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Aflatoxin-M1 in dairy products and detection methods

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Abstract

Molds that produce aflatoxins which are dangerous chemicals include Aspergillusflavus and Aspergillusparasiticus. Aflatoxin is a term used to describe four types of mycotoxins produced: B1 B2 G1 G2 M1 and M2. When ruminants absorb aflatoxins B1 and B2 the poisons are metabolized and excreted in milk as AFM1 and M2. Aflatoxin B1 (AFB1) the most hazardous is a potent carcinogenic that has been related to major health impacts in both animals and people including liver cancer. Biotransformation of ingested AFB1 occurs primarily in the liver. AFM1 has a lower hazardous potency than AFB1 but it is significantly more thermal stability. AFM1 was initially classified as a group 2B carcinogen by the International Agency for Research on Cancer (IARC) suggesting that it is a probable human carcinogen. To maintain food safety and human health the Food Safety and Standards Authority of India (FSSAI) and the Codex Alimentarius Commission (CAC) have set a maximum residual limit for AFM1 in dairy products at 500 ng/kg. As a result detecting AFM1 requires a sensitive robust and dependable technique. Chromatographic methods such as Thin Layer Chromatography Gas Chromatography and High-Performance Liquid Column Chromatography are now employed to detect AFM1 in dairy products and provide confirming results.

1.1 Aflatoxins

The word "mycotoxin" comes from the Greek words "mukos" which means "fungus" and "toxikon" which means "poison." The fungal kingdom produces secondary metabolite products. Three primary fungusAspergillusPenecillium and Fusarium are known to create mycotoxins among the many kinds of fungi found in nature. Aspergillusparasiticus and Aspergillusflavus are the most common aspergillus species that produce aflatoxins. Fusarium species like F. verticillioides create fumonosin while Penecillium species like P.

verrucosum produce ochratoxin. The majority of difficulties in tropical countries particularly in the south and southeast are due to aflatoxins pollution. Nuts corn cotton seed wheat chocolate cereals legumes and spices peanut ground chile and animal feeds are among the foods that these fungi may grow on. Some of Moldsare AspergillusPenicilliumAspergillusFusariumand their mycotoxins of concern for publichealth areAflatoxinOchratoxin/Patulin and Trichothecenes/Zearalenone/Fumonisins respectively

Aflatoxin the toxin of concern or analyte in this investigation is a heterocyclic molecule that is closely connected to each other. The phrase was coined in 1960 in London in response to a wide-scale outbreak of aflatoxicosis (also known as turkey "X" sickness) caused by contaminated ground nut feed which killed a large number of poultry (Blount 1961). Aflatoxin has now been discovered in a variety of foods including maize (Chakrabarty 1981) and cottonseed (Sharma et al. 1997). Aspergillusflavus which produced aflatoxins and ideal temperature for Aspergillusflavus is 30 °C. It can however grow in temperatures ranging from 10 to 45°C. Aspergillus thrives in an environment with an average relative humidity of 80%. As a result aflatoxin generation is a bigger problem in humid tropical areas like Brazil Uganda Nigeria and India where grains aren't stored properly(Janardhana*et al.*1999).

The many kinds of aflatoxins such as B1 B2 G1 G2 M1 and M2 are structurally similar yet differ somewhat (Figure 1.1). Lactating animals create AFM1 and AMF2 which are hydrolyzed when they consume feed contaminated with aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2). Aspergillusflauvus and Aspergillusparasiticus are the principal producers of these chemicals. Under a variety of climatic conditions these fungi contaminate a wide range of food and agriculture items. As a result most food items are concern to afla-toxigenic fungus contamination at various stages of manufacturing processing and duringstorage.

