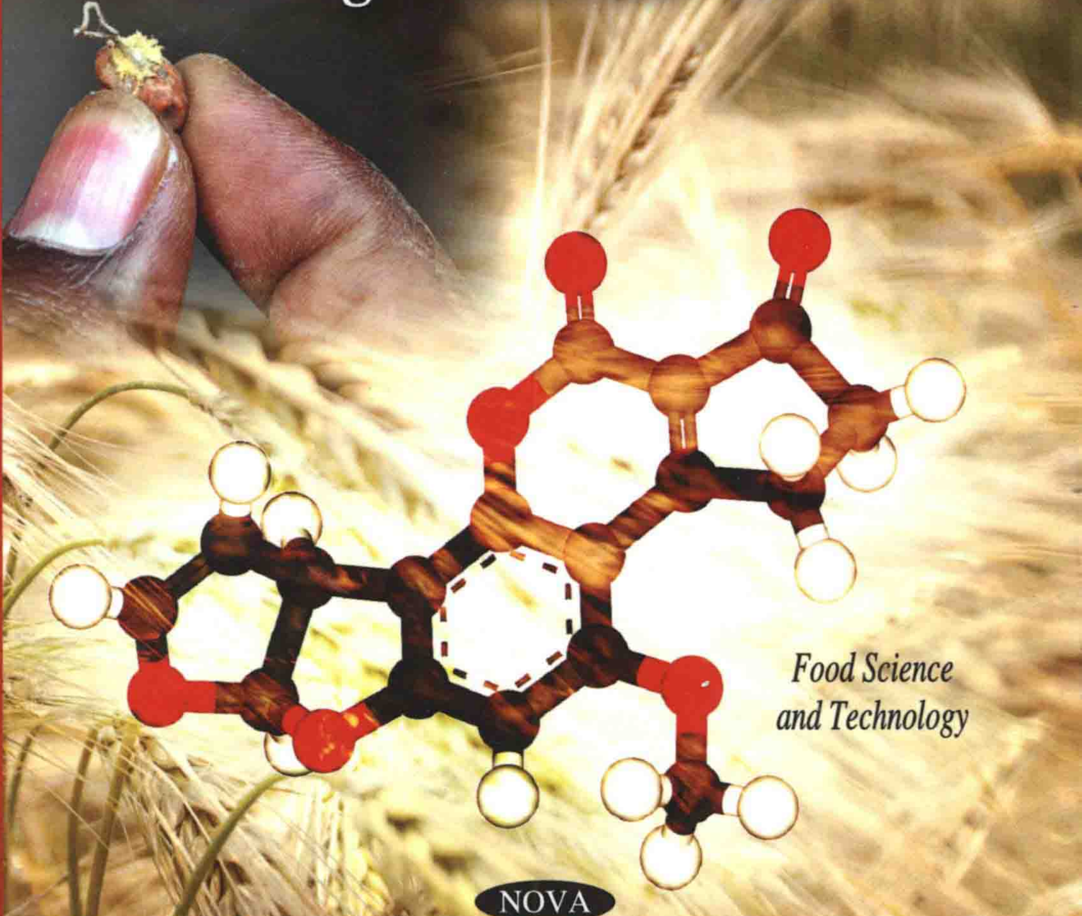
Adina G. Faulkner
Editor

Aflatoxins

Food Sources, Occurrence and Toxicological Effects



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AFLATOXINS

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ADINA G. FAULKNER
EDITOR

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PREFACE

Progress in understanding the biology of Aspergillus has greatly improved with the new techniques in genome sequencing and the developed molecular tools that enable rapid genetic analysis of individual genes. Particularly, the genetics of aflatoxin synthesis is regarded as a model to gain insight into fungal secondary metabolism. This compilation discusses topics that include the prevalence of aflatoxin B1 in cereals; contamination exposure, toxicology and control of aflatoxins in food and feed; immunosuppressive actions of aflatoxin; hazards and regulations; toxicological effects, risk assessment and legislation for aflatoxins; and the threat aflatoxins have on the economy and health.

