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Review

Aflatoxin B1: Toxicity, characteristics and analysis: Mini review

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Aflatoxins (AF) are mycotoxins derived by Aspergillus flavus and Aspergillus parasiticus and are listed as Group I carcinogens by the International Agency for Research on Cancer (IARC), a body of the World Health Organization. The main aflatoxins are the B1 (AFB1), B2, G1 and G2 together with their metabolites, among which the most important is the aflatoxin B1. AFB1 is the molecule with the highest toxic significance. It is commonly found in any foodstuff or animal feed which can support fungal growth during growth, harvest, or storage. This paper is a mini review of AFB1, its toxicity, its characteristics and its different methods of analysis.

Keywords: AFB1, WHO, AFB2, IARC, HPLC.

INTRODUCTION

Aflatoxins are typically found as secondary metabolites of *Aspergillus parasiticus and Aspergillus flavus* (Maragos and Pohland, 2001). Aflatoxins frequently contaminate cereal crops such as corn, beans, peanuts, and dried fruits (Hussein and Brasel, 2001). Among all aflatoxin B1 (AFB1) has the highest toxicity. Epidemiological studies have shown that with prolonged exposure to AFB1 liver cancer may develop, especially in persons with hepatitis B antigens (Sun and Chen, 2003; Lu, 2003). Consequently, the *World Health Organization (WHO)* classifies AFB1 as a human carcinogen and proposes no safe dose (Anklam et al., 2002). The induction of cancer by AFB1 has been extensively studied. *International Agency for Research on Cancer (IARC)* has produced sufficient evidence of carcinogenicity of aflatoxin B1 in experimental animals .

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Toxicity

A study was conducted by N. Jindal *et.al* (Jindal et al., 1994) on 60 broiler chicks, of the effect of activated charcoal (200ppm) on the toxicity of 0.5 ppm aflatoxin B1 (AFB1) in feed fed from day 1 to day 42. Activated charcoal was found to be moderately effective in reducing the harmful effects of AFB1 as assessed by growth response and various biochemical parameters. The feeding of activated charcoal along with AFB1 reduced the inhibitory effect of AFB1 on bodyweights and feed intake. In the presence of there was also a significant improvement in the serum aspartate aminotransferase, alkaline phosphatase, total proteins, calcium and phosphorus levels. However, no significant improvement was observed in cholesterol levels.

The effects of aflatoxin B1 (AFB1) in rats (Rogers and

Table 1. Acute toxicity of aflatoxin B1 expressed as a single oral dose LD_{50} (FAO web library).

Species	LD_{50} mg/kg body weight
Rabbit	0.30
Duckling (11 day old)	0.43
Pig	0.60
Cat	0.55
Dog	0.50-1.00
Chicken	6.30
Rat (male)	5.50-7.20
Rat (Female)	17.90
Mouse	9.00
Hamster	10.20
Rainbow trout	0.80
Babbon	2.00
Sheep	1.00-2.00
Guinea pig	1.40-2.00
Macaque(female)	7.80

Newberne, 1971) are markedly influenced by diet. Fischer and Sprague-Dawley male rats were fed either a complete synthetic diet or a synthetic diet marginally deficient in lipotropes, which had enhanced AFB1 carcinogenesis in earlier studies. Deficient rats of both strains were resistant to the toxicity of a single dose of AFB1 (7 or 9 mg/kg), which killed 60–100% of rats fed the complete diet. In contrast, the deficient rats were more sensitive to the acute toxicity of repeated small doses of AFB1. The dietary influence may be explained by the low resting levels of the hepatic drug-metabolizing enzymes and by the failure of the enzyme levels to respond to repeated doses of AFB1 in the livers of deficient rats.

No animal species is resistant to the acute toxic effects of aflatoxin B1. A wide variation in LD_{50} values has been obtained in animal species tested with single doses of aflatoxins (Table1). For most species, the LD_{50} value ranges from 0.5 to 10-mg/kg body weight. Animal species respond differently in their susceptibility to the chronic and acute toxicity of aflatoxins. Environmental factors, exposure level, and duration of exposure beside age, health, and nutritional status of diet can influence the toxicity (FAO web library 2000).