1.2 Aflatoxin M1 in Milk

The hepatic microsomal mixed function oxidase system bio converts the fungal toxin Aflatoxin B1 to trigger a carcinogenic response. When a breastfeeding animal consumes AFB1-contaminated feed it is transformed to AFM1. Many findings on the conversion of AFB1 to AFM1 are contradictory. The amount of absorbed AFB1 that converts to AFM1 in dairy cow milk varies between 0.003 %- 5%(Rodricks and Stoloff 1976).After consuming

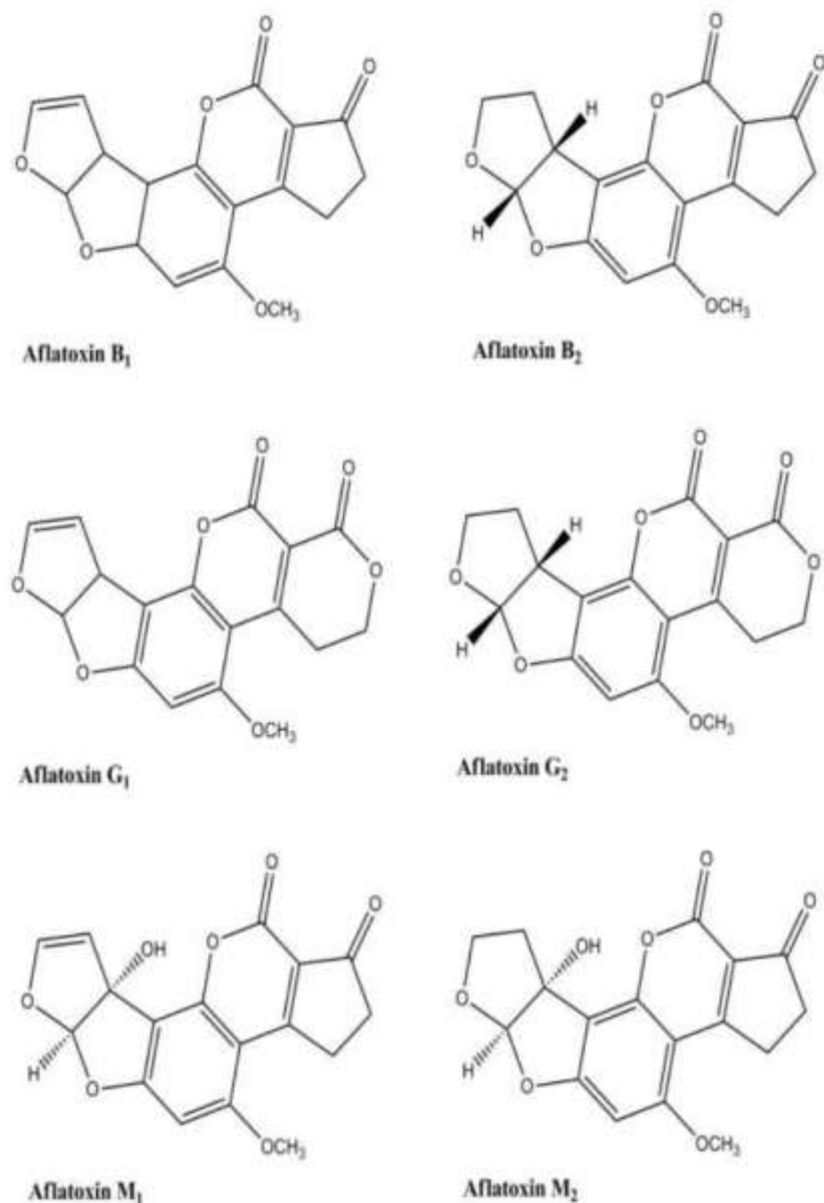

Figure1.1: Structure of Aflatoxins

aflatoxin B1-contaminated feed the conversion takes anywhere from 12 to 72 hours (Egmond 1983; Lopez et al. 2003). AFM1 levels are seasonal due to dairy cattle is been fed stockpiled fodder feed during the cold season or pastureland naturally on fresh grass during the warmer months (Bakirci 2001; Henry et al. 1997; Lopez et al. 2003). Because the carcinogenicity of AFM1 1/10 AFB1 and the genotoxicity is estimate 1/10-1/3 (Neal et al. 1998).