Chapter 1 - Moulds of *Aspergillus* genus are among the most important causes of food and feed spoilage and can produce mycotoxins as toxic secondary metabolites when under adverse conditions. Aflatoxins are a group of mycotoxins that commonly contaminate maize and groundnuts, and are categorized by the International Agency for Research on Cancer under Class 1A human carcinogens. From the food safety standpoint, one of the most important mycotoxins is aflatoxin B_1 (AFB_1). Due to its potent carcinogenic, teratogenic and mutagenic effects dependent on the level and length of exposure, the presence of this contaminant in food and feed should be kept as low as achievable. In order to investigate the occurrence of AFB_1, determine its concentrations and explore the possibility of its reduction using different methods, samples of maize, wheat, barley and oat were collected from different cultivation fields during a three-year period. The immunoassay (ELISA) as a screening method and high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) as a confirmatory method were used to determine AFB_1 concentrations. Maize contamination seen with AFB_1

concentrations higher than permitted was associated with climate conditions established in the period of concern, which was extremely warm and dry, and might had favored mould production and AFB_1 formation. Substantial to almost absolute AFB_1 reduction in the maize samples was achieved using gamma radiation. A strong antifungal effect was also obtained upon the use of essential oils and lactic acid bacteria as biological AFB_1-reduction alternatives. As the presence of AFB_1 in cereals could be dangerous for human and animal health, in order to prevent its harmful effects and huge economic problems, the prevention of formation of this contaminant and consistent control over it are of major interest. Based on these substantiated grounds, possibilities of implementing new methods of AFB_1 determination and reduction within the frame of safe food production are virtually countless.

Chapter 2 - Toxigenic fungi in crops have been divided historically into two groups, field and storage fungi. Mycotoxins are produced by toxigenic fungi at the fields and in the storage. Although many compounds are termed as "mycotoxin", there are only five agriculturally-important fungal toxins: deoxynivalenol, zearalenone, ochratoxin A, fumonisin and aflatoxin. *Penicillium* and *Aspergillus* species are the most important storage fungi. However, they can also invade stressed plants in the field. The main mycotoxins produced by *Aspergillus* species are aflatoxins, citrinin and patulin. The word 'aflatoxin' comes from '*Aspergillus flavus* toxin', based on the fact that *A. flavus* and *A. parasiticus* are the predominant species responsible for aflatoxin contamination of crops prior to harvest or during storage. Aflatoxins B1, B2, G1, and G2 are the four major isolated aflatoxins from food and feed commodities.

A. flavus and *A. parasiticus* have distinct affinity for nuts and oilseeds including peanuts, maize and cotton seed. Cereals are a general substrate for growth of *A. flavus* but, unlike nuts, small grain cereal spoilage by *A. flavus* is the result of poor handling. Moreover, aflatoxin M1 as a milk contaminant has potential risk for animal and human health. The character of the aflatoxin problem varies by region. For instance, aflatoxin accumulation in stored maize in subtropical Asia has risen rapidly in post-harvest conditions whereas in the US, the issue is pre-harvest condition of maize. Therefore, the exposure to aflatoxins differs between countries particularly due to different diets. Food contamination with *Aspergillus* is associated with warm and dry climates. However, in variable environmental conditions, the aflatoxin contamination may differ from one year to another at the same location.

Progress in understanding the biology of *Aspergillus* has greatly improved with the new techniques in genome sequencing and the developed molecular tools that enable rapid genetic analysis of individual genes. Particularly, the genetics of aflatoxin synthesis is regarded as a model to gain insight into fungal secondary metabolism. Well-designed research on production of the aflatoxin precursor sterigmatocystin with the genetic model *A. nidulans*, has contributed greatly to our knowledge of the aflatoxin pathway and the global regulatory mechanisms. According to the recent studies, fungal pathogenesis is related to lipid-mediated fungal-host crosstalk, suggesting that secondary metabolism may be controlled by oxylipins at the transition level. Also, some oxylipins have been reported to be engaged in the signalling mechanism like quorum sensing responses in *Aspergillus*. Quorum sensing molecules and their genes which are responsible for intra and inter kingdom communications could be applied in the future aflatoxin bio-control strategies.

