.85% at 10%), Pongamia cake (81.35% at 5% and 75.17% at 10%), *Withania somnifera* (78.11% at 10%), *C. sinensis* (70.84% at 10%) and *C. medica* (73.65% at 5%).

Leaf powder of *Ocimum* has been successfully used in inhibiting mould development and aflatoxin production on stored soyabean (Awuah, 1996). Neem leaf extracts were found to be very effective in controlling growth of mould and aflatoxin production (Bankole, 1997). Ghorbaman *et al.* (2007) studied the effect of neem (*Azadiracta indica* A. juss) leaf extract on the growth of *A. parasiticus* and production of aflatoxin. They found maximum inhibition i.e. 80-90% at 50% concentration. Similar finding was also reported by Bhatnagar and McCormic (1988) and Allameh *et al.* (2001). In 1977, Bullerman *et al.* established that Cinnamon and clove oil and their principle components, cinnamic aldehyde and Eugenol, inhibited the growth and toxin of *A. parasiticus.*

Chandra *et al.* (2007) studied the synergistic effect of *Pongamia pinnata* (Bark) and *Tamarindus indica* (fruit) against *A. flavus* and found significant reduction in growth *i.e.* 62%. Sanchez *et al.* (2005) studied the effect of ethanolic, methanolic and aqueous extract of *Agave asperrima* and *Agave striata* on growth and production of aflatoxin and observed remarkable reduction in aflatoxin synthesis. Hazare *et al.* (2005) reported that aqueous extract of Ajowan (*Trachyspermum ammi*) has aflatoxin Inactivation Factor (IF). Essential oil of *Thymus eriocalyx* and *Thymus x. porlock* showed

inhibitory effect when exposed to *A. parasiticus*. The oil from above plants was found to be strongly fungicidal and inhibitory to aflatoxin production (Rasooli and Abyaneh, 2004). Chloroform extracts of *Garcinia indica* rinds was tested for the inhibition of *A. flavus* and Aflatoxin production using peanut powder as model food system. Aflatoxin was estimated by spectroflurophotometric and Thin Layer Chromatographic methods. At lower concentration i.e. 500-1000 ppm, the aflatoxin inhibition was much higher than the growth inhibition. At 3000 ppm the Aflatoxin production was completely inhibited (Tamil Selve *et al*, 2003). As evident from the above account, the Botanicals have immense potential to control fungal growth and toxin and thus there is need of more research on purification of active fraction for commercialization as an alternate to chemicals.

Heat

Normal home cooking such as boiling and frying (approx. 150°C) failed to destroy AFB_1 and AFG1 in the solid state (Kamimura, 1989). Aflatoxins have high decomposition temperature ranging from 237°C to 306°C. Solid AFB_1 is quite stable to dry heating at temperature of 267°C (Behna, 1989). Degradation of aflatoxin by heat is also governed by the moisture content, ionic strength and pH of the food. The moisture content is a critical factor; contaminated food that contains more moisture can more easily be inactivated by heat. Mann *et al.* (1967) observed that heating a cottonseed meal containing 30% moisture at 100°C for 1h degraded 74.8% of aflatoxins ($B_1 + B_2$) present in meal, whereas only 32.7% of the toxins were destroyed after heating a similar meal containing 6.6 % moisture under the same condition.

Farah *et al.* (1983) cooked raw unshelled peanuts in 5% NaCl solution in an autoclave at 116°C, 0.7 bar for 30 min. This treatment reduced the total content of aflatoxins ($B_1 + G_1 + B_2 + G_2$) by 80-100%. Rustom *et al.* (1993) studied the effect of pH (5.0, 8.0, 10.2), temperature (121°C, 130°C, 140°C) and heating time (5s, 20s, 15 min). Heat treatment at pH 8.0 was not effective in reducing mutagenic activity. On the other hand, the treatment pH 10.2, 130°C, 20s and pH 10.2, 121°C, 15 min reduced the mutagenic activity by 78% and 88% respectively. Harish *et al.* (2002) studied the effect of roasting at different moisture levels on the inactivation of Aflatoxin B_1 of food grains. The initial content of AFB_1 in inoculated groundnut kernel sample was 2836.4 ppb. Roasting at 150°C for 10 min at 10% moisture level reduced AFB_1 by 48%; whereas at the same temperature and time and 20% and 30% moisture AFB_1 was significantly reduced by 53.03% and 56.02% respectively.

Antagonistic microorganism

Ciegler *et al.* (1966) have screened approximately 1000 microorganisms representing yeasts, molds, mold spores, actinomycetes, bacteria and algae for their ability either to destroy or to transform aflatoxin B_1 andG_1. Some molds and mold spores partially transformed aflatoxin B_1 to new fluorescing compounds. Only one of the bacteria *Flavobacterium aurantiacum*, NRRL B-184 removed aflatoxin from solution. Detoxification by cells of this microorganism was tested on milk, corn oil, peanut butter, corn, soybeans and peanuts. The milk, corn oil, and peanut butter were artificially contaminated by adding 600, 700 and 700 $\mu g\ kg^{-1}$ of aflatoxin B_1 respectively, to 50 ml of milk and corn oil and 50 gm of peanut butter. When 2.0 x 10^{13} viable cells of *F. aurantiacum*, NRRL B-184 were added to each of these food products aflatoxin levels were reduced to 0 and a trace after 3 hours of incubation. Viable cells of *F. aurantiacum* were mixed with soybeans, corn, and peanuts contaminated seeds which were then incubated for 12 hours at 28°C. Aflatoxins were completely removed from corn and peanuts but only 86% were removed from soybeans.