It is suggested that its formation of AFM1 is a detoxifying process (Henry et al. 1997). The amount of AFB1 eaten by an animal and converted to AFM1 varies from animal to animal. After the animal has consumed AFB1 the AFM1 in milk can be detected after 24 hours.When AFB1 is terminated the amount of Aflatoxin M1 identified in milk diminishes and eventually becomes negligible after 72 hours (Egmond et al. 1989). Aflatoxins are a crucial factor in the development of hepatocellular carcinoma. Aflatoxin M1 is classified as a

class 2B carcinogen by the International Agency for Research on Cancer. Toxicological responses vary by species whereas infants are more susceptible to the toxicological effects of AFM1 because milk is their primary source of nutrition. The human population consumes a lot of milk and milk products which increases the risk of AFM1 exposure. In vivo AFM1 is genotoxic in mammalian systems. AFM1 is a Group 2B agent which means it has the potential to cause cancer in humans. During the production and storage of various dairy products AFM1 remains relatively stable. Heat processing employed in the dairy business such as pasteurisation and sterilisation cannot inactivate thisAFM1 toxin (Prandini2009 ;Fallah 2010; Ghanem&orfi 2009;Tekinsen&Ucar 2008).

Aflatoxin exposure in neonates has been linked to stunted growth (Gong et al. 2004). Aflatoxinis danger in every developed country and so set maximum allowable amounts of AFM1 in milk and milk products is important. The European Union has set a maximum permitted level of 50 ng AFM1/kg in liquid dried or processed milk but the Codex Alimentarius Commission and the FSSAI allow up to 500 ng AFM1/kg milk. According to the Joint FAO/WHO Committee on Food Additives (2001) the European, Latin American,Far Eastern,Middle Eastern and African countries dietconsumed AFM1 6.8 ng/day, 3.5ng/day, 12 ng/day, 0.7 ng/day and 0.1 ng/day for per person respectively. As a result numerous countries have enacted legislation and proposed to regulate the levels of AFB1 in feed. AFM1 Maximum Permissible Levels (MPL) in milk have been established to limit the danger. The legal regulations concerning AFM1 levels in milk and dairy products vary from country to country.

Acetonitrile methanol chloroform and dimethylsulfoxide are moderately polar solvents that are soluble in AFM1. AFM1 has a limited solubility in water ranging from 10 to 30 mg/L and is insoluble in non-polar solvents. Several procedures for extracting AFM1 from milk call for centrifugation to remove the fat followed by syringe filters and immune-affinity columns to remove the intermediate layer. (Shundoet al.2006)

1.3 Existing detection techniques for AFM1

Because of the great vulnerability of infants and children to aflatoxin the presence of AFM1 in milk and dairy products is a worldwide issue (Horn et al. 2009). AFM1 estimate methods are discussed in detail (Table 1.2). TLC (Stubblefield et al. 1980) or HPLC (Stubblefield et al. 1986) ELISA (Rastogi; 2004; Rodriguez; 2003; Rubio; 2009 and Thirumala-Devi : 2002) HPLC-immunoaffinity (Dragacci et al. 2001). The detection limit and method limitations are also summarised in the following table 1.2. The majority of them necessitate the painstaking extraction of AFM1 from its source followed by purification and cleanup to remove other interfering chemicals and ultimately measurement.