Chapter 3 - Aflatoxins (AFs) are secondary metabolites produced by various fungal species of the genus *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus*. The most important compounds are aflatoxins B1, B2, G1 and G2, as well as two metabolic products secreted in milk, M1 and M2.

The worldwide occurrence of aflatoxins contamination in raw agricultural products has been well documented; such contamination occurs in a variety of food and feed, such as cereals, nuts, dried fruits, spices and also in milk as a consequence of the ingestion of contaminated feed. However, pistachios, peanuts and corn are the most frequently contaminated food items reported in the Rapid Alert System for Food and Feed (RASFF) of the European Union. The occurrence of aflatoxins is mainly affected by environmental factors such as climatic conditions, geographic location, agricultural practices, and susceptibility of the products to fungal growth during harvest, storage and processing. High contamination levels of aflatoxins are mainly associated with post-harvest growth of *Aspergillus* moulds in poorly stored commodities.

Aflatoxins can cause adverse effects to the health of animals and humans. These toxins have been reported to be associated with acute liver damage, liver cirrhosis, induction of tumors and teratogenic effects. Aflatoxin B1 (AFB1) is usually predominant and the most toxic among aflatoxins because it is responsible for hepatocarcinoma in animals and strongly associated with the incidence of liver cancer in humans. AFB1 is a genotoxic and mutagenic chemical, and it has been classified by the International Agency of Research on Cancer (IARC) as human carcinogen (group 1). The toxic effects of the ingestion of aflatoxins in both humans and animals depend on several factors including intake levels, duration of exposure, metabolism and defense mechanisms, and individual susceptibility. Aflatoxins affect not only the health of humans and animals but also the economics of agriculture and food.

Because of the multiple adverse health effects to humans and animals caused by aflatoxin consumption, many nations worldwide have regulatory standards on aflatoxin in food and feed. The European Union (EU) regulation on aflatoxins in foodstuffs is among the strictest in the world (Commission Regulation (EC) n° 1881/2006 and successive amendments). Maximum contents of aflatoxins in feeds are also established by Commission Regulation (EU) n° 574/2011 on undesirable substances in animal feed.

Throughout the world there are many advisory bodies concerned with food safety, including the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), the Codex Alimentarius Joint Expert Committee for Food Additives and Contaminants (JECFA), and many others, which regularly assess the risk from mycotoxins, advise on controls to reduce consumer exposure and establish different regulations for these toxins in different countries.

Chapter 4 - Aflatoxins are secondary metabolites produced by fungi of the *Aspergillus* species. They occur as contaminants in a variety of food and feed stuffs that have been infected with the producing fungi. Aflatoxin exposure is known to cause a number of acute and chronic effects in both humans and animals, including immunosuppression, liver and other cancers, and failure of vaccination regimens. The immunomodulatory effects of the aflatoxins have been shown to affect cell-mediated immunity more than humoral immunity. In particular, aflatoxin exposure modulates secretion of inflammatory cytokines and phagocytic function. Decreases in phagocytosis and inflammation observed following aflatoxin exposure may reduce the effectiveness of the host immune response to infection, thereby increasing susceptibility to infection in individuals exposed to these toxins. The aim of this chapter is to summarise the immunomodulatory effects of aflatoxin exposure in order to better understand its potential immunosuppressive effects in humans and animals. The relationship between these immunosuppressive actions and susceptibility to infection will also be discussed.

Chapter 5 - Brazil nut is an important non-timber forest product produced in Amazon region. This nut is used as food with high value in the international market, due to its high nutritional and flavor characteristic and to their association with environmental conservation and alleviation of poor people living from Amazonia. Annually, several hundred tons of Brazil nuts are produced in Brazil. However, they are susceptible to aflatoxins (AF) contamination. Because of the detection of unacceptable level of AF in Brazil nuts consignments arriving in European Union ports, in 2003, special conditions were imposed on Brazil nuts entering the European Union, decreasing the acceptable levels of AF. In 2010, the European Union revised AF regulation on nuts; these new limits are more adequate when considering the complexity of Brazil nut chain and the low risk related to its low consumption. This chapter points data on the occurrence of AF in Brazil nuts, as reported by the Rapid Alert System for Food and Feed (RASFF), and evaluates the efforts made by all sectors involved in the agribusiness of Brazil nuts, in Brazil, in order to contribute to protection of both domestic and international consumers from possible health hazard caused by AF.