Contamination Conditions

Aflatoxin B1 producing members of *Aspergillus* are common and widespread in nature. They can colonize and contaminate grain before harvest or during storage. Host crops are particularly susceptible to infection by *Aspergillus* following prolonged exposure to a high humidity environment or damage from stressful conditions such as drought, a condition which lowers the barrier to entry. The native habitat of *Aspergillus* is in soil, decaying vegetation, hay, and grains undergoing microbiological deterioration and it invades all types of organic substrates whenever conditions are favorable for its growth. Favorable conditions include high moisture content (at least 7%) and high temperature.

Detection in humans

There are two principal techniques that have been used most often to detect levels of AFB1 in humans.

1. The first method is measuring the AFB1-guanine adduct in the urine of subjects. Presence of this breakdown product indicates exposure to aflatoxin B1 in the past 24 hours. However, this technique only measures recent exposure, and due to the half-life of this metabolite, the level of AFB1-guanine measured can vary from day to day, based on diet, and thus is not ideal for assessing long term exposure.
2. Another technique that has been used is a measurement of the AFB1-albumin adducts level in the blood serum. This approach provides a more integrated measure of exposure over several weeks/months.

Analysis

Sampling and Sample Preparation

Sampling and sample preparation remain a considerable source of error in the analytical identification of Aflatoxin B1. Thus, systematic approaches to sampling, sample preparation, and analysis are absolutely necessary to determine aflatoxin B1 at the parts-per-billion level.

In this regard, specific plans have been developed and tested rigorously for some commodities such as corn,

peanuts, and tree nuts; sampling plans for some other commodities have been modelled after them. A common feature of all sampling plans is that the entire primary sample must be ground and mixed so that the analytical test portion has the same concentration of toxin as the original sample.

Solid-Phase Extraction

All analytical procedures include three steps: extraction, purification, and determination. The most significant recent improvement in the purification step is the use of solid-phase extraction. Test extracts are cleaned up before instrumental analysis(thin layer or liquid chromatography) to remove coextracted materials that often interfere with the determination of target analytes.

Thin-Layer Chromatography

Thin layer chromatography (TLC), also known as flat bed chromatography or planar chromatography is one of the most widely used separation techniques in aflatoxin B1 analysis. Since 1990, it has been considered the AOAC official method. The TLC method is also used to verify findings by newer, more rapid techniques.

Liquid Chromatography

Liquid chromatography (LC) is similar to TLC in many respects, including analyte application, stationary phase, and mobile phase. Liquid chromatography and TLC complement each other. For an analyst to use TLC for preliminary work to optimize LC separation conditions is not unusual. Liquid chromatography methods for the determination of AFB1 in foods include normal-phase LC (NPLC), reversed-phase LC (RPLC) with pre- or before-column derivatization (BCD), RPLC followed by postcolumn derivatization (PCD), and RPLC with electrochemical detection.

Immunochemical Methods

Thin layer chromatography and LC methods for determining AFB1 in food are laborious and time consuming. Often, these techniques require knowledge and experience of chromatographic techniques to solve separation and interference problems. Through advances in biotechnology highly specific antibody-based tests are now commercially available that can identify and measure AFB1 in food in less than 10 minutes. These tests are based on the affinities of the monoclonal or polyclonal antibodies for AFB1. The three types of immunochemical methods are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunoaffinity column assay (ICA).

A method has been developed by J.A.Van Rhijin *et al*. for determination of AFB1 in cattle feed, uses a two column SPE clean up with intermediate solvent evaporation and is coupled on line to a high performance liquid chromatographic system. This method is the Automated version of the method which was tested by Europian community. Although automated version has lower recovery, but this method may be used for screening purposes due to its good reproducibility.