Destruction of aflatoxins in solution by *F. aurantiacum* was confirmed by duckling tests. Aflatoxin contamination by the toxic strains of *A. flavus* inhibited by an atoxigenic strain of *A. flavus* in vivo and in liquid fermentation, and the atoxigenic strain was equally effective when applied at spore concentration either equal to or one-half those of the toxigenic strain (Cotty and Bayman, 1993). Displace toxigenic strains of *A. flavus* from agricultural fields with strains of *A. flavus* that do not produce aflatoxins; strategy is possible because of the great variability of phenotypes of *A. flavus* in agricultural fields and the common occurrence of atoxigenic strains (Cotty, 1989). Several atoxigenic strains of *A. flavus* isolated from agricultural fields in Arizona can reduce the aflatoxin contamination of developing cotton bolls caused by toxigenic strains (Cotty, 1990). *Lactobacillus casei pseudoplantarum* 371 isolated from a silage inoculant was found to inhibit aflatoxin B_1 and G_1 biosynthesis by *A. flavus* sub sp parasiticus NRRL 2999 in liquid medium (Gourama and Bullerman, 1997).

Radiation

Food irradiation is becoming a technique of potential application on commercial scale as it renders the food product sterile (Diehl, 1990).

Gamma rays

The toxicity of Peanut meal contaminated with AFB_1 was reduced by 75% and 100% after irradiation with gamma rays at a dose of 1 and 10 kGy, respectively (Temcharoen and Thilly, 1982). Dose higher than 10kGy inhibited the seed germination and increased the peroxide value of the oil in gamma irradiated peanuts (Chiou *et al.*, 1990). A dose of 10 kGy completely inactivated AFB_1 and 95% of AFG_1 in Dimethylsulphoxide-Water (1:9 v/v) solution (Mutluer & Erkoe, 1987). Addition of 1 ml of 5% hydrogen peroxide to an aqueous AFB1 solution (50 µg/ml) resulted in 37-100% degradation of the toxin at a lower dose (2 kGy). The final degradation products showed no biological activity in Ames mutagenic test. The same treatment reduced the level of AFB_1 in peanut kernel by 73-80% (Patel *et al.*, 1989). El-Bazza *et al.* (2001) exposed the *A. flavus* isolate to gamma radiation dose level from 0.0 to 3.0 kGy. The gradual decrease in the growth occurred by increasing the irradiation dose upto 2.5 kGy. Low doses of gamma radiation did not affect its production upto 0.5 kGy, the mycelial weight markedly increased the total production reaching 3000 µg/L. Thereafter a decrease in its production was observed by increasing the dose. El-Bazza *et al.* (2001) found that increased dose of ionizing radiation for the spore of *A. flavus* led to an increase in the aflatoxin production reaching the maximum at a dose level of 1.0 kGy. No detectable aflatoxin was observed at 3.0 kGy. Aziz and Youssef (2002) showed that application of radiation at 10 kGy significantly detoxify aflatoxin B_1 by 82-88%. Aziz *et al.* (2004) showed that at 4 kGy gamma rays in maize significantly destroyed 60.9%-66.7% of aflatoxin.There is number of reports which suggest that the molds are very sensitive to gamma radiation and the mycotoxin production decreased after irradiation of food (Refai *et al.*, 1996). It appears that the fungal strains, condition of storage, humidity, inoculum size and irradiation dose affect mold growth and toxin production (Mahrous *et al.*, 2002; Aziz *et al.*, 2004).

UV rays

Aflatoxin B_1 absorbs UV light at 222, 265 and 362 nm with maximum absorption occurring at 362 nm, which lead to the formation of 12 photodegradation products (Samarjeeva *et al.*, 1995). The photodegradation products were less toxic to chick embryo than the parent toxins (Andrello *et al.*, 1967). Treatment of

peanut oil with UV light for 2 h destroys 40-45% aflatoxin (Shantha and Murthy, 1977). UV Radiation (30 min) treatment of dried figs artificially contaminated with AFB_1 (250 µg/kg) reduced the aflatoxin level by 45.7% (Altug *et al.*, 1990).

Solar Radiation

Efficacy of solar energy has been studied in different countries. In India, Shantha and Murthy (1981) exposed the peanut cake in sunlight for 6h and found 50% reduction in aflatoxin content. Naturally contaminated peanut flakes were exposed to sunlight for 14h and 50% reduction in aflatoxin content was found (Shantha and Murthy, 1981).

In USA, Mahjabeb & Bullermann (1988) exposed the olive oil for 10 and 40 min and found the 55% and 95% reduction of aflatoxin respectively.