Chapter 6 - Aflatoxin B_1 (AFB_1) is an important genic toxin produced by the moulds Aspergillus parasiticus and Aspergillus flavus. AFB_1 is metabolized by cytochrome P450 enzymes to its reactive form, AFB_1-8,9-epoxide (AFB_1-epoxide), which covalently binds to DNA and induces DNA damage. DNA damage induced by AFB_1, if not repaired, may cause such genic tox toxicological Effects as DNA adducts formation, gene mutations and hepatocellular carcinoma (HCC). During the repair process of DNA damage produced by AFB_1, DNA repair genes play a central role, because their function determines DNA repair capacity. In this study, the authors investigated the association between seven polymorphisms (including rs25487, rs861539, rs7003908, rs28383151, rs3734091, rs13181, and rs2228001) in DNA repair genes XPC, XRCC4, XRCC1, XRCC4, XPD, XRCC7, and XRCC3, and toxicological effects of AFB_1 using a hospital-based case-control study. Toxicological effects of AFB_1 were analyzed by means of the levels of AFB_1-DNA adducts, the mutant frequency of TP53 gene, and the risk of AFB_1-related HCC. The authors found that the mutants of XPC, XRCC4, XRCC1, XRCC4, XPD, XRCC7, and XRCC3 had higher AFB_1-DNA adducts levels, compared with the wilds of these genes (3.276 vs 3.640 μmol/mol DNA for rs25487, 2.990 vs 3.897 μmol/mol DNA for rs861539, 2.879 vs 3.550 μmol/mol DNA for rs7003908, 3.308 vs 3.721 μmol/mol DNA for rs28383151, 3.229 vs 3.654 μmol/mol DNA for rs3734091, 2.926 vs 4.062 μmol/mol DNA for rs13181, and 3.083 vs 3.666 μmol/mol DNA for rs2228001, respectively). Furthermore, increasing risk of TP53 gene mutation and HCC was also observed in these with the mutants of DNA repair genes. These results suggested that polymorphisms of DNA repair genes might modify the toxicological effects of AFB.

Chapter 7 - Studies in typical and new Argentinean peanut areas showed that toxigenic *Aspergillus* section *Flavi* strains are widely distributed in soils and seeds, with high probability of being transferred to the storage ecosystem. Mycological analyses of soil showed that *Aspergillus* section *Flavi* population were present in the two areas at similar counts ($3.2x10^2$ cfu g^{-1}). Within this section, two fungal species were frequently isolated with isolation percentages of 73 and 90% for *A. flavus* and of 27 and 9% for *A. parasiticus* in soil samples from traditional and new areas, respectively. The percentages of the different *A. flavus* phenotypes from both peanut-growing areas showed that L strains were recovered in the highest percentage and represented 59 and 88% of the isolates with variable ability to produce aflatoxins (AFs). Peanut kernels collected at harvest time from different localities of Córdoba and Formosa provinces showed *A. flavus* and *A. parasiticus* contamination. The 42.8 and 70% were classified as type L and the percentages of aflatoxigenic *A. flavus* strains were 68.6 and 80.0% in samples from traditional and recent peanut-growing areas, respectively. Highly toxigenic *A. flavus* S strains were isolated with major frequency from soil and kernel samples coming from traditional peanut-growing area. Aflatoxin contamination was detected in peanut kernels from typical peanut growing area. Harvested peanut were stored during 5 months in three storage systems (big bags, wagons of conditioning and drying and stockpiled warehouse) and mycological population succession was analyzed. Fungal isolation was greater from pod (95%) than from kernel tissues. The most common fungi identified included *Penicillium*, *Aspergillus*, *Eurotium* and *Fusarium* spp. Within *Aspergillus* genus, the section *Flavi* had the greatest mean counts of $1.4x10^4$, $9.4x10^2$, $5.2x10^2$ cfu g^{-1} for big bags, wagon and warehouse, respectively. *A. flavus* and *A. parasiticus* strains with variable ability to produce AFs were isolated from peanut kernels stored in the three systems at all sampling periods in the order of $1.5x10^2$, $2.3x10^2$ and 4.5 cfu g^{-1}, respectively. .*A. flavus* S and L strains contributed to silo community toxigenicity during all storage period. Total AF levels ranging from 1.1 to 200.4 ng g^{-1} were registered in peanuts conditioned at the higher a_W values (0.94–0.84 a_W) and stored in big bags. Despite the water stress conditions registered in the stockpiled warehouse throughout the storage period, AFB_1 levels ranging between 2.9 and 69.1 ng g^{-1} were registered from the third sampling.