Table 1.2 Methods available for determination of AFM1

Method	Detection limit (ng/L)	Drawbacks	Approval status	Reference
TLC	300	Require sample preparation;time consuming; does notcover EU limits	Yes (by AOAC)	Stubblefield *et al.* 1980
HPLC	80-130	Expensive; require sample preparation; laborious and time consuming; requires Well equippedlaboratory; does not cover EU limits	Yes (by AOAC)	Stubblefield *et al.* 1986
HPLCImmuno - affinity	20-100	Expensive; require sample preparation; laborious and time consuming; require antibodies; requires well equipped laboratory.	Yes (by AOAC)	Dragacci*et al.* 2001
ELISA	5	Antibodies can show considerable cross-reactivity for structural analogs; require samplepreparation	Not yet approved	Rastogi*et al.*2004 Rodriguez *et al.*2003 Thirumala-Devi *et al.* 2002
LC-MS	0.59-0.66	Require sample preparation which is laborious and time consuming; requires specialized equipments	Not yet approved	Chen *et al.* 2005
Charmassay	25	Requires expensive equipment	Yes (by AOAC)	Charm Sciences Inc.

SporeInhibition basedassay	50	Not yet published.	Not yet approved	Naresh Kumar (unpublished)
Aflasensor	50	Requires use of antibodies	Not known	Mayasan food industries Turkey

*Recommended limits: Europeon Union- 50 ng/Kg US regulatory limit- 500 ng/Kg

1.3.1 Thin-Layer Chromatography (TLC)

Thin-Layer Chromatography is a frequently used chromatographic technique for the separationand identification of non-volatile mixtures similar to paper chromatography. Aflatoxincan be detected via TLC and can detectquantify aflatoxinlevels as low as 1 ng/g(Kamkar2006). It has two phases: a stationary phase made up of a thin layer adsorbent such alumina silica gel or cellulose immobilised on a glass or plastic plate and a mobile phase made up of solvent. A spot on the stationary phase is applied with a sample that should be in liquid state. After that, the chromatographic plate is placed vertically in a solvent reservoir, and the solvent is pulled up onto the plate by capillary action.The separated spots are easily visible with ultraviolet light or an appropriate reagent spray. Because of variances in partitioning behaviour the rate of movement of distinct components in a mixture varies. For each point the retardation factor (R_f) is determined. In centimetres it's the ratio of the sample spot's distance travelled to the solvent's distance travelled and thenR_f values of standards are compared to those of unknown samples for analyte identification. For the isolation of aflatoxins this method is simple quick sensitive and selective.

TLC was significantly less expensive than HPLC and did not require highly skilled operators yet it was less accurate than HPLC. The original extraction procedure was a six hour soxhlet extraction with acetone, chloroform and water followed by treatment with lead acetate petroleum ether and finally spotting onto TLC plates. With times original extraction procedure improved and used to extractedaflatoxins in milk with chloroform and spotted onto silica plates using a combination of toluene ethyl acetate and formic acid followed by diethyl ether methanol and water.(Gilbert and Anklam 2002)

1.3.2 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is a process for separating identifying and quantifying each component in a mixture that is highly selective sensitive exact and automated (Kim et al. 2000). HPLC is mostly utilised for aflatoxin detection and final separation (Reiter et al. 2009). Extraction and clean-up operations must be carried out prior to HPLC detection. The sample is moved through the column by a pressured liquid solvent which is packed with an immobilised liquid stationary phase in the HPLC process. As the analyte passes through the column it is partitioned between the two phases resulting in compound separation due to difference in partitioning coefficients. The two most prevalent types of chromatography are normal phaseHPLC and reversed phase HPLC.UV detectors diode array detectors (DAD) and fluorescence detectors (FLD) are some of the detectors utilised in HPLC (Alcaide-Molina et al. 2009). For aflatoxin detection,fluorescence detection uses emission of light (435 nm) from molecules that have been exicted to

higher energy levels by electromagnetic radiation. Elizalde-Gonzalez et al. (1998) used HPLC with an amperometric detector to evaluate aflatoxins and found that it is possible to detect aflatoxins up to 5 ng.