Chemicals

Ghosh *et al.* (1996) studied the effect of propionic acid, sodiumbisulfite and sodium hydroxide on the biosynthesis of aflatoxin on groundcake. The aflatoxin free ground cake were treated with 0.25, 0.50, 0.75 and 1.0% each of propionic acid, sodiumbisulfite or sodium hydroxide at three moisture levels of 10, 15 and 20%. Out of three chemicals, the propionic acid was found to be most effective followed by sodium sulfite and sodium hydroxide. Acetic acid and propionic acid which are used in animal feeding are effective mold inhibitor (Sauer, 1997). Several studies have dealt with the use of propionates to control mold growth and toxin production by *A. flavus, A. parasiticus, A. ochraceus* and *Penicillium viridicatum* in artificially inoculated corn up to 29 weeks of storage (Vandergraft *et al.*, 1975). Propionic acid inhibits aflatoxin formation largely through inhibition of growth of *A. flavus* and *A. parasiticus* (Tsai *et al.*, 1984). Davis and Diener (1967) reported that sulfite inhibits a number of metabolic pathways in fungi. Therefore, it reduces the growth of fungus which leads to less aflatoxin production. Moerck *et al.* (1980) reported that sodium hydroxide at 2% concentration was effective for reducing aflatoxin level. Aflatoxin in the maize grain with an initial concentration of 29 ng/g was completely degraded and 96.7% degradation occurred in maize contaminated with 93 ng/g when treated with citric acid. Aflatoxin fluorescence strength of acidified samples was much weaker than untreated sample when observed in HPLC chromatogram (Mendez-Albores *et al.*, 2005).

Ammonia is one of the most effective reagents proposed for chemical inactivation of aflatoxin in contaminated peanut and cottonseed meals. Masri (1965) treated a toxic meal (Aflatoxin B_1 content about 1 ppm) with ammonium hydroxide solution and biological test of treated meal indicated elimination toxicity. Dollear and Gardner (1966), reported inactivation of aflatoxin in cottonseed and peanut meals with anhydrous ammonia under pressure in the range of 20 to 43 psig. It has been observed that AFB_1 molecular structure is irreversibly altered if exposure to ammonia lasts long enough. The disadvantage of ammonia treatment is mainly related to the need to build special plant as ammonia corrodes metal and becomes explosive in the air at mixture over 15% volume. Some effect on the chemical and qualitative characteristics of the feed i.e. undesirable brown color of the treated feed has also been observed.

Feuell (1966) reported that treatment of peanut meal with chlorine reduced its toxicity to duckling but did not prevent liver lesion. Aly *et al.* (2004) used the commercially hydrated sodium calcium alumino silicate (HSCAS) and Egyptian monmorillonite (EM) and found that it has capability of absorbing AFB_1 and

Fumonisin B_1 in aqueous solution. HSCAS was reported to have high affinity to AFB_1. The HSCAS removed more than 80% of the toxin from solution. In vivo studies demonstrated the role of HSCAS in preventing the mutagenicity and toxicity of AFB_1 (Phillips *et al.*, 1998).

Mckenzie *et al.* (1997) treated aqueous equimolar (32 μM) solution of Aflatoxin B_1, B_2, G_1, G_2, cyclopiazonic acid, Fumonisin B_1, Ochratoxin A, Patulin, Secalonic acid and Zearalenone with 2, 10 and/or 20 weight % Ozone (O_3) over a period of 5 min and analysed by HPLC. AFB_1 and AFG_1 were rapidly degraded using 2% O_3, while AFB_2 and AFG_2 were more resistant to oxidation and required higher dose of O_3 (20%) for rapid degradation. O_3 gas generated by corona discharge has been reported to degrade the aflatoxin in corn and cottonseed meals (Dollear *et al.*, 1968; Dwarkanath *et al.*, 1968) and in aqueous solution. Akacid® plus, a new member of guanidine based polymeric disinfectant was recently introduced for the first time as a potent inhibitor of *A. parasiticus* growth and its aflatoxin productivity (Razzaghi-Abyaneh *et al.*, 2006). Akacid® plus was developed by POC polymer Production GmbH, Vienna, as a new member with enhanced broad antimicrobial activity while significantly less toxicity compared to the former compound of this class. Akacid® plus is water soluble, nonflammable, nonexplosive, colorless and odorless formulation, which is composed of a mixture of two different polymeric guanidine compounds. It was accepted as biocide according to the new EU guidelines and it has in vitro antimicrobial activity against some important pathogenic bacteria and fungi (Kratzer *et al.*, 2006a, b; Buxbaum *et al.*, 2006).

Analysis of aflatoxin is difficult task because only trace amount of toxin are present in the animal feed and other food commodities. However, rapid progress in the development of new aflatoxin analytical methodology has been made during the last few years. Researchers have attempted to simplify and improve the existing analytical methods. New more versatile and sensitive methods have come to the market. After many years of laboratory research, immunoassay technique has gained wide acceptance as analytical method for aflatoxin and other toxins. The mycotoxin problem is serious in developing countries where conditions and agricultural practices are conductive to fungal growth and toxin production. The control of Aflatoxin can be achieved by good farm management and use of effective detoxification process which we have discussed in this review.

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