N.Bradburn et al compared efficiency of a phenyl bonded phase column and two different immunoaffinity columns for the extraction, clean-up and quantification of aflatoxin Bl from sorghum and maize. For quantification of aflatoxin B1, fluorodensitometry of high performance thin-layer chromatograms was used. The sorghum matrix was complex and the bonded-phase procedure was the most precise and accurate method. There was evidence to suggest that recovery from sorghum by immunoaffinity columns is a solvent extraction problem. Maize had a simple matrix, and comparable precisions and accuracies were obtained for each of the methods. Better aflatoxin B1 recoveries were obtained from naturally contaminate sorghum and maize when acetonitrile was replaced with acetone as the extraction solvent.

For the detection of aflatoxin B1 (AFB1) S.R. Garden and N.J.C. Strachan used a rapid colorimetric sequential injection immunoassay (SIIA) Figure 1 system utilising a jet ring flow cell packed with a solid phase of polymethylmethacrylate beads. This method is proven in its ability to detect AFB1 down to a level of 0.2 ng/mL in artificially contaminated food materials, which is comparable to the sensitivity of ELISA. The automated SIIA system takes under 10 min per sample and uses a methanol–water (80:20) and hexane extraction procedure that takes approximately 15 min to perform.

E. Calleri et al developed and characterized an anti-aflatoxin B1 (anti-AFB1) immunoaffinity monolithic disk. Polyclonal anti-AFB1 was covalently immobilized by a one-step reaction via epoxy groups of the polymer surface in a batch of an epoxy-activated monolithic Convective Interaction Media (CIM) disk (12mm×3mmi.d.). The binding capacity of the CIM disk was determined by frontal analysis it was found that 0.96 mg of antibody were immobilized. The CIM disk was coupled through a switching valve to a reversed-phase column. A fully automated HPLC method with fluorescence detection for the determination of aflatoxin B1 in aqueous solution was developed. The total analysis time with the integrated system is 46 min and the retention time of AFB1 is approximately 29 min. The binding capacity of the immunoaffinity disk was evaluated in terms of linearity, precision and accuracy of the extraction procedure. The immunoaffinity support was stable after repeated runs.

Analytical-scale supercritical fluid extraction (SFE) has

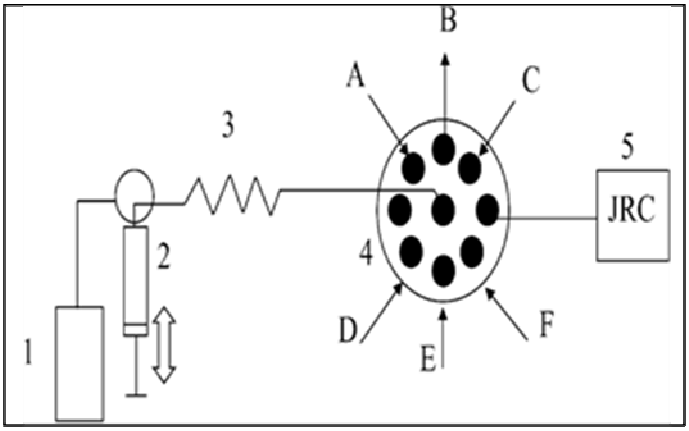


Figure 1. Schematic diagram of the SIIA system showing (1) carrier solution, (2) syringe pump, (3) holding coil, (4) multi-position valve (connecting (A) bead suspension, (B) waste out, (C) bleach solution, (D) sample solution, (E) labelling solution and (F) substrate solution and (5) jet ring cell.

been applied for the removal of aflatoxin B1 (Taylor et al., 1993) from field-inoculated corn samples, analysis of extracts was performed with high-performance liquid chromatography coupled with fluorescence detection of the trifluoroacetic acid derivative. The SFE method yielded over 90% analyte recovery when compared to conventional solvent extraction procedures. Attempts to remove coextracted lipid matter by a supercritical fluid-based cleanup method are also described (Taylor et al., 1993). The precision of the method is affected by the sample size and the homogeneity of the corn sample.