Therefore, the interaction between biological and abiotic factors and substrate may promote the *Aspergillus* contamination and the subsequent AF accumulation in peanut from sowing to storage, highlighting the need to promote good practices in order to avoid the risk of these metabolites contamination in peanut food chain.

Chapter 8 - Aflatoxins are toxic metabolites produced by the fungus *Aspergillus*. The main representatives are aflatoxins B1, B2, G1, G2. Their occurrence in food like nuts, cereals and cereal-derived products is a result of fungal contamination before harvest and during storage. Milk can also be

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contaminated by aflatoxin M1 (main metabolite of B1) as a result of animals' exposure to feed contaminated by the aflatoxin B1.

Aflatoxins manifest acute and chronic toxicity. Evidence of acute aflatoxicosis in humans involving a range of symptoms from vomiting to death has been reported mainly in Third World Countries. In relation to chronic toxicity aflatoxins are well known for their genotoxic and carcinogenic properties while recent studies evident a series of other possible effects like reprotoxicity, impaired growth in children, intestinal functions, chronic fatigue syndrome, compromise immunity and interfere with protein metabolism and multiple micronutrients that are critical to health.

The critical step for aflatoxins' risk assessment is the estimation of the real exposure. For this reason a number of surveys are conducted globally using tools like biomarkers of exposure and modeling. In addition new parameters like the climate change are now taken into consideration in order to predict possible current and future changes of exposure to aflatoxins. As aflatoxins are compounds of natural origin and their presence in food cannot be totally eliminated the risk management is based on keeping the total exposure as low as reasonably achievable taking into account the social-economic impact of crop and livestock losses. Exposure reduction is achieved mainly by reducing the number of highly contaminated foods reaching the market by regulatory control but also applying detoxification strategies. According to the EU regulatory framework minimization of the exposure to aflatoxins is based on setting maximum levels of aflatoxins in different foodstuffs (4 – 10 μg/kg total aflatoxins) and feed (EC/1881/2006, Directive 2002/32/EC). Products exceeding the maximum levels should not be placed on the EU market. Methods of sampling and analysis for the official control of aflatoxins, are also set (EC/401/2006) in order to ensure common sampling criteria to the same products and that certain performance criteria are fulfilled. The United States Food and Drug Administration (FDA) has established the action levels for aflatoxin present in food to the 20 μg/kg (0.5 μg/kg for milk) and up to 300 μg/kg for feed. Finally an action level of 10 μg/kg total aflatoxins is also used from Japan authorities.

Chapter 9 - This paper presents a review of the occurrence of aflatoxins in different food commodities in Greece, based both on results represented in literature as well as results derived from monitoring programs of the Center of Toxicology Science & Research, Medical School, University of Crete. Aflatoxins, can pose a severe threat to food safety, since they are characterized carcinogenic to humans, IARC Group 1. They may be formed or developed in any stage of the agricultural production (primary production, processing and storage) as a result of transitional weather conditions or of poor storage. Studies, monitoring programs and surveys, which have been carried out in Greece, are mainly focused in milk and dairy products. In this context, several studies have been conducted in animal feeds as well, since there is notable evidence that they are potential sources of aflatoxins in milk production. Additionally, both black and green olives have been examined for possible contamination by aflatoxins, due to the fact that they are damaged during harvest and processing and thus providing a substrate for aflatoxin development. Finally, a limited number of studies investigate the presence of aflatoxins in different processed products like breakfast cereals. The above foodstuffs have been studied on account of their high nutritional value and the fact that they are consumed by different population groups. Results indicate that residue levels of aflatoxins which are presented in fresh as well as processed agricultural products, do not pose any considerable risk for the Greek population groups. The most important factors influencing the levels of aflatoxins in major agricultural products appear to be the growing and cultivation techniques, as well as the food safety parameters during harvesting, storage and processing. An additional issue, which seems to raise concern internationally, is the fact that climate change in combination with modifications in the cultivation techniques may affect the frequency and severity of aflatoxin residues in agricultural products.