Kim et al. (2000) used ELISA and HPLC to analyse 180 samples form milk and dairy products collected in Seoul, South Korea. The results showed that AFM1 was present in pasteurised milk (76%) infant formula (85%) powdered milk (75%) and yoghurt (75%).

Iqbal et al. (2013) investigated the prevalence of aflatoxin M1 (AFM1) in milk and dairy products produced in Pakistan's Punjab province. Aflatoxin M1 was detected in milk samples and dairy products using high-performance liquid chromatography with a fluorescence detector. The findings revealed that AFM1 was detected in 71% of milk samples with 58 percent of those samples of dairy products above the European Union's allowed level (EU).

1.3.3 Liquid Chromatography with Mass Spectrometric Detection (LC-MS)

A new technique for detecting aflatoxins is liquid chromatography with mass spectrometric detection (LC-MS). High skills are required before performing a mass spectrometric analysis extraction and clean-up methods must be used. An ionisation chamber receives the HPLC effluent in LC-MS. Electrospray thermal spray chemical ionisation and rapid atom bombardment are some of the ionisation processes employed. Fragmentation is achieved in a collision chamber. The fragments are then detected in the MS's high vacuum area. For aflatoxins LC-MS procedures have been developed with electrospray (ESI) as the ionisation source in the majority of cases. The LC-MS method was used to determine aflatoxin levels in milk products (Sorensen and Elbaek 2005). The selection-ion-monitoring (SIM) mode is useful for detecting very low levels (pico-gram levels).

1.3.4 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA most extensively used method for detecting aflatoxin since it is a simple sensitive and versatile technology. ELISA is a cost-effective technology for quick and sensitive analysis (Pei; 2009; Parker&Tothill 2009). This approach has been used to detect AFM1 in milk product like UHT milk, yoghurt and newborn formula (Kim et al. 2000; Thirumala-Devi et al. 2002; Rastogi et al. 2004). Although antibodies have great specificity and sensitivity the matrix effect caused by the binding of other comparable chemicals that interact with antibodies creates cross reactivity which causes results to be altered (Trucksess and Koeltzow 1995). Furthermore the use of ELISA is restricted to matrices for which it has been validated (Gilbert and Anklam 2002). Direct competitive ELISA and indirect competitive ELISA are the two most common types of ELISA. The quantitative determination of aflatoxin present in the sample is done by comparison with the standard curve

1.3.5 Lateral flow assay

The lateral flow assay also known as the lateral flow immunochromatographic assay is a simple device for detecting the target analyte in a sample matrix that does not require any expensive equipment. Sample pad conjugate pad nitrocellulose membrane and absorbent pad are the main components. The test line (AFM1-protein conjugate) and the control line are coated on the nitrocellulose membrane in this approach (goat anti-

rabbit IgG antibodies). As a detection reagent colloidal GNP coated with polyclonal antibodies is commonly utilised. The AFM1 immobilised on the test line of the membrane competes with the analyte present in the sample. It is feasible to undertake a more rapid and accurate point-of-care diagnostic using a combination of the one-dot LFA and the smartphone-based reading method. Anfossi et al. (2013) developed a semi-quantitative lateral flow immunoassay for the detection of AFM1 in milk that was ultrasensitive.The LOD of the suggested approach is within the range permitted by EU legislation in milk however it falls short of the limit established for baby food which is 25 ppt. To detect AFB1 in maize samples a dipstick assay format based on a competitive response of the biotin-modified aptamer specific to AFB1 between target and cy5-modified DNA probes was devised. The nitrocellulose membrane (NC) and absorbent pad are used in the dipstick assay. Corn samples were analysed using LC-MS to ensure that they were free of AFB1[DNA probe 1 (14 NT) and DNA probe 2 (23 NT)] were employed with a biotinylatedaptamer and two Cy5 modified DNA probes. NC was treated with streptavidin before the test line and control line were coated with anti Cy5 antibodies to generate the dipstick.After drying the NC membrane was placed on stiff polyethylene sheets. AFB1 free samples as well as aptamer (0.1mM) and DNA probes (0.5mM) were added to a well and incubated. The fluorescence of a dipstick was detected using the ChemicDoc MP system. One fluorescent dot was found in the presence of AFM1 whereas two fluorescent dots were observed in the absence of AFM1. For corn samples the dipstick assay has a sensitivity of 0.3 ng/g ABF1 (Chen et al. 2014).