An immunoassay-based lateral flow dipstick for the rapid detection of aflatoxin B1 in pig feed was developed (Delmulle et al., 2005). The test consisted of three main components: conjugate pad, membrane, and absorbent pad. The membrane was coated with two capture reagents,*i.e.* aflatoxin B1–bovine serum albumin conjugate and rabbit anti-mouse antibodies. The detector reagent consisted of colloidal gold particles coated with affinity-purified monoclonal anti-aflatoxin B1 antibodies, which saturated the conjugate pad. A comparison of several extraction methods for the pig feed matrix is presented. A mixture of methanol/water (80:20, v/v) gave the best recoveries. After sample extraction and dilution, the dipstick was put in the sample solution at the conjugate pad side and developed for 10 min. Analyte present in the sample competed with the aflatoxin B1 immobilized on the membrane for binding to the limited amount of antibodies in the detector reagent. Thus the line color intensity of an aflatoxin B1-positive dipstick is visually distinguishable from that of an aflatoxin B1-negative sample. The visual detection limit for aflatoxin B1 is 5 µg/kg. The major advantages of this one-step strip test are that results can be obtained within 10 min and that all reagents are immobilized on the lateral flow dipstick.

Egner et al., 2006 developed first isotope dilution mass spectrometry method for determination of aflatoxin B1-N7-guanine (AFB1-N7-Gua), a major human aflatoxin–DNA adduct that is excreted in the urine. It has helped identify AFB1 as a risk factor in the development of hepatocellular carcinoma, a common cancer worldwide. Triple-quadrupole mass spectrometry coupled with the use of a stable isotope-labeled internal standard (AFB1-N7-15N5-Gua) and better solid phase extraction and immunoaffinity column chromatography, have greatly improve accuracy, precision, specificity, and sensitivity. The limit of quantitation for AFB1-N7-Gua was 0.8 pg/20 mL urine (0.07 pg/mg creatinine). The method was validated for accuracy and precision over the range of 0.8–25 pg/20 mL urine, with between-day and within-day reproducibility for analysis of six aliquots of a human urine sample containing 6.0 pg/20 mL measured at <6% coefficient of variation.. The mean concentration of AFB1-N7-Gua, measured in 16/20 urine samples with levels above the method's limit of quantitation, was 2.9 pg/20 mL urine (0.28 pg/mg creatinine) with a range of <0.8–7.2 pg/20 mL urine (0.04–65 pg/mg creatinine). This sensitive biomarker will be especially useful for measuring the efficacy of planned interventions to reduce aflatoxin-related liver cancer in AFB1-exposed populations.

Korde et al., 2003 developed a radioimmunoassay (RIA) procedure to measure aflatoxin B1 (AfB1) in agricultural commodities by synthesising AFB1 oxime derivative, and used for preparation of 125I-labeled AFB1. Anti-aflatoxin B1 serum was raised in-house using AFB1–bovine serum albumin conjugate as immunogen. The assay system was optimized in the range of 0.2–5 ng/mL using a liquid phase (Polyethylene gycol) as well as a solid phase (coated polystyrene beads) separation system. Inter-assay and intra-assay variations, recovery, and parallelism studies validated the assay. AFB1

Table 2. AOAC Aflatoxin B1 Extraction Methods For some Food Products

Food Products	AOAC method	Extraction Solvent
Cottonseed Products, mixed feed	989.06	Methanol: Water(55:45)
Eggs	978.15	Water/sodium chloride/acetone
Soyabeans	972.27	Chloroform
White & yellow corn, peanut & cottonseed meals,	975.36	Acetone:water (85:15)
peanut butter, almonds, mixed feeds, pistachio nuts		
Peanuts & peanut products	968.22	Chloroform
Corn	972.26	Chloroform
	990.32	Methanol
Liver	982.24	Methylene Chloride

analysis was carried out in nearly 130 samples of different agricultural commodities. The correlation coefficient was determined using commercial ELISA and in-house-developed RIA methods.