Chapter 10 - This review deals with the aflatoxins especially with their food sources, wide occurrence and toxicological effects on animals and humans. Aflatoxins are highly oxygenated, heterocyclic, difuranocoumarin compounds and are an important group of mycotoxins produced by the fungi. There are almost 20 different types of aflatoxins identified till now; among these AFB_1 is considered to be the most toxic. Aflatoxins persist to some extent in food even after the inactivation of the fungi by food processing methods, such as ultra-high temperature products, due to their significant chemical stability. Aflatoxins can affect a wide range of commodities including cereals, oilseeds, spices, and tree nuts as well as milk, meat, and dried fruits. Twenty-five percent of the world's crops are affected with mycotoxins. On a worldwide scale, the aflatoxins are found in stored food commodities and oil seeds. Some of the foods on which aflatoxin producing fungi grow well include cereals (maize, sorghum, pearl millet, rice, wheat, corn, oats, barley), oilseeds (peanut, soybean, sunflower, cotton), spices (chile peppers, black pepper, coriander, turmeric, ginger), and tree nuts (almond, pistachio, walnut, coconuts), sweet potatoes, potatoes, sesame, cacao beans, almonds, etc., which on consumption pose health hazards to animals, including aquaculture species of fish, and humans. Food commodities affected by aflatoxins are also susceptible to other types of mycotoxins and multiple mycotoxins can co-exist in the same commodity. Various cereals affected by aflatoxins are also susceptible to contamination by fumonisins, trichothecenes (especially deoxynivalenol), zearalenone, ochratoxin A and ergot alkaloids.

More than 5 billion people in developing countries worldwide are at risk of chronic exposure to naturally occurring aflatoxins through contaminated foods. Aflatoxin is a potent liver toxin causing hepatocarcinogenesis, hepatocellular hyperplasia, hepatic necrosis, cirrhosis, biliary hyperplasia, and acute liver damage in affected animals. Effects of aflatoxins in animals depend on age, dose and length of exposure, species, breed and nutritional status of the animal. Health effects occur in fish, companion animals, livestock, poultry and humans because aflatoxins are potent hepatotoxins, immunosuppressants, mutagens, carcinogens and teratogens. Aflatoxin– B_1 has been shown to cause significant morphological alterations along with reduced phagocytic potential in chicken and turkey macrophages. Aflatoxin- B_1 exposure to chicken embryos causes significant suppression in macrophage phagocytic potential in chicks after hatch. Aflatoxin intercalates into DNA and alkylates the DNA bases through its epoxide moiety resulting in liver cancer. Other effects include mutagenic and teratogenic effects. Exposure of biological systems to harmful levels of aflatoxin results in the formation of epoxide, which reacts with proteins and DNA leading to DNA-adducts, thus causing liver cancer. The primary target of aflatoxins is the hepatic system. Acute effects include hemorrhagic necrosis of the liver and bile duct proliferation while chronic effects include hepatocellular carcinoma (HCC). HCC is the sixth most prevalent cancer worldwide with a higher incidence rate within developing countries. Preliminary evidence suggests that there may be an interaction between chronic aflatoxin exposure and malnutrition, immunosuppression, impaired growth, and diseases such as malaria and HIV/AIDS. Outbreaks of acute aflatoxin poisoning are a recurrent public health problem. The discussion of this problem and its remedies must be held in the context of the associated question of food insufficiency and more general economic challenges in developing countries. Aflatoxin constitutes a serious health concern to the entire food chain, necessitating a multidisciplinary approach to analysis, action, and solution.

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