1.3.6 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) biosensor used to detect AFM1 in milk has beenreported. This approach can be used to identify AFM1 quickly and accurately. AFM1 is studied utilising an indirect competitive immune assay that is amplified by secondary antibodies that are Au nanoparticle conjugated. An interface of poly(2-hydroxyethylmethacrylate) brush was used to prevent milk components from fouling the sensor surface during analysis. On a mixed thiol self-assembled monolayer the study compares the performance characteristics of a p(HEMA)-based sensor with a commonly used polyethylene glycol-based design. The surface mass density of immobilised AFM1 conjugate as well as affinity bound primary and secondary antibodies are measured in both sensors. With 55 minutes of analysis time the biosensor enabled for high sensitivity with detection limit as low as 18 pg/ml (Karczmarczyk et al. 2016).

1.3.7 Charm ROSA

Rapid One Step Assay (ROSA) created by Charm Sciences in the United States is a lateral flow-based assay for detecting AFM-1 in bovine milk. Test strips for measuring AFM1 concentration in parts per trillion (ppt) are quantitatively interpreted by a ROSA reader. Concentrations below this level are viewed as negative hence the detection limit was determined to be 400 ppt. The consistency of results among laboratories is relatively low. Five laboratories' HPLC examination of the research sample revealed 38 % false negatives with the 500 ppt and 550 ppt AFM1 samples. This approach has a poor level of reliability yet it produces results quickly.

1.4 Human health risk associated with aflatoxin M1

Aflatoxin M1 is a hazardous strong carcinogenic chemical according to Sun et al. (1999). It causes liver cancer. According to Creppy (2002) the International Agency for Research on Cancer (IARC) has classed AFB1 and AFM1 as class 1 (carcinogenic to humans) with AFM1 having a carcinogenicity of 2-10% as compared AFB1. AFM1 was less harmful to living organisms (as a known carcinogen in Group 1) than AFB1, but it exhibited a high level of genotoxic action and constituted a considerable health risk due to its potential accumulation and binding to DNA, according to Shundo and Sabino (2006). AFM1 is a potential human carcinogen, according to Sugiyama et al. (2008), and the cancer risk linked with AFM1 contaminants in milk is a severe food safety problem.

Conclusion of aflatoxin

Aflatoxins are harmful fungal compounds generated mostly by Aspergillusflavus and Aspergillusparasiticus. Aflatoxin refers to four different types of mycotoxins that are produced: B1 B2 G1 G2 M1 and M2 which are metabolic products of B1 and B2 respectively. The most dangerous Aflatoxin B1 (AFB1) is a strong carcinogen that has been linked to significant health effects in both animals and humans including liver cancer. The Food Safety and Standards Authority of India (FSSAI) has established a permitted limit for Aflatoxin concentration in milk and milk products of 0.5 g/kg (FSSAI 2011). Currently chromatographic methods such as Thin Layer Chromatography Gas Chromatography and High-Performance Liquid Chromatography are used to detect AFM1 in milk and milk products which provides confirmatory results; however these techniques are expensive and require time-consuming sample extraction and cleanup procedures.Other analytical procedures make use of ligands that are unique to the target molecule. Antibody is one type of ligand that is employed in the development of ELISA and immunological sensors. As a result continual monitoring throughout the milk production chain is required to reduce health risks associated with the presence of aflatoxin in milk. Implementing appropriate agricultural and storage techniques to reduce the risk of toxigenic fungi aids in the lowering of aflatoxin levels in milk.

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