A study was pursued for the simultaneous determination of mold, aflatoxin B1 (AFB1), and fumonisin B1 (FB1) levels in white and yellow corn (Medina-Martínez and Martínez, 2000). Mold levels were determined using potato dextrose agar and identification of the main genus of molds present in corn, AFB1 levels by immunoaffinity chromatography, and FB1 levels by a Bond-Elut SAX (strong anion exchange) cartridge and HPLC. AFB1 and FB1 occurrences were 16.6 and 83.78% respectively. The yellow corn presented higher mold incidence than the white corn.

A surface plasmon resonance (SPR)-based immunoassay was developed (Daly et al., 2000) by using two different anti-aflatoxin B1 antibodies to examine aflatoxin B1. A conjugate consisting of aflatoxin B1–bovine serum albumin (BSA) was immobilized on the dextran gel surface. Competition between immobilized aflatoxin B1 conjugate and free aflatoxin B1 in solution for binding to antibody injected over the surface formed the basis for the assay. Regeneration of the antibody from the immobilized conjugate surface is essential for the development of such an inhibitive immunoassay. Problems were encountered with the regeneration of the sensor surface due to the high-affinity binding of the antibodies. Conventional regeneration solutions consisting of low concentrations of NaOH and HCl worked to a degree, but regeneration was at the expense of the integrity of the immobilized conjugate. A polyclonal anti-aflatoxin B1 antibody was produced and was found to be regenerable using an organic solution consisting of 1 M ethanolamine with 20% (v/v) acetonitrile at pH 12.0. This combined high ionic strength and extreme pH as well as chaotrophic properties and allowed the development of an inhibitive immunoassay. The assay had a linear range of 3.0–98.0 ng /mL with good reproducibility.

Elisa Technology developed a Flow-Through Rapid Test (FTRT) Kit for determination of aflatoxin B1which is very simple to operate and does not require laboratory environment and trained persons.

Advantages of Elisa (FTRT)

- A precise and highly sensitive assay
- Simple sample preparation
- Results within 10 minutes

Screening of Aflatoxin B1 in Food and Feed

In order to test for AFB1 analytical methods such as HPLC, GC-MS, LC-MS, ELISA and TLC require trained personnel, laboratory environment, expensive equipment and analytical time takes hours or days. The Flow-Through Rapid Test kit offers rapidity and flexibility for screening AFB1 under non-laboratory environments in conformity to the EU regulations.

A Precise and highly sensitive assay

The Flow-Through Rapid Test for AFB1 can detect AFB1 in different matrices with sensitivities down to 2 ppb.

Sample Preparation

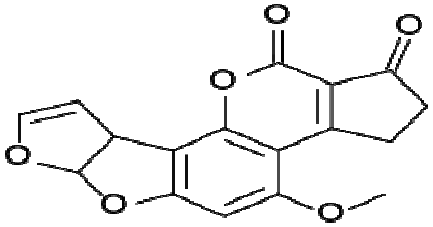
Food samples are prepared for analysis by simply shaking the sample by hand in the presence of an extraction solution. An aliquot of the supernatant is diluted and used in the assay.

Results within 10 minutes

(a) The Flow-Through Rapid Test for AFB1 has a detection limit of 2 ppb (ng/g).

(b) Configured for 1 – 10 tests at a time.

Table 3. General characteristic of Aflatoxin B1

Description	**Aflatoxin B1 is a potent hepatotoxic and hepatocarcinogenic mycotoxin. It is also mutagenic, teratogenic, and causes immunosuppression in animals.**
Source	Aspergillus flavus
Common name	Aflatoxin B1
Chemical name	2,3,6aα,9aα-tetrahydro-4-methoxycyclopenta[c]furo[2',3':4,5]furo[2,3-h]chromene-1,11-dione
Synonyms	Aflatoxin B1 AFB1
Chemical Formula	$C_{17}H_{12}O_6$
Structure	
Solubility Information	May be dissolved in DMSO, DCM or Methanol.
Appearance	Off - white powder; blue fluorescence
Molecular weight	312.3g/mol
Melting point	268-269^{0}C
Reduction Product	Aflatoxin B2
Molar Absorptivity	For molar absorptivity maximum near 350 nm in benzene:acetonitrile (98:2) 19,800
Toxicity	Primarily an hepatotoxin and in some species, such as the rat and the rainbow trout, an hepatocarcinogen and mutagen. Toxic response depends on how the molecule is metabolised in the liver. Oral LD50 for adult male rat 7.2 mg/kg, adult dog 0.5 mg/kg and day-old duckling 0.35 mg/kg body-weight, respectively. Possibly associated with human liver cancer in association with hepatitis B infection.
Storage/Stability	+4℃, Protect from light
Pharmacological Action	Carcinogens, mutagens, teratogens.
Related Compounds	Aflatoxin B1, B2, G1, G2, M1, M2.
Aflatxoin B1 Exposure	Liver damage, Liver Necrosis, Liver Cirrhosis, Fever, Progressive Jaundice, Limb Swelling, Pain, Vomiting, Enlarged Liver.
Exposure Route	Inhalation, Eye, Skin , Ingestion.
Classification	Furano-furano-Benzopyranmycotoxin Aflatoxin
Use	Studies of carcinogenesis
Major Metabolite	Aflatoxin Q_1 in Monkey Liver (7)

(c) Reagents are added drop-wise.
(d) Results are visually interpreted.
Validated Matrices: Cereals, soya beans, nuts and derived products.

Biosensors and nanotechnology

To date the development in nanomaterials and biosensors fabrications technology is moving rapidly with

new and novel nanobiorecongnition materials being developed which can be applied as the sensing receptors for AFB1 analysis. Interest in nanotechnology relies on the new properties that materials exhibit when reduced to the nanometer scale compares to the bulk materials (Maragos, 2009). Lab-on-a-chip devices are examples of micro/nanotechnology systems approaches which can be used for the analysis of food toxins. These devices can be cost effective and highly beneficial for food industry in ensuring high safety and quality of food and also for risk assement and management. The high surface to volume ratio offered by nanomaterials makes these devices very sensitive and can allow a single molecule detection which is very attractive in contaminants monitoring such as toxins. Quantum dots show distinct advantages over other markers due to their spectroscopic properties and narrow emission peaks and therefore their use in multiplexed analysis of toxins is increasing. To date the use of quantum dots in sensing application have been expanding across a diverse range of analysis. Xiulan *et al* 2005 and Liu *et al* 2006 used gold nanoparticles as tags for the analysis of AFB1 (Xiulan et al., 2005). Other researchers such as Tsai and Hsieh (2007), reported the development of QCM sensor for AFB1 and detection of 0.01-10.0µg/L. Gaag *et al* 2003 developed an SPR biosensor array to monitor AFB1 (Zourob, 2010).

AOAC International Methods

The AOAC method of analysis, manual lists a number of collaboratively studied methods for the analysis of aflatoxin B1 in variety of Food products. Food products, AOAC method numbers, extraction solvents used for the AFB1 are listed in table 2.
The general properties of the toxin Aflatoxin B1 has been summarised in the table 3.

CONCLUSION

Due to the presence of mycotoxins in wide range of foodstuffs and their harmful effects to human health, research groups have devoted much effort to finding suitable mycotoxin detection techniques that fulfill the requirements optimal analytical performance. Current concerns regarding food safety and quality requires a multidisciplinary approach based on a new generation of innovative and advanced technologies and tools to be used along the food chain for contaminants monitoring. Mycotoxins (AFB1) are one of the contaminants that require detection in food and feeds to maintain good foods quality and to reduce their negative impact on human health. To control AFB1 from contaminating food products there is a need for monitoring and control at different critical steps of the food chain to ensure food safety. These include monitoring of raw materials and food supply, monitoring during food processing monitoring of final products and also during storage. In this review, we discuss the various techniques and tools used for the analysis of AFB1 in milk food samples. In this review, an overview emerging technologies available to date and nanotechnology based materials and biosensors which are being developed and can be applied for AFB1 analysis will be covered in this review article